Mn Ions Pass through Calcium Channels

A Possible Explanation

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ABSTRACT The divalent transition-metal cations Fe, Co, and Ni were used to test the hypothesis that Mn ions pass through calcium channels because Mn ions have a relatively low energy of hydration. The test ions were applied to the bath and comparisons were made of their effects on Ca or Mn spikes elicited from myoepithelial cells of the proventriculus of the polychaete worm *Syllis spongiphila*. Control experiments showed that (*a*) results obtained using deoxygenated solutions (required to stabilize Fe^{2+} ions) could be compared with those using solutions containing oxygen, and (*b*) the test cations did not measurably affect the electrical coupling between cells. Ca spikes were reversibly abolished by the test cations in the order of effectiveness: Fe (16.1 mM ± 1.0, SE; $n = 15$) = Co (14.6 mM ± 0.8; $n = 27$) < Ni (8.3 mM ± 0.7; $n = 16$). The test cations diminished Mn spikes by decreasing maximum rates of rise (Fe = Co < Ni) and overshoot amplitudes (Fe < Co < Ni). The test cations also increased the current intensity required for Ca (Fe = Co < Ni) or Mn spike initiation (Fe < Co < Ni). Since the energies of hydration of Fe, Co, and Ni increase stepwise from that of Mn, and the effectiveness of these ions in diminishing Ca and Mn spikes increased in the order Fe ≤ Co < Ni, these data support the hypothesis that Mn ions pass through Ca channels because they shed waters of hydration relatively easily. An additional observation was that, at below-blocking concentrations, the test cations caused decreased duration of Mn spikes and increased duration of Ca spikes.

INTRODUCTION

The myoepithelial cells that make up the proventriculus of the marine polychaete worm *Syllis spongiphila* generate regenerative, overshooting action potentials of several hundred milliseconds duration; these spikes appear to result from an influx of calcium ions (Anderson, 1979*a*). As in other Ca-spiking cells, spikes can be maintained in Ca-free solutions containing Sr^{2+} or Ba^{2+}; in addition, the myoepithelial cells also generate spikes in Ca-free solutions containing Mn^{2+}, and several electrophysiological and pharmacological results (Anderson, 1979*a*) strongly suggest that Mn^{2+} ions specifically pass through the calcium channels.

Mn^{2+} ions are reported to pass through the calcium channels of other preparations. Ochi (1970, 1975) reported a slow inward current that was Mn dependent and tetrodotoxin (TTX) resistant in guinea pig papillary muscle.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/83/06/0805/23 $1.00 805
Volume 81 June 1983 805–827

Fukuda and Kawa (1977) showed that Mn^{2+} as well as Be^{2+}, Sr^{2+}, Ba^{2+}, Zn^{2+}, or Cd^{2+} could replace Ca^{2+} in the generation of spikes by muscle fibers of larval beetles. Hagiwara and Miyazaki (1977) showed a small inward Mn^{2+} current in voltage-clamped starfish egg cells, and Keatinge (1978) reported that slow discharges could be elicited from smooth muscle cells of sheep carotid arteries by high concentrations of potassium in Ca- and Na-free solutions containing 5 mM Mn or Mg.

It is of interest to determine the characteristic(s) of Mn^{2+} ions that permits them to pass through certain types of calcium channels, since such information may provide further understanding of the general nature of calcium channels. A preparation that exhibits calcium channels that are permeable to Mn^{2+} is useful because it permits comparison of the actions of Ca ions both with those of cations in the same (alkali-metal) group as Ca and with those of (transition-metal) cations of the same period. The myoepithelial cell preparation of *Syllis spongiphila* seems especially well suited for such a study, because except for its permeability to Mn^{2+}, it appears to exhibit characteristics similar to those of other preparations that generate calcium spikes (see Hagiwara, 1973, 1975; Hagiwara and Byerly, 1981; Reuter, 1973, for reviews).

Of the divalent, first-row, transition-series metals, Mn^{2+} ions exhibit the lowest energy of hydration (Noyes, 1962). A simple explanation of the ability of Mn^{2+} ions to pass through calcium channels is that they can relatively easily substitute sufficient waters of hydration with ligands of the channel to permit them to pass through the channel. On the basis of this hypothesis, similar transition-metal divalent cations with higher energies of hydration should block the calcium channel with an effectiveness that would increase as the energy of hydration increases. Thus, the predicted sequence of effectiveness of blocking the channel by the ions used in this study would be $Fe < Co < Ni$. The experiments described in this paper used the *Syllis* myoepithelial cell preparation to test the relative effects of these three ions on regenerative events elicited by directly applied intracellular stimuli. Fe, Co, and Ni were chosen because they are divalent; like Mn, they form octahedral aqueous complexes (Burgess, 1978); their energies of hydration increase stepwise from that of Mn (Noyes, 1962); and, finally, there are relatively small, definable chemical differences among these cations, compared with the large differences between each and the members of its vertical subgroup (Sienko and Plane, 1974). The effectiveness of the non-transition-metal cations Cd^{2+} and Zn^{2+} in blocking Ca spikes was also briefly examined because they are reported to pass through a calcium channel (Fukuda and Kawa, 1977). The results obtained using Fe, Co, and Ni support the hypothesis that the energy of hydration of Mn^{2+} ions is sufficiently low to permit their passage through the syllid calcium channel. Abstracts of some of the results presented here have been published previously (Anderson, 1979*b*, 1980).

MATERIALS AND METHODS

Specimens of *Syllis spongiphila* were collected in the harbor of San Juan, Puerto Rico, and mailed to Massachusetts, where they were maintained in natural seawater for periods of up to 2 mo.

For experiments in which the effects of the applied cations were tested on Ca spikes, an artificial seawater (ASW) was used with the following ionic composition (mM): 457 Na; 9.7 K; 10 Ca; 52.5 Mg; 534 Cl; 2.5 HCO_3; 27.7 SO_4 (Welsh et al., 1968). For experiments in which the effects of the cations were tested on Mn spikes, $CaCl_2$ was omitted from the ASW and 20 mM $MnCl_2$ was added. The test cations were added to the ASW in concentrations ranging from 1 to 25 mM (all as chlorides except Fe^{2+}, which was added as sulfate). The slight increase in tonicity produced by the added ions was considered acceptable because results obtained from earlier experiments using $MnCl_2$ added to ASW up to a concentration of 50 mM did not differ in any obvious way from results obtained using $MnCl_2$ in solutions in which the ionic strength was kept constant.

