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

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Abstract. We investigated the cytosolic free calcium concentration ($[Ca^{2+}]_i$) of leech Retzius neurons in situ while varying the extracellular Ca^{2+} concentration via the bathing solution ($[Ca^{2+}]_B$). Changing $[Ca^{2+}]_B$ had only an effect on $[Ca^{2+}]_i$ if the cells were depolarized by raising the extracellular K^+ concentration. Surprisingly, raising $[Ca^{2+}]_B$ from 2 to 10 mM caused a decrease in $[Ca^{2+}]_i$, and an increase was evoked by reducing $[Ca^{2+}]_B$ to 0.1 mM. These changes were not due to shifts in membrane potential. At low $[Ca^{2+}]_B$ moderate membrane depolarizations were sufficient to evoke a $[Ca^{2+}]_i$ increase, while progressively larger depolarizations were necessary at higher $[Ca^{2+}]_B$. The changes in the relationship between $[Ca^{2+}]_i$ and membrane potential upon varying $[Ca^{2+}]_B$ could be reversed by changing extracellular pH. We conclude that $[Ca^{2+}]_B$ affects $[Ca^{2+}]_i$ by modulating Ca^{2+} influx through voltage-dependent Ca^{2+} channels via the electrochemical Ca^{2+} gradient and the surface potential at the extracellular side of the plasma membrane. These two parameters are affected in a counteracting way: Raising the extracellular Ca^{2+} concentration enhances the electrochemical Ca^{2+} gradient and hence Ca^{2+} influx, but it attenuates Ca^{2+} channel activity by shifting the extracellular surface potential to the positive direction, and vice versa.

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

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Introduction

The flux of ions through voltage-dependent ion channels depends on the electrochemical gradient of the permeating ions as well as on the activity of the channels (*see* Hille, 1992). The activity of voltage-dependent ion channels is controlled primarily by the electrical field across a specific portion of the channel protein, the voltage sensor. This electrical field is determined by the membrane potential (E_m) and by local fields in the vicinity of the voltage sensor. The sources of the local fields may be charged groups on the channel protein, on membrane lipids, or on proteins associated with the channel.

The Retzius neurons of the leech nervous system possess slowly inactivating voltage-dependent Ca^{2+} channels (Stewart, Nicholls & Adams, 1989; Gottmann, Dietzel & Lux, 1989; Bookman & Liu, 1990). In these neurons, a membrane depolarization induced by raising the extracellular K^+ concentration ($[K^+]_o$) leads to Ca^{2+} channel activation and hence to an increase in the cytosolic free calcium concentration ($[Ca^{2+}]_i$; Hochstrate, Piel & Schlue, 1995; Dierkes, Hochstrate & Schlue, 1997). The Ca^{2+} influx through the Ca^{2+} channels is affected by extracellular pH and ionic strength in a fashion that is consistent with a modulation by the extracellular surface potential (Hochstrate et al., 2001).

We investigated the effect of the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) on the $[Ca^{2+}]_i$ of leech Retzius neurons in situ at normal and at increased $[K^+]_o$. The results show that $[Ca^{2+}]_o$ markedly affects $[Ca^{2+}]_i$, provided that the voltage-dependent Ca^{2+} channels are activated. It is concluded that $[Ca^{2+}]_o$ modulates the Ca^{2+} influx through the Ca^{2+} channels by affecting two flux-controlling parameters in a counteracting way: the electrochemical Ca^{2+} gradient and the surface potential at the extracellular side of the plasma membrane.