All solutions containing Fe^{2+} were prepared using distilled water or ASW that was deoxygenated by bubbling with 100% nitrogen through a glass dispersion tube for at least 15 min. All Fe-containing solutions were bubbled with nitrogen before use, manipulations of deoxygenated solutions were performed under nitrogen, syringes were used to transfer deoxygenated solutions through environments containing oxygen, and, in most experiments, nitrogen was blown over the experimental chamber when deoxygenated solutions were applied to the preparation. (Solutions that were not bubbled with nitrogen are referred to as "nondeoxygenated.")

The pH of all solutions was usually adjusted to 7.5–7.7 with dilute HCl. In some experiments, the deoxygenated ASW (without added cations) was used at pH 8, the value that resulted from bubbling with 100% nitrogen, without subsequent adjustment of the pH.

The cuticular body wall in the region of the proventriculus was removed and the posterior portion of the animal was cut off. The preparation was pinned with two stainless-steel insect pins to the bottom of a Silgaard-lined plexiglass chamber of 0.35 ml capacity; the pins pierced the proboscis and the intestine near the anterior and posterior margins of the proventriculus (see Haswell, 1890; Smith et al., 1973; Anderson and del Castillo, 1976, for detailed descriptions of the proventriculus and the preparation).

Glass 3 M KCl-filled microelectrodes of 5–15 MΩ resistance were used according to standard methods to record from and apply current pulses to the myoepithelial cells. Because the cells are electrically coupled (Anderson and del Castillo, 1976), the stimulating electrode was placed either in the same cell as the recording electrode or in an adjacent cell; in the latter case, the electrodes were separated by a distance that did not exceed 10% of the length constant of the proventriculus (Anderson and del Castillo, 1976). The trends of the results were consistent from one preparation to the next, regardless of whether both electrodes were placed in the same cell or in adjacent cells. The current applied was monitored by recording the voltage drop across a 10-kΩ resistor between the calomel cell (connected to the bath by a tube of agar gel made with Ca-free ASW) and ground. Because of the extensive electrical coupling between cells, large current pulses of 0.5–4.0 μA and 50–200 ms duration were required to elicit regenerative responses.

Bathing solutions were changed by drawing off the original solution and washing the chamber thoroughly (at least five times) with the new solution. Solutions containing increasing concentrations of a given test cation were applied successively. The characteristics of responses elicited 1 min after the application of a test solution were usually similar to those elicited after periods of up to 10 min. However, to control for possible changes over time in a given solution, all three cations were applied in the same pattern in any one experiment. In most experiments, successive test solutions were applied for periods of 3.5–5 min each in order of increasing concentration, and

stimuli were applied to the preparation at 1-min intervals. Between the applications of series of test solutions, the preparation was bathed in and washed frequently with control ASW for periods of at least 10 min. All experiments were performed at room temperature.

Examples of records are shown in Figs. 1, 3, and 4. Throughout any one set of tests using a series of different test cations, the positions of the stimulating and recording electrodes were kept constant. All records show superimposed traces (current pulses on bottom traces and voltage responses on top traces). For each test, square, depolarizing current pulses of a given, constant duration were applied successively at increasing intensities until a regenerative event was or was not elicited. Thus, each record shows a series of consequent subthreshold responses that ended either with a regenerative event in below-blocking concentrations of the test ions or with no such event in blocking concentrations. In some records, the peaks of the larger current pulses are interposed between the smaller electrotonic responses. Projected images of all records were measured on the grid of a digitizing tablet (HIPAD; Houston Instrument, Austin, TX) that had a resolution, for these measurements, of 0.4×10^{-3} V and 0.2×10^{-7} A on the y axis and $4–10 \times 10^{-3}$ s (depending on the sweep speeds used) on the x axis. Points from the grid of the tablet were interfaced to a microcomputer programmed to record the amplitudes and durations of current pulses and spikes; the rate of rise of a given spike was determined from points taken from the steepest slope of the rising phase.

RESULTS

Controls

To prevent their oxidation, Fe^{2+} ions were applied to the preparation in deoxygenated solutions. Two types of control experiments were performed to test the effects of deoxygenated solutions. First, proventriculi were bathed in deoxygenated ASW at pH 7.6 or 8.1 for up to 1 h; during this time, changes were seen neither in the resting potentials of the myoepithelial cells nor in the characteristics of the regenerative events elicited by directly applied intracellular stimuli. Thus, it was concluded that deoxygenated solutions do not exert adverse effects. Second, the effects of Co^{2+} ions on Ca spikes and Mn spikes were tested in deoxygenated solutions as well as in the usual nondeoxygenated solutions. Fig. 1 shows intracellular recordings from a preparation bathed in Ca-containing ASW to which $CoCl_2$ was added in increasing concentrations. All of the records in this figure were taken from one cell and show multiple sweeps resulting from the successive application of current pulses of constant duration and increasing intensity. The figure illustrates the effects on Ca spikes of increasing concentrations of $CoCl_2$ applied first in solutions containing oxygen, then in deoxygenated solutions, and again in solutions containing oxygen. In all three series, the regenerative Ca spike was reversibly abolished at a concentration of 15–20 mM $CoCl_2$. The overshooting spikes of several hundred milliseconds duration elicited in both deoxygenated and nondeoxygenated control solutions are typical of those recorded intracellularly from the myoepithelial cells of the proventriculus. In other experiments, Mn spikes were also reversibly abolished by Co^{2+} at similar concentrations in both deoxygenated and nondeoxygenated solutions. These results indicate that the