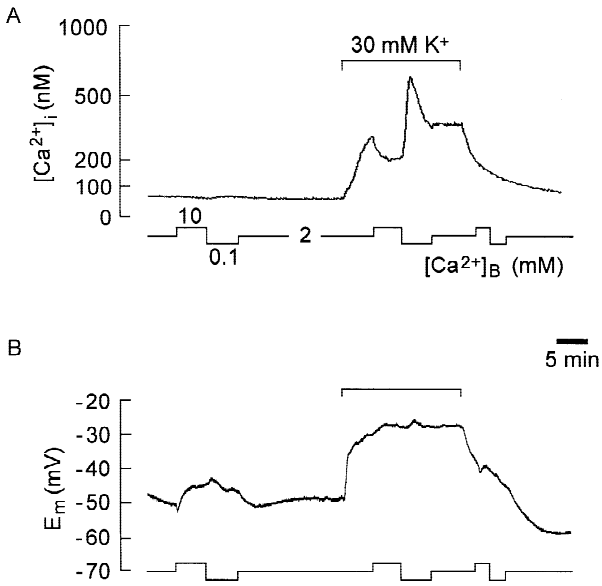

Fig. 1. Effect on $[Ca^{2+}]_i$ and E_m of changing $[Ca^{2+}]_B$ to 10 and 0.1 mM before, during and after raising $[K^+]_B$ to 30 mM. (*A*) Variation of $[Ca^{2+}]_B$ in physiological solution had no significant effect; at raised $[K^+]_B$, however, $[Ca^{2+}]_i$ decreased upon augmenting $[Ca^{2+}]_B$ and it transiently increased upon $[Ca^{2+}]_B$ reduction. (*B*) Raising $[Ca^{2+}]_B$ in physiological solution evoked a slight membrane depolarization (+3.5 ± 4.1 mV, $n = 13$), while reducing $[Ca^{2+}]_B$ had no significant effect on E_m (−1.0 ± 5.3 mV). At $[K^+]_B = 30$ mM changing $[Ca^{2+}]_B$ had virtually no effect on E_m.

Materials and Methods

Preparation and Experimental Setup

The experiments were done on Retzius neurons in intact segmental ganglia of the leech *Hirudo medicinalis* (*see* Muller, Nicholls & Stent, 1981). Except ganglia 5 and 6, all segmental ganglia were used for the experiments. The preparation and the experimental setup have been described in the companion paper (Hochstrate et al., 2001) and in previous papers (Hochstrate et al., 1995; Dierkes et al., 1997). In short, the Retzius neurons were iontophoretically loaded with Fura-2 (Molecular Probes, Eugene, OR, USA) by way of single-barrelled microelectrodes. 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 volume was exchanged 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 (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 of 10 to 50 μm edge length by means of a variable diaphragm.

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

In order to obtain the fluorescence of the injected Fura-2 (F_{340}, F_{380}), the 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 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 $Ca^{2+} \cdot$ Fura-2 complex. The parameters R_{min}, R_{max} and F_f/F_s were determined by using aqueous Fura-2 solutions that were either Ca^{2+}-free or contained 10 mM Ca^{2+}. For K_d a value of 135 nM was used, a value that was measured under similar conditions by Grynkiewicz et al. (1985). Mean values of $[Ca^{2+}]_i$ were determined by averaging the ratios R(340nm/380nm) measured in the single experiments.

Electrophysiological Recordings

The electrophysiological recordings were performed in a different setup, with the exchange rate of the bath medium similar to that in the fluorescence measurements. $[Ca^{2+}]_o$ was measured with double-barrelled Ca^{2+}-sensitive microelectrodes of the twisted type (Schlue, 1987), filled with Ca^{2+} ionophore II-cocktail A (Fluka, Buchs, Switzerland) followed by 0.1 M $CaCl_2$. The reference barrel was filled with 3 M KCl. The electrodes were calibrated in solutions having the same ionic composition as the physiological solution, except that the Ca^{2+} concentration was changed. To obtain stable recordings the tips of the electrodes were broken by gently touching the bottom of the experimental chamber. The tip diameter of the broken electrodes used for the experiments varied between 3 and 5 μm. The electrodes were positioned between the cell bodies of the two Retzius neurons, about halfway between the outer and inner capsule of the segmental ganglion. E_m recordings were carried out by the use of conventional single-barrelled microelectrodes filled with 3 M KCl. The $[Ca^{2+}]_o$ recordings were low-pass filtered with 0.5 Hz, the E_m recordings with 5 Hz.

Solutions

The physiological solution had the following composition (in mM): 85 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]; Roth, Karlsruhe, Germany), adjusted to pH 7.40 with NaOH; the pH adjustment increased the Na^+ concentration by 4 mM. In solutions with raised K^+ concentration, equimolar amounts of NaCl were replaced by KCl. The Ca^{2+} concentration of the solutions was varied by omitting or adding appropriate amounts of $CaCl_2$ without osmotic compensation.