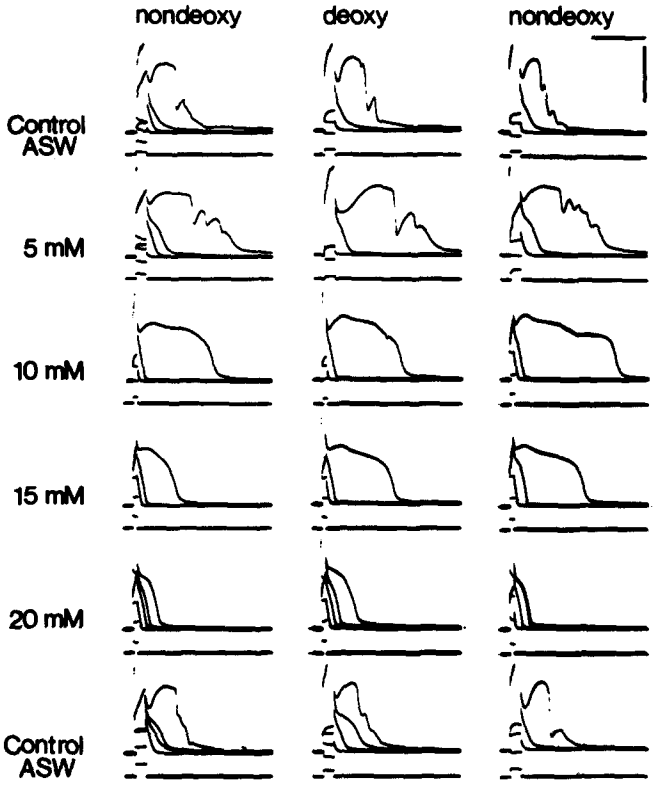


FIGURE 1. Increasing $[Co]_o$ affects directly elicited spikes similarly in both nondeoxygenated and deoxygenated solutions. Intracellularly recorded responses (top traces) were elicited by directly applied current pulses (bottom traces). Three series of solutions containing increasing $[Co]_o$ (5, 10, 15, and 20 mM) were applied to the preparation. The series of nondeoxygenated solutions were applied before (left column) and after (right column) the deoxygenated series (middle column). Each test solution was applied for a period of 2.5 min and the record shown was taken 2 min after application. Control solutions (top and bottom records of each column) were applied for at least 15 min between the series of test solutions. The resting potential in nondeoxy control ASW upon removal of the recording electrode after the final nondeoxy series was −63 mV. The positions of the two oscilloscope traces were not changed during any one experiment; thus, changes in resting potential can be noted from the relative positions of the voltage and current traces. In some records, small electrotonic responses occurred within the envelope occupied by the current pulses. In the top left record (control ASW, nondeoxy), an electrotonic response (to the smallest current pulse) can be seen between the third and fourth current pulses; the record is comprised of four superimposed sweeps. To ensure that blockade was achieved in a test solution, current pulses larger than those required to elicit a response in the immediately preceding test solution were applied. For example, in 15 mM Co, nondeoxy solution (left), the maximum current pulse applied was 3.34×10^{-6} A, whereas that in 20 mM Co, nondeoxy solution (left) was 5.7×10^{-6} A. Calibrations: vertical, 80 mV and 4×10^{-6} A; horizontal, 800 ms for control ASW and 5 mM $CoCl_2$; 2 s for 10, 15, and 20 mM $CoCl_2$.

effects of competing cations applied in deoxygenated solutions can be compared with the effects of competing cations applied in solutions containing oxygen.

Further control experiments were performed to test for possible effects of the applied test cations on the electrical coupling between cells. In both control ASW and Ca-free, Mn-containing solutions, hyperpolarizing current pulses of 500–800 ms duration and increasing intensities were applied to a cell with a current-passing microelectrode; voltage responses were recorded simultaneously with a recording microelectrode in the same cell (R1) and with a second recording electrode (R2) in a cell 4–10 rows of cells distant from the stimulated cell. In the presence of Ni^{2+} or Co^{2+}, the slopes of the current-voltage (*I-V*) relationships of both recording sites reversibly increased, relative to controls, to about the same degree. Fig. 2 illustrates one of the largest increases of slopes observed in all tests made using eight animals. To compare the sets of experimentally obtained points, the 95% confidence intervals for the regression coefficients (slopes) were computed for each recording site in the control solutions (both pre-test and post-test) and in the test solutions. The ratio of the slopes (R2/R1) and the 95% confidence intervals for the ratios were then found for test and control conditions. The 95% confidence intervals of the ratios obtained from control solutions overlapped those of ratios obtained from test solutions. These results indicate that the ratios of the slopes of the two recording sites were not significantly different from each other even when the values of the slopes themselves changed; thus, it can be concluded that the coupling coefficient ($VR2/VR1$; Bennett, 1966) was not significantly changed in the presence of the test cation. Had the coupling decreased, the slope for R1 would have increased and that for R2 decreased; had the coupling increased, the converse would have occurred. On the basis of this analysis, it would appear that the increase in slope at both R1 and R2 can be accounted for by the test cations blocking channels in the plasma membrane. It is possible that blockade of channels by the test cations obscured an additional effect of increased coupling; however, if this were the case, the slope of R2 would have appeared to increase more than that of R1. Thus, these results strongly suggest that the applied cations used in the following experiments did not significantly affect electrical coupling.

Reversible Abolition of Ca Spikes

All three first-order, transition-metal test cations reversibly blocked the regenerative events elicited by directly applied intracellular pulse stimuli. Fig. 3 shows records of Ca spikes recorded from a single cell in one experiment in which four series of test solutions were applied in the order shown from left to right. At concentrations below that required to abolish the Ca spike, the presence of each test cation was associated with a reversible prolongation of the spike. Partial blockade was associated with a sloping of the plateau of the spike, and complete blockade with a rapid fall of the voltage response to the resting level. In this experiment, Co^{2+} blocked the regenerative spike at a concentration of 15 mM and Ni^{2+} at 10 mM; Co^{2+} applied a second time blocked the spike somewhat at 15 mM and completely at 20 mM; Fe^{2+},