Results

Effect of $[Ca^{2+}]_B$ on the K^+-induced $[Ca^{2+}]_i$ Increase

The effect of varying the Ca^{2+} concentration of the bathing solution ($[Ca^{2+}]_B$) on the $[Ca^{2+}]_i$ of leech Retzius neurons is shown in Fig. 1. In physiological solution, changes in $[Ca^{2+}]_B$ had no effect on $[Ca^{2+}]_i$, but they markedly affected it after the K^+ concentration of the bathing solution ($[K^+]_B$) had been raised, which activated voltage-dependent Ca^{2+} channels (Hochstrate et al.,

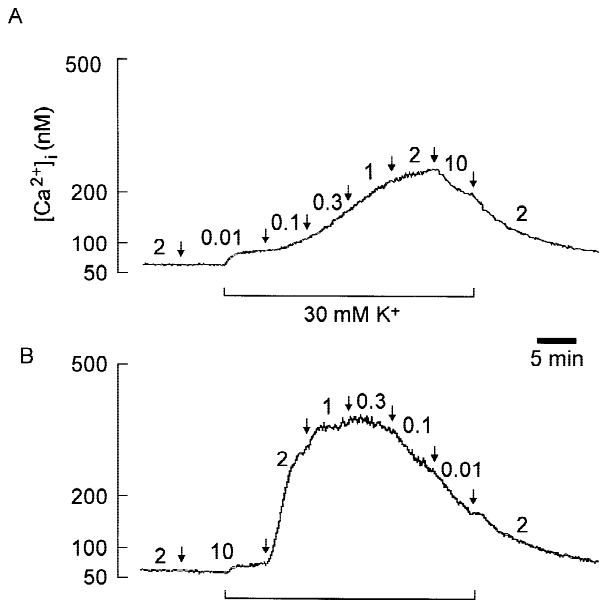

Fig. 2. Effect on $[Ca^{2+}]_i$ of gradually increasing (*A*) or decreasing $[Ca^{2+}]_B$ (*B*) at $[K^+]_B$ = 30 mM. When $[Ca^{2+}]_B$ was gradually decreased (10, 2, 1, 0.3, 0.1, and 0.01 mM), $[Ca^{2+}]_i$ increased significantly more (381 ± 57 nM *vs.* 258 ± 48 nM; n = 5), and reached its maximum at a lower $[Ca^{2+}]_B$ (0.3 *vs.* 2 mM). Note the different $[Ca^{2+}]_i$ reached at $[Ca^{2+}]_B$ = 10 mM during the two protocols: when $[Ca^{2+}]_B$ was raised to 10 mM after a prolonged elevation of $[K^+]_B$ at various lower $[Ca^{2+}]_B$, the $[Ca^{2+}]_i$ remained markedly increased (196 ± 44 nM, n = 9, *see* Fig. 1*A*). However, if $[Ca^{2+}]_B$ was first raised and then $[K^+]_B$, the $[Ca^{2+}]_i$ increased only slightly (73 ± 11 nM, n = 8).