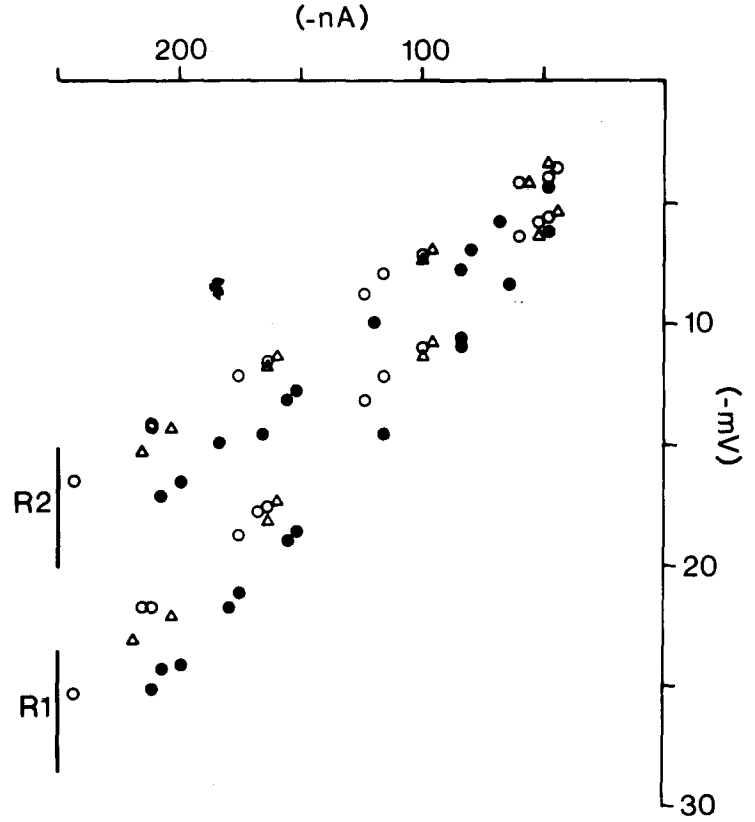


FIGURE 2. Ni^{2+} ions reversibly increase the slope of the current-voltage relationship recorded at two sites along the proventriculus. Square hyperpolarizing current pulses of 800 ms duration and increasing intensities were applied with a microelectrode. The voltage responses were recorded simultaneously with microelectrodes at two different positions. One electrode (R1) was placed in the same cell as the current passing electrode; the other (R2) was placed in a cell six rows of cells distant from R1 (~150 μm) along the long axis of the proventriculus. Closed symbols represent responses recorded in ASW containing 10 mM $NiCl_2$ (5 min exposure) and open symbols responses in control ASW (circles: before application of Ni^{2+} ions; triangles: 4–6 min after return to control ASW). In this experiment, the ratio of the slopes at R2 and R1 in control ASW preceding the test solution was 0.6481 (95% confidence interval [CI], 0.6100–0.6885), that in the presence of 10 mM Ni^{2+} ions was 0.6947 (95% CI, 0.6561–0.7352), and that upon return to control ASW was 0.6832 (95% CI, 0.6385–0.7315). Because the 95% confidence intervals overlap, it was concluded that the electrical coupling between cells was not significantly affected by the Ni^{2+} ions. See text for details.

applied in deoxygenated solutions, blocked the Ca spike at 20 mM, but to a somewhat lesser extent than did Co^{2+} at this concentration. The relative effectiveness of the three cations in reversibly abolishing the Ca spike was Fe $\leq$ Co < Ni.

The results obtained from 15 experiments similar to that illustrated in Fig. 3 showed that Ni^{2+} consistently abolished the Ca spike at lower concentrations than did Co^{2+} or Fe^{2+}. In 8 of the 15 experiments, the mean $[Co]_o$ required to abolish the spike was greater than the $[Fe]_o$; in 4 experiments Co^{2+} and Fe^{2+} were equally effective and in 3 experiments, Fe^{2+} abolished the spike at a lower concentration than did Co^{2+}. The mean blocking concentration for each

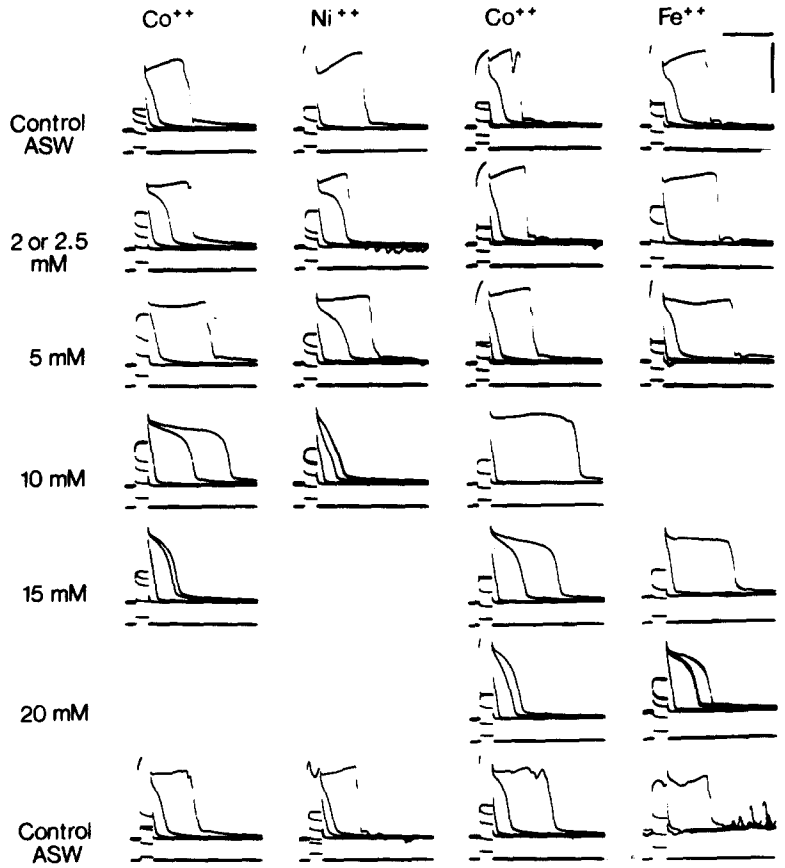


FIGURE 3. Ni^{2+}, Co^{2+}, and Fe^{2+} ions reversibly abolish directly elicited Ca spikes (top traces) at different concentrations. The three cations were tested in the order shown from left to right. Each ion was applied in increasing concentrations (at the lowest test concentration, Ni and Co were used at 2.0 and Fe at 2.5 mM); each test solution was applied for a period of 1.5 min, and the record shown was taken 1 min after application. Ni^{2+} ions reversibly blocked the Ca spike at a concentration of 10 mM, Co^{2+} ions at 15 (first test) and 15–20 mM (second test), and Fe^{2+} ions at 20 mM. Control solutions were applied for periods of 10 min or longer between series of test solutions. The resting potential in control ASW upon removal of the recording electrode after the Fe^{2+} series was −61 mV. Calibrations: vertical, 80 mV (top traces) and 4×10^{-6} A (bottom traces); horizontal, 800 ms.