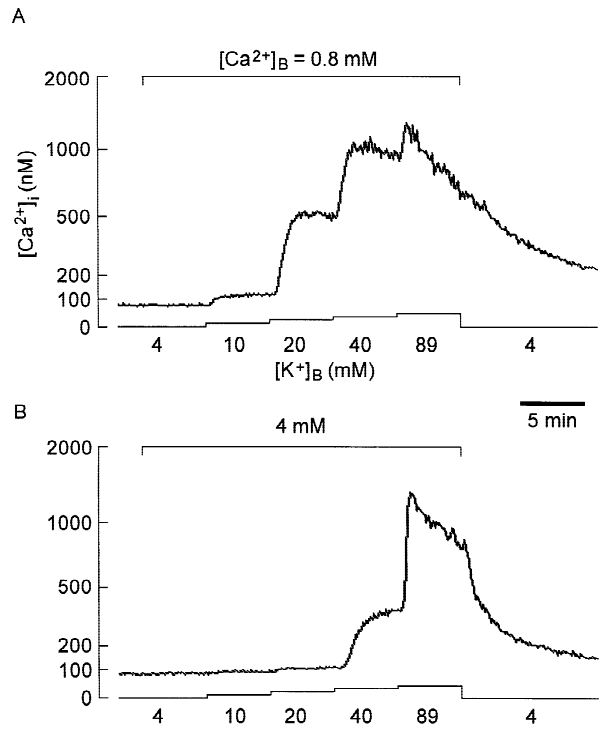

Fig. 3. Effect of raising $[K^+]_B$ on $[Ca^{2+}]_i$ at two different $[Ca^{2+}]_B$. (*A*) At $[Ca^{2+}]_B$ = 0.8 mM, raising $[K^+]_B$ to 10, 20, and 40 mM evoked distinct increases in $[Ca^{2+}]_i$, while the further rise to 89 mM caused a drop in $[Ca^{2+}]_i$. (*B*) Contrastingly, at $[Ca^{2+}]_B$ = 4 mM, raising $[K^+]_B$ up to 20 mM had no effect on $[Ca^{2+}]_i$, while $[Ca^{2+}]_i$ increased markedly upon raising $[K^+]_B$ to 40 and 89 mM.

1995; Dierkes et al., 1997). Surprisingly, in the presence of 30 mM K^+, raising $[Ca^{2+}]_B$ from 2 to 10 mM caused a marked decrease in $[Ca^{2+}]_i$, while reducing it to 0.1 mM evoked a transient overshoot of the $[Ca^{2+}]_i$ level recorded at $[Ca^{2+}]_B$ = 2 mM (Fig. 1*A*). In physiological solution, raising $[Ca^{2+}]_B$ evoked a slight membrane depolarization, while reducing it had no significant effect on E_m; at increased $[K^+]_B$ changing $[Ca^{2+}]_B$ had virtually no effect on E_m (Fig. 1*B*).

In physiological solution, the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$), as recorded by Ca^{2+}-sensitive microelectrodes between the cell bodies of the two Retzius neurons, was 3.7 ± 1.2 mM (n = 7), which was significantly higher than $[Ca^{2+}]_B$ (2 mM; *compare* Rose, Lohr & Deitmer, 1995; Lohr, Rose & Deitmer, 1996). Changing $[Ca^{2+}]_B$ to 10 or 0.1 mM caused corresponding changes in $[Ca^{2+}]_o$ to 7.1 ± 3.1 mM or 0.5 ± 0.4 mM (n = 7), each measured after 5 minutes in the respective solution. Raising $[K^+]_B$ to 30 mM had no significant effect on $[Ca^{2+}]_o$. It is noted that in leech segmental ganglia the extracellular concentrations of K^+ and H^+ were also found to be slightly higher than those in the physiological bathing solution (Schlue, Wuttke & Deitmer, 1985; Frey & Schlue, 1993).

Raising $[Ca^{2+}]_B$ gradually every 5 min at $[K^+]_B$ = 30 mM led to a slow increase in $[Ca^{2+}]_i$, which revealed no distinct steps corresponding to the steps in $[Ca^{2+}]_B$ (Fig. 2*A*). The $[Ca^{2+}]_i$ was maximally increased at $[Ca^{2+}]_B$ = 1 or 2 mM, and it dropped upon raising $[Ca^{2+}]_B$ to 10 mM. When $[Ca^{2+}]_B$ was changed in the reverse order, $[Ca^{2+}]_i$ was also maximally increased at intermediate $[Ca^{2+}]_B$ (Fig. 2*B*). In most experiments, $[Ca^{2+}]_i$ reached a transient maximum at $[Ca^{2+}]_B$ = 0.3 mM, but subsequently it fell below the level recorded before at $[Ca^{2+}]_B$ = 1 mM. Again steady-state levels were not reached, but the changes in $[Ca^{2+}]_B$ were clearly reflected by changes in the slope of the $[Ca^{2+}]_i$ recordings. Electrophysiological recordings that were performed analogously to the $[Ca^{2+}]_i$ recordings in Fig. 2 showed that E_m was independent of the order in which $[Ca^{2+}]_B$ was changed. Five minutes after raising $[K^+]_B$ to 30 mM E_m was −21.6 ± 6.5 mV at $[Ca^{2+}]_B$ = 0.01 mM and −22.4 ± 2.6 mV at 10 mM Ca^{2+} (for each, n = 5). Over the whole period at 30 mM K^+ the cells continued to depolarize slightly (+4.1 ± 1.9 mV, n = 10), independent of the order in which $[Ca^{2+}]_B$ was changed.