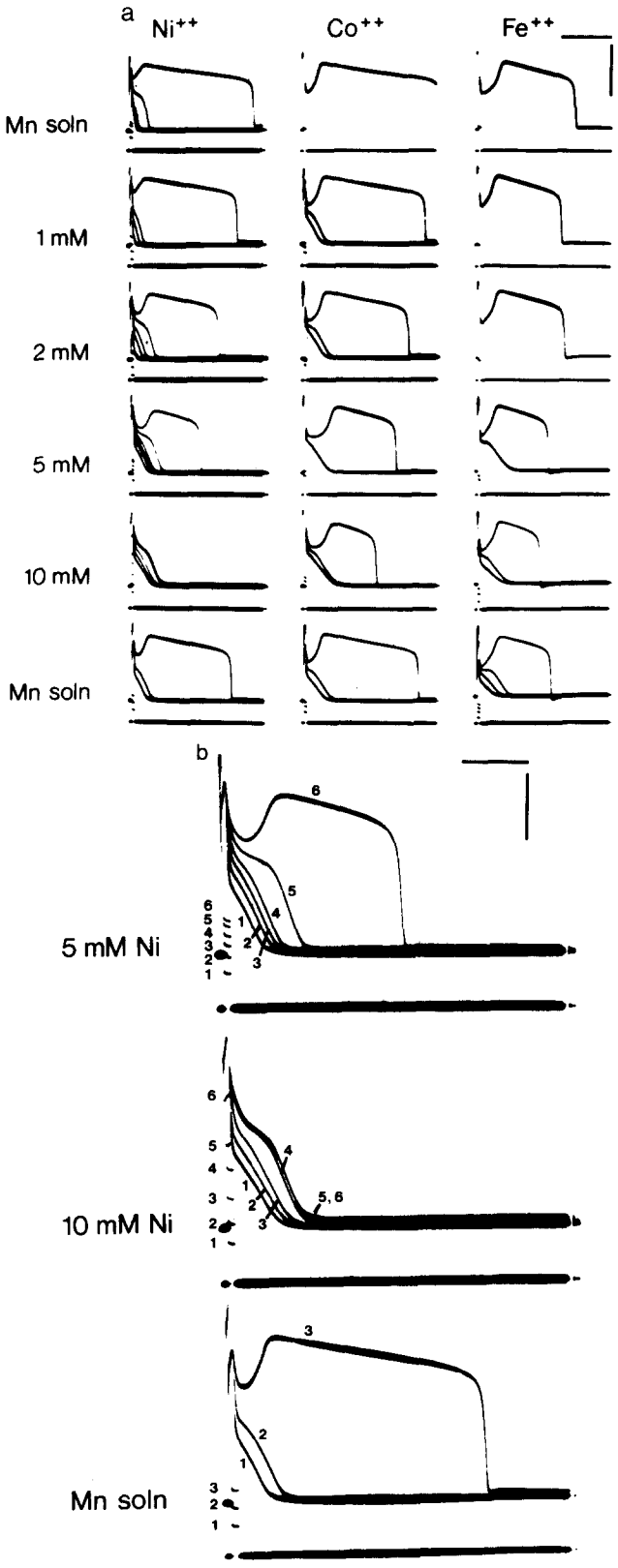
of the three cations tested in the 15 preparations was Ni, 8.3 mM ± 0.7 (SE; $n = 16$); Co, 14.6 mM ± 0.8 ($n = 27$); and Fe, 16.1 mM ± 1.0 ($n = 15$).

In the three experiments in which the Ca spike was abolished most effectively in Fe-containing solutions, there was an accompanying decline in the resting potential, and recovery upon return to Fe-free solution was often incomplete. These effects may have resulted from contamination of the Fe-containing solutions by oxygen. The Fe^{3+} ions resulting from oxidation, although they occurred in low concentration in solution (Peters et al., 1974), were associated with a significant decrease in pH (presumably due to hydrolysis; see Huheey, 1978, for hydrolysis constants), which could have been responsible for the adverse effects observed.

Maximum Rates of Rise

If the competing cations abolish the spike by blocking the influx of Ca^{2+} through the Ca channel, then below-blocking concentrations of these cations should slow the influx of Ca^{2+} and, therefore, slow the maximum rate of rise (MRR) of the spike. As can be seen in Figs. 1 and 3, the rising phases of the Ca spikes are irregular; thus, it was seldom possible to compare the MRRs of Ca spikes recorded in a series of different test solutions. For this reason, the effects of the applied transition-metal cations were tested on Mn spikes. Although Mn^{2+} ions appear to replace Ca^{2+} ions in generating spikes, they do not replace Ca^{2+} ions in initiating contraction, and cells undergoing Mn spikes are quiescent (Anderson, 1979*a*). Although some inconsistencies in the rising phases of Mn spikes are apparent, it was nevertheless possible to compare the means of the MRRs of several spikes recorded in each of a series of test solutions. Fig. 4 illustrates spikes recorded from a single cell of a preparation bathed in Ca-free solution containing 20 mM Mn. Series of solutions containing Ni^{2+}, Co^{2+}, or Fe^{2+} were applied (in the order shown from left to right) in increasing concentrations from 1 to 10 mM. In this experiment, Ni^{2+} reversibly abolished the Mn spike at a concentration of 10 mM. This concentration is in the same range of concentrations in which Ni^{2+} was effective in abolishing the Ca^{2+} spike. Co^{2+} and Fe^{2+} applied at 10 mM were not present in sufficient concentration to abolish the Mn spike; higher concentrations of these ions were also usually required to abolish the Ca spike.

The mean MRR of spikes recorded in a given test solution was compared with the mean MRR of spikes recorded in the immediately preceding (usually) or following (occasionally) Mn solution. The effects of the three competing cations on the MRRs from a single preparation (bottom left) and from five different preparations (bottom right) are plotted in Fig. 5. In both plots, Ni^{2+} ions were most effective in decreasing the MRR. Both Co^{2+} and Fe^{2+} caused a decrease in the MRR at a concentration of 10 mM; although Co^{2+} ions were more effective than Fe^{2+} ions in this single preparation, the pooled data do not distinguish between the effects of these two ions. The top graph shows the relative increase in MRR of spikes recorded in Ca-free solutions containing concentrations of Mn^{2+} from 10 to 50 mM. The data shown in this graph were obtained from the same preparation as the data shown in the lower left


a
Ni++
Co++
Fe++
Mn soln
1 mM
2 mM
5 mM
10 mM
Mn soln
b
5 mM Ni
10 mM Ni
Mn soln

graph. Thus, the MRRs are increased as the $[Mn]_o$ is increased and are decreased at a constant $[Mn]_o$ in the presence of competing cations, with a relative effectiveness of the order Fe = Co < Ni (pooled data).

The top graph of Fig. 5 also illustrates that lower-intensity pulses were required to elicit responses as the $[Mn]_o$ was increased. This observation is of interest in view of the stabilizing function exerted by Ca^{2+} in many preparations (Frankenhaeuser and Hodgkin, 1957; Hille et al., 1975). If Mn^{2+} ions competed with but could not replace the stabilizing Ca^{2+} ions, the expected result would be increased excitability.

Overshoots

The amplitudes of overshoots of spikes elicited in Ca-free solutions containing Mn^{2+} increase ~27 mV with a 10-fold increase in $[Mn]_o$ (Anderson, 1979*a*). This value approaches that predicted by the Nernst equation for a membrane permeable to a divalent cation. Although overshoot amplitude increases as the $[Ca]_o$ is increased, consistent values have not been obtained because the preparation contracts more vigorously in solutions containing higher-than-control concentrations of Ca^{2+}, and electrode placement is difficult to maintain (Anderson, 1979*a*). In the present experiments, the overshoots of the Ca spikes were variable in amplitude, and consistent trends in the effects of the applied cations were not discernible. However, for Mn spikes, the test cations clearly caused a reversible decrease in the amplitudes of the overshoots. Fig. 6 shows the relative amplitudes of overshoots of Mn spikes recorded in five different preparations plotted against the concentrations of the applied cations. The relative effectiveness of the three cations in decreasing the overshoot amplitudes of Mn spikes and the three slopes determined from pooled data are Fe (−0.37) < Co (−3.2) < Ni (−6.3). The points for low concentrations of Co^{2+} and Ni^{2+} fall above unity on the *y* axis, which suggests that Ni and Co may exert a potentiating effect on overshoot amplitude at low concentrations and a suppressing effect at higher concentrations; however, further experiments are required to clarify this result.

FIGURE 4. *(opposite)* (*a*) Ni^{2+}, Co^{2+}, and Fe^{2+} ions reversibly diminish directly elicited Mn spikes (top traces). The series of solutions for each test cation were applied in the order shown from left to right. Each ion was applied in increasing concentrations; each test solution was applied for a period of 3.5 min and the record shown was taken 2 min after application. Control Mn solutions (Ca-free ASW containing 20 mM Mn) were applied for periods of ≥20 min between series of test solutions. The resting potential in Mn solution upon removal of the recording electrode after the Fe^{2+} series was −67 mV. Calibrations: vertical, 80 mV (top traces) and 4×10^{-6} A (bottom traces); horizontal, 2 s. (*b*) Enlargements of the last three records of the Ni^{2+} test series (5 and 10 mM Ni and Mn solution). The successive current and voltage traces are numbered in each record. In the record of 10 mM Ni, the responses to the fifth and sixth current pulses are superimposed. Calibrations: vertical, 40 mV and 2×10^{-6} A; horizontal, 1 s.