The maximum $[Ca^{2+}]_i$ reached upon increasing $[Ca^{2+}]_B$ in regular amounts was significantly smaller than that upon reducing $[Ca^{2+}]_B$ in the reverse order,

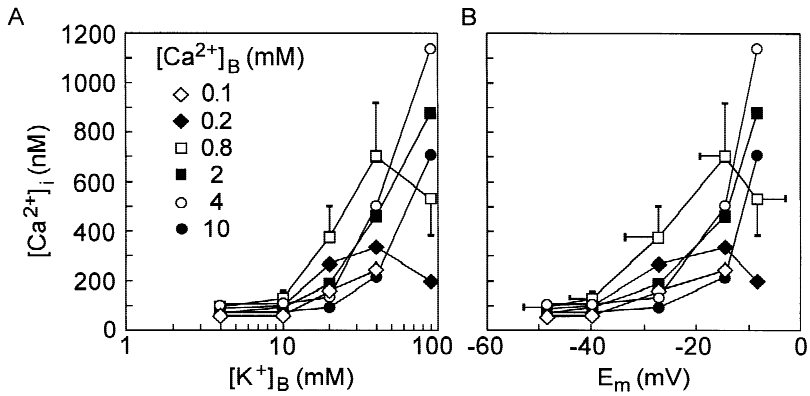

Fig. 4. Relationship between $[Ca^{2+}]_i$ and $[K^+]_B$ (*A*) and between $[Ca^{2+}]_i$ and E_m (*B*) at different $[Ca^{2+}]_B$. Data from experiments performed in the same way as shown in Fig. 3. The $[Ca^{2+}]_i$ values (mean of $n = 5$ to 11 experiments) and the corresponding E_m ($n = 22$ to 45) were determined after 5 min at the respective $[K^+]_B$. SD exemplified only for one data set for reasons of clarity.

which shows that $[Ca^{2+}]_i$ was not only determined by the composition of the bathing solutions but also by the bath composition at an earlier time. This was particularly evident when comparing the $[Ca^{2+}]_i$ levels that were recorded at $[Ca^{2+}]_B = 10$ mM in the presence of 30 mM K^+. Thus, $[Ca^{2+}]_i$ remained considerably increased if $[Ca^{2+}]_B$ was raised to 10 mM after $[K^+]_B$ had been raised before at a lower $[Ca^{2+}]_B$ (Figs. 1*A*, 2*A*). However, $[Ca^{2+}]_i$ increased only slightly when $[Ca^{2+}]_B$ was raised before raising $[K^+]_B$ (Fig. 2*B*).

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

$[Ca^{2+}]_B$ affected the $[K^+]_B$ that was necessary to evoke a detectable $[Ca^{2+}]_i$ increase (Figs. 3, 4). At low $[Ca^{2+}]_B$ of 0.1–0.8 mM, raising $[K^+]_B$ to 10 mM was usually sufficient to evoke a $[Ca^{2+}]_i$ increase, and $[Ca^{2+}]_i$ increased in proportion to $[Ca^{2+}]_B$. The $[Ca^{2+}]_i$ increase was enhanced by raising $[K^+]_B$ to 20 or 40 mM, but at 89 mM the increase was transient and within 5 min $[Ca^{2+}]_i$ dropped below the steady-state level at 40 mM K^+ (Fig. 3*A*). At high $[Ca^{2+}]_B$ of 2–10 mM, larger $[K^+]_B$ were necessary to evoke a $[Ca^{2+}]_i$ increase, and the increase was continuously augmented up to $[K^+]_B = 89$ mM (Fig. 3*B*).

The result that $[K^+]_B$ had to be raised more at high than at low $[Ca^{2+}]_B$ in order to evoke a detectable $[Ca^{2+}]_i$ increase reflects a shift in the threshold E_m for the activation of the voltage-dependent Ca^{2+} channels. At low $[Ca^{2+}]_B$ a membrane depolarization to about −40 mV was sufficient to raise $[Ca^{2+}]_i$, while at high $[Ca^{2+}]_B$ a depolarization to −30 mV or more positive was necessary (Fig. 4*B*).

The data in Fig. 4 also show that at a given $[K^+]_B$ or E_m the $[Ca^{2+}]_i$ was maximally increased at intermediate $[Ca^{2+}]_B$, which is in line with the results shown in Figs. 1 and 2. Furthermore, the $[Ca^{2+}]_B$ at which $[Ca^{2+}]_i$ was maximally increased was the larger the more $[K^+]_B$ was raised or the more the cells were depolarized. Thus, at membrane potentials between −30 and −20 mV the $[Ca^{2+}]_i$ was maximally increased at $[Ca^{2+}]_B = 0.8$ mM, while at membrane potentials around −10 mV the maximum $[Ca^{2+}]_i$ increase occurred at $[Ca^{2+}]_B = 4$ mM.

The changes in the relationship between $[Ca^{2+}]_i$ and E_m caused by varying $[Ca^{2+}]_B$ could be reversed by changing the pH of the bathing solution. As shown in Fig. 5*A*, an extracellular acidification shifted the negative threshold E_m at low $[Ca^{2+}]_B$ to more positive values and, conversely, an extracellular alkalinization shifted the positive threshold E_m at high $[Ca^{2+}]_B$ back in the negative direction (Fig. 5*B*). The data in Fig. 5 also show that the effect of the pH of the bathing solution was more pronounced at low than at high $[Ca^{2+}]_B$.

Discussion

Modulation of Ca^{2+} Influx by $[Ca^{2+}]_o$

The results show that $[Ca^{2+}]_B$ and hence $[Ca^{2+}]_o$ markedly affect the $[Ca^{2+}]_i$ of leech Retzius neurons, provided that the cells are depolarized and voltage-dependent Ca^{2+} channels are activated (Hochstrate et al., 1995; Dierkes et al., 1997). The continuous influx of Ca^{2+} through the Ca^{2+} channels must be compensated by active Ca^{2+} extrusion, because $[Ca^{2+}]_i$ reached a steady state far below the electrochemical equilibrium. It appears unlikely that Ca^{2+} extrusion is significantly modulated by $[Ca^{2+}]_o$ (*see* Kratje, Garrahan & Rega, 1985; Wolosker & de Meis, 1994), because the recovery of a K^+-induced $[Ca^{2+}]_i$ increase, which essentially reflects Ca^{2+} extrusion, was unaffected by $[Ca^{2+}]_B$ (Fig. 1*A*). Furthermore, in contrast to the experimental results shown in Figs. 1 and 2, an increase in $[Ca^{2+}]_o$ should reduce Ca^{2+} extrusion and hence cause an increase in $[Ca^{2+}]_i$. Therefore, it is concluded that the changes in $[Ca^{2+}]_i$ upon varying $[Ca^{2+}]_B$ were mainly due to a modulation of Ca^{2+} influx through the voltage-dependent Ca^{2+} channels.

The $[Ca^{2+}]_o$ close to the cell bodies of the Retzius

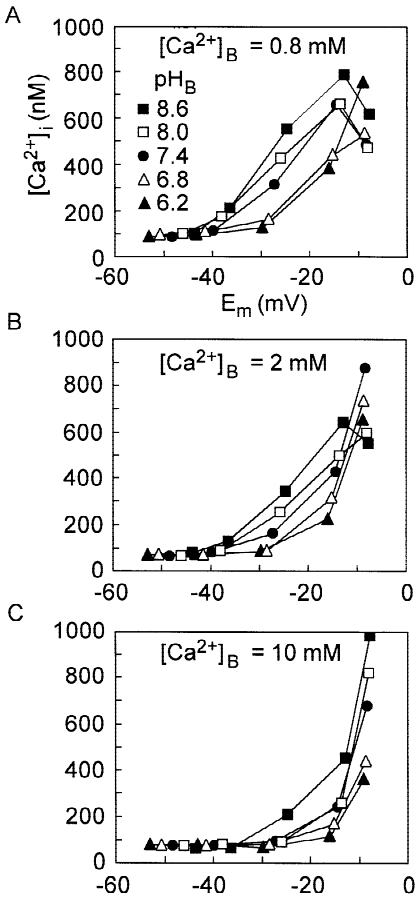

Fig. 5. Effect of the pH of the bathing solution on the $[Ca^{2+}]_i/E_m$ relationship at $[Ca^{2+}]_B$ = 0.8, 2, or 10 mM. Data from experiments performed as shown in Fig. 3, except that in addition to $[Ca^{2+}]_B$ the pH of the bathing solution was also changed before raising $[K^+]_B$. Data points are the mean of n = 5 to 7 experiments. SDs were similar in size to those shown in Fig. 4, but are omitted for reasons of clarity.