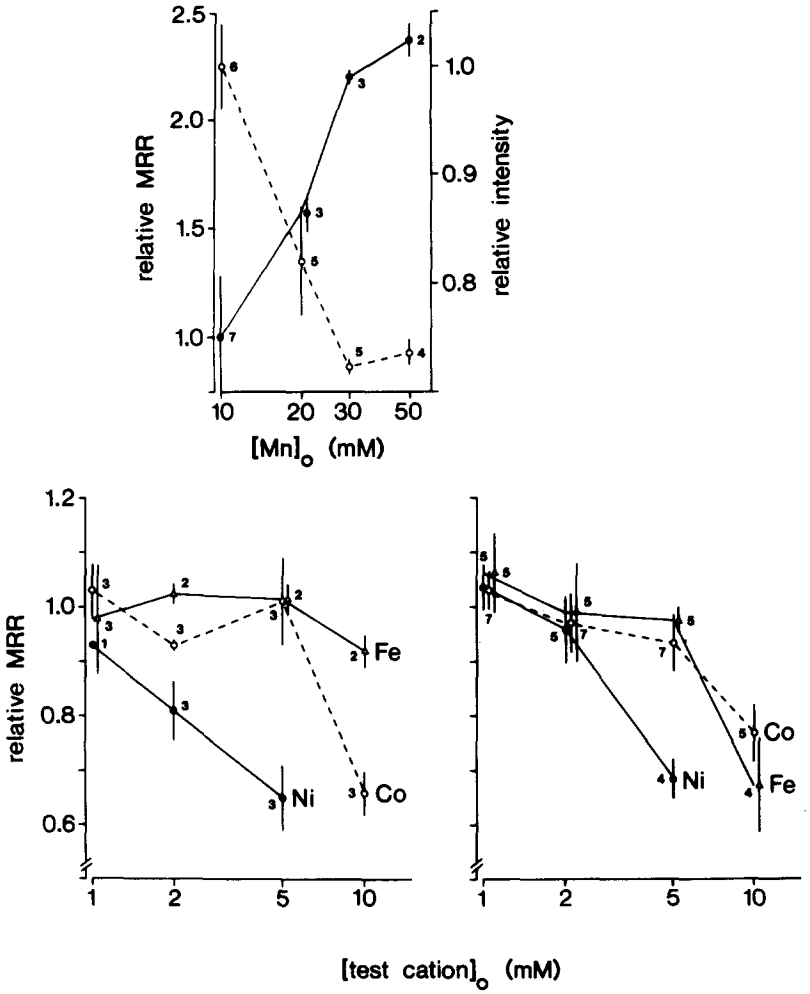


FIGURE 5. Relative effects of Ni^{2+}, Co^{2+}, and Fe^{2+} ions on the maximum rates of rise (MRR) of Mn spikes. Top graph: the mean relative MRR (solid line) and the mean relative stimulus intensity required to elicit a regenerative event (interrupted line) are plotted against the $[Mn]_o$. The data (mean ±1 SD) are from the same experiment as that shown in the bottom left graph. The values plotted are relative to those obtained in Ca-free solutions containing 10 mM Mn. Bottom graphs: the values of mean relative MRR of spikes elicited in test solutions are plotted against the concentration of the competing cation. The results of one experiment are shown on the left (mean ±1 SD) and the pooled results from five different animals on the right (mean ±1 SEM). Except for Ni in the left graph, the values plotted are relative to those obtained in the control Mn solution immediately preceding each test series; in the case of Ni, the values are relative to those obtained in the control Mn solution immediately following the test series. In all graphs, the number near each point indicates the number of observations made. Overlapping symbols were spread laterally for clarity.

Current Intensities

With increasing concentrations of the applied test cations, current pulses of greater intensity were required to elicit regenerative responses. This result is shown in Fig. 7 for Ca spikes (top) and Mn spikes (bottom). At any given concentration, current pulses of greater amplitude were required to elicit

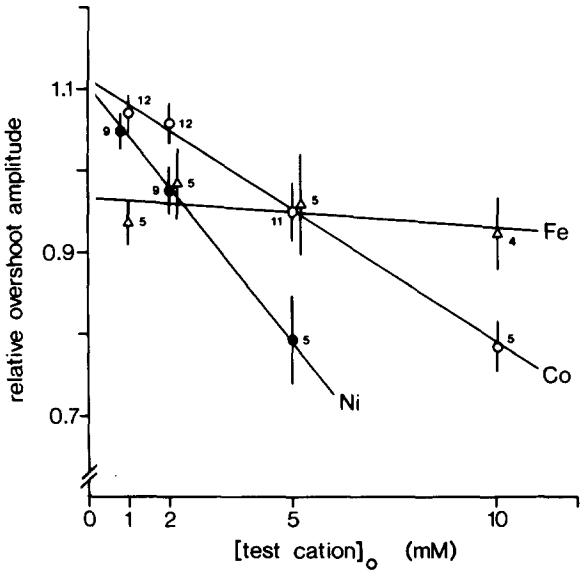


FIGURE 6. Relative effects of Ni^{2+}, Co^{2+}, and Fe^{2+} ions on overshoot amplitude of directly elicited Mn spikes. The mean relative overshoot amplitudes of spikes elicited in test solutions are plotted against the concentrations of the applied competing cations. The points represent values relative to those obtained in the control solution immediately preceding a given test series. The bars indicate standard errors of the mean; the number near each point indicates the number of observations made under a given experimental condition. Overlapping symbols were spread laterally for clarity. Values were obtained from seven animals for Ni and Co and five animals for Fe. The lines were fitted by linear regression analysis.

spikes in the presence of Ni than in the presence of Fe or Co; although the effects on Ca spikes of Fe^{2+} and Co^{2+} were not distinguishable, Co^{2+} ions were more effective than Fe^{2+} ions in suppressing Mn spikes. The slopes of the regression lines determined from pooled measurements of Mn spikes were Fe (−2.32) < Co (+5.5) < Ni (+14.4).

Durations

The Ca spikes in Fig. 2 and the Mn spikes in Fig. 3 are of distinctly different durations. Previous studies showed that whereas Ca spikes are usually a few hundred milliseconds in duration, Mn spikes are consistently longer (Ander-

son, 1979*a*). The transition-metal test cations affected the durations of the two types of spike in opposite ways; in increasing concentrations, they caused a reversible lengthening of the Ca spikes and a reversible shortening of the Mn spikes. This result is shown in Fig. 8; the relative durations of Ca spikes (closed

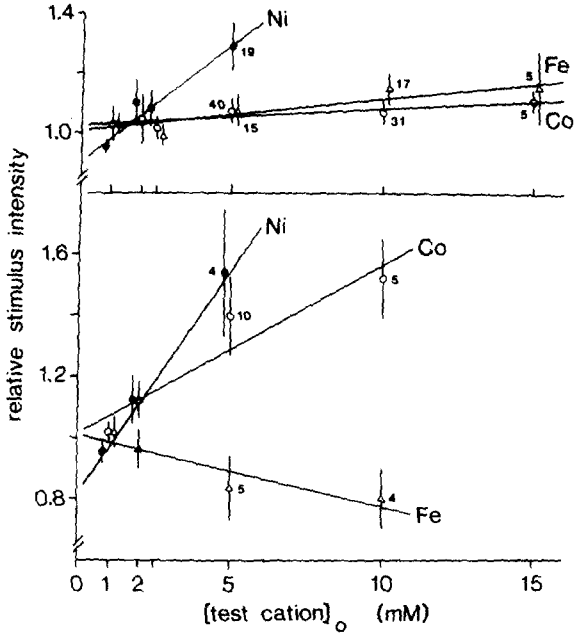


FIGURE 7. Relative effects of Ni^{2+}, Co^{2+}, and Fe^{2+} ions on the intensity of stimulus pulses required to elicit regenerative events. Successive pulse stimuli of constant duration were applied in increasing intensities until a regenerative Ca spike (top graph) or Mn spike (bottom graph) was elicited. The mean relative intensities required to elicit suprathreshold events are plotted against the concentrations of the test cations. The points (which were spread laterally for clarity) represent values relative to those obtained in the immediately preceding control solution. The numbers near each point for concentrations of 5–20 mM represent the number of observations made; comparable numbers of observations were made at lower concentrations. Ca spikes: data from 20 animals are plotted for Ni and Fe and from 22 animals for Co; Mn spikes: data from seven animals are plotted for Ni, eight for Co, and five for Fe. The lines were fitted by linear regression analysis; bars represent standard errors of the mean.

symbols) and Mn spikes (open symbols) are plotted against the concentrations of the competing cations. As they are plotted here, the pooled data do not reveal any obvious difference among the three cations in affecting the durations of Ca spikes. The small SE bars suggest a possible difference among the three cations in affecting the durations of Mn spikes. The slopes from pooled data indicate a relative effectiveness in the order Fe (−4.26) < Co (−5.01) < Ni (−7.74).

Cd^{2+} and Zn^{2+} Ions

The effects on Ca spikes of series of increasing concentrations of Zn^{2+} ions were tested in a total of 10 trials, using 10 animals, at concentrations of 0.01, 0.1, 0.2, 0.5, and 1.0 mM. The Zn^{2+} ions caused reversible abolition of Ca

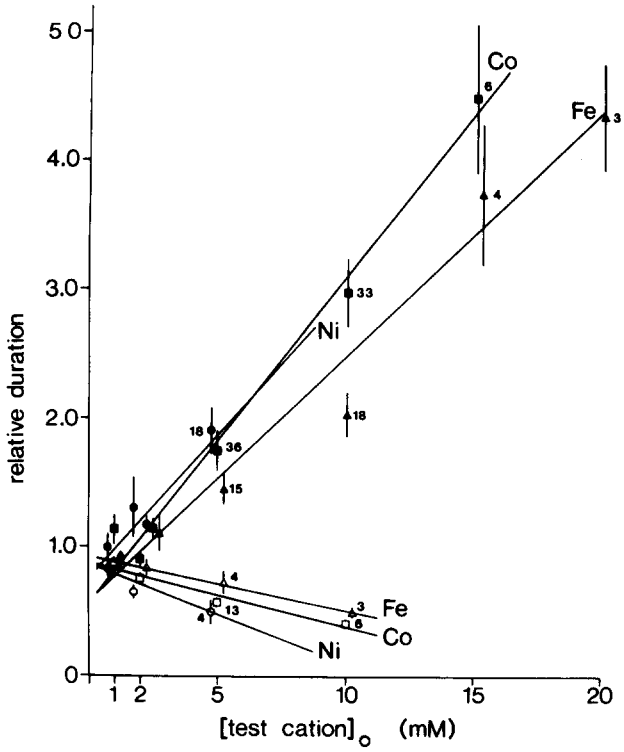


FIGURE 8. Relative effects of Ni^{2+}, Co^{2+}, and Fe^{2+} ions on durations of directly elicited Ca spikes (closed symbols) and Mn spikes (open symbols). The mean relative durations of spikes elicited in test solutions are plotted against the concentrations of the applied test cations. Points represent the means of at least three observations and indicate values relative to those obtained in the control solution immediately preceding a test series. Overlapping data for a given concentration were spread laterally. The numbers near each point for concentrations of 5–20 mM indicate the actual number of observations; comparable numbers of observations were made at 1 and 2 mM. Bars represent standard errors of the mean. Ca spikes: results from 20 animals are plotted for Ni, 22 for Co, and 20 for Fe. Mn spikes: results from seven animals are plotted for Ni, eight for Co, and four for Fe. All lines were fitted by linear regression analysis.

spikes at a concentration of either 0.5 mM (n = 7) or 1.0 mM (n = 3). These definitive results are different from previously published (Anderson, 1979*a*) observations of irreversible abolition. Cd^{2+} ions, applied in five test series on three animals at concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 mM, caused reversible abolition of Ca spikes at 10 (n = 4) or 20 mM (n = 1). The

fact that Cd^{2+} ions form complexes with chloride ions (Sillén and Martel, 1971; Edwards, 1982) must be taken into account in the interpretation of these results (see below). Prior to blockade, increasing concentrations of both cations were associated with decreasing amplitudes of the overshoots of the spikes. Whereas increasing concentrations of Cd^{2+} ions appeared to cause an increase in duration of the Ca spikes similar to the effect exerted by the transition-metal cations, increasing concentrations of Zn^{2+} ions did not cause such an increase.

DISCUSSION

The experiments in this study were performed to test the hypothesis that Mn^{2+} ions can pass through the calcium channels of the myoepithelial cells of *Syllis spongiphila* because they have a relatively low energy of hydration. The effects on Ca and Mn spikes of Fe^{2+}, Co^{2+}, and Ni^{2+} were tested because these first-row, transition-metal cations are divalent, their energies of hydration increase stepwise from that of Mn, and, like Mn, they form octahedral aqueous complexes. (The last characteristic thus distinguishes them from Cr^{2+} and Cu^{2+}, cations of the same first-row transition series, which have tetragonally distorted primary hydration spheres [Burgess, 1978].)

In these experiments an effort was made to avoid the oxidation of Fe^{2+} ions by using deoxygenated solutions; control experiments demonstrated (*a*) that it is possible to compare the effects exerted by the test ions in both nondeoxygenated and deoxygenated solutions, and (*b*) that the applied test cations did not significantly affect the electrical coupling between the myoepithelial cells. The known solubility products of the hydroxides of the test cations and Mn are lower than that of Ca (Peters et al., 1974); calculations of solubility in ideal solutions show that, at the concentrations and pH values used, the possibility for the formation of precipitate exists mainly for Ni^{2+} and Zn^{2+}. However, the solutions, which were freshly prepared for each experiment, appeared clear; thus, it seemed acceptable to compare directly the relative effects of the ions tested at different concentrations. Except for the slightly lower value for Cd, the activity coefficients of the chlorides of the test metal ions are similar to those of the alkaline-earth metals; the activity coefficients of the sulfates are estimated to be lower than those of the chlorides (Robinson