neurons appears to be not in equilibrium with $[Ca^{2+}]_B$ (*compare* Rose et al., 1995; Lohr et al., 1996). Although the absolute $[Ca^{2+}]_o$ values measured with the Ca^{2+}-sensitive microelectrodes should be regarded with caution, because the tissue might be severely disturbed by the rather large electrode tips, the data suggest that the shifts in $[Ca^{2+}]_o$ that caused the observed changes in $[Ca^{2+}]_i$ were substantially smaller than the experimentally performed shifts in $[Ca^{2+}]_B$. It is noted that a marked discrepancy between $[Ca^{2+}]_o$ and $[Ca^{2+}]_B$ was also found in the vertebrate retina (*see* Dmitriev, Pignatelli & Piccolino, 1999).

Possible Mechanism of Ca^{2+} Channel Modulation

An increase in $[Ca^{2+}]_o$ augments the electrochemical Ca^{2+} gradient and should, therefore, enhance the influx of Ca^{2+} and hence the K^+-induced $[Ca^{2+}]_i$ increase. However, under certain experimental conditions raising $[Ca^{2+}]_B$ caused a marked $[Ca^{2+}]_i$ decrease, and conversely, $[Ca^{2+}]_i$ increased upon reducing $[Ca^{2+}]_B$ (Figs. 1, 2). These effects may be mediated by shifts in the surface potential of the cell membrane (McLaughlin, 1977; 1989), which contributes to the electric field across the channels' voltage sensor and thereby affects its gating (*see* Hille, 1992). Since an increase in $[Ca^{2+}]_o$ 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 the activity of the Ca^{2+} channels will be smaller. The data in Fig. 4 are consistent with this view and suggest that the surface potential is significantly affected if $[Ca^{2+}]_B$ is $\geq$0.8 mM. It is noted that the same conclusions have been drawn from similar results obtained in isolated inner segments of salamander photoreceptors (Baldridge, Kurennyi & Barnes, 1998).

The effects of $[Ca^{2+}]_o$ on the surface potential and on the electrochemical Ca^{2+} gradient affect the Ca^{2+} influx in a counteracting way, which could explain why the K^+-induced $[Ca^{2+}]_i$ increase was maximal at intermediate $[Ca^{2+}]_B$ (Fig. 2). The latter result might also provide an explanation for the finding in snail neurons that an intracellular acidification, which was probably caused by Ca^{2+} entry, was maximum at intermediate $[Ca^{2+}]_B$ (Thomas, 1989). However, the observation that $[Ca^{2+}]_i$ was maximal at different $[Ca^{2+}]_B$, depending on the order in which the solutions were applied, is hard to explain. When $[Ca^{2+}]_B$ was changed in decreasing order, $[Ca^{2+}]_i$ reached a transient maximum upon reducing $[Ca^{2+}]_B$ from 1 to 0.3 mM, suggesting that $[Ca^{2+}]_o$ passed through an optimum during the solution exchange (Fig. 2*B*). This closely matches the data in Fig. 4, according to which $[Ca^{2+}]_i$ was maximal at $[Ca^{2+}]_B$ = 0.8 mM, if $[K^+]_B$ ranged in between 10 and 40 mM. The passing through a $[Ca^{2+}]_o$ optimum also explains why the $[Ca^{2+}]_i$ increase upon switching $[Ca^{2+}]_B$ from 10 to 0.1 mM in the presence of 30 mM K^+ was transient (Fig. 1*A*).

When changing $[Ca^{2+}]_B$ in increasing order, $[Ca^{2+}]_i$ did not reach its maximum upon changing $[Ca^{2+}]_B$ from 0.3 to 1 mM, but at higher concentrations (Fig. 2A). This result cannot be explained by differences in E_m, because the shifts in E_m were independent of the order of changing $[Ca^{2+}]_B$, and in both experimental protocols the change of $[Ca^{2+}]_B$ from 0.3 to 1 mM, or vice versa, was performed at the same time, 15 min after raising $[K^+]_B$. One may speculate that Ca^{2+} diffusion into the tissue was slowed down due to the period in 0.01 mM Ca^{2+} at the beginning of the experiment, which might have caused the emptying of extracellular high-affinity binding sites for Ca^{2+} that must be refilled during the subsequent periods of increased $[Ca^{2+}]_B$. A slowed Ca^{2+} diffusion would also explain why the $[Ca^{2+}]_i$ increase was both retarded and reduced in amplitude. On the other hand, slight changes in E_m may explain the different $[Ca^{2+}]_i$

levels at $[Ca^{2+}]_B$ = 10 mM in the presence of 30 mM K^+ (Figs. 1*A*, 2). Thus, E_m was around −22 mV within the first five minutes of the period in 30 mM K^+, which at $[Ca^{2+}]_B$ = 10 mM should have only a small effect on $[Ca^{2+}]_i$ (*see* Fig. 4B). However, the slight further depolarization that occurred upon prolonged exposure to 30 mM K^+ might be sufficient to induce a significant rise in $[Ca^{2+}]_i$ due to the steep relationship between $[Ca^{2+}]_i$ and E_m.

The modulations of Ca^{2+} influx by $[Ca^{2+}]_o$ could be reversed by changing the pH of the bathing solution, such that the effect of raising $[Ca^{2+}]_o$ was reduced by an extracellular alkalinization and that of decreasing $[Ca^{2+}]_o$ by an extracellular acidification (Fig. 5). This suggests that extracellular Ca^{2+} and H^+ ions compete for the same binding sites at the outer side of the plasma membrane, which is in line with results obtained in heart myocytes (Kwan & Kass, 1993). This view is supported by the result that the efficacy of the extracellular pH to modulate the Ca^{2+} influx was larger at low than at high $[Ca^{2+}]_o$.

The effect of $[Ca^{2+}]_o$ on the extracellular surface potential might explain why the threshold E_m for Ca^{2+} channel activation found in voltage-clamp experiments was more positive than that determined by monitoring $[Ca^{2+}]_i$ at different $[K^+]_o$. The voltage-clamp experiments were performed at raised $[Ca^{2+}]_o$ (Gottmann et al., 1989; Bookman & Liu, 1991), while the $[Ca^{2+}]_i$ measurements were done at normal $[Ca^{2+}]_o$ (Hochstrate et al., 1995; Dierkes et al., 1997).

Physiological Implications

The modulation of Ca^{2+} influx by extracellular Ca^{2+} may be functionally important, because relatively small changes in $[Ca^{2+}]_B$ close to its physiological value were sufficient to affect $[Ca^{2+}]_i$ significantly (Figs. 1, 2). Furthermore, as noted above, the changes in $[Ca^{2+}]_o$, which are relevant for the control of Ca^{2+} influx were probably smaller than those in $[Ca^{2+}]_B$. The results show that a drop in $[Ca^{2+}]_o$ following Ca^{2+} channel activation does not necessarily lead to a reduced net Ca^{2+} influx, because the attenuation of the electrochemical Ca^{2+} gradient might be compensated by an enhanced Ca^{2+} channel activity due to the negative shift in the surface potential at the outer side of the plasma membrane (*see* Dmitriev et al., 1999). It is questionable whether a significant depletion of extracellular Ca^{2+} and hence a modulation of Ca^{2+} channel activity occurs in response to single action potentials, but this may well take place during periods of enhanced action potential activity.

The changes in $[Ca^{2+}]_o$ caused by neuronal activity are difficult to estimate quantitatively. Theoretical considerations suggest a substantial decrease in $[Ca^{2+}]_o$ in mammalian neural tissue during normal activity (Egelman & Montague, 1999), in particular in the synaptic cleft (Vassilev et al., 1997), and there is also experimental support for such a Ca^{2+} depletion (Stanley, 2000; *but see* Borst & Sakmann, 1999). We found no change in $[Ca^{2+}]_o$ close to cell bodies of the Retzius neurons during a K^+-induced Ca^{2+} influx, and only a small $[Ca^{2+}]_o$ decrease was measured in the leech neuropile upon electrical stimulation (Rose et al., 1995). However, marked drops in $[Ca^{2+}]_o$ were observed in the leech neuropile upon application of excitatory neurotransmitters or after raising $[K^+]_B$ (Lohr et al., 1996). These changes in $[Ca^{2+}]_o$ are sufficiently large to contribute significantly to the control of Ca^{2+} influx during neuronal activity.

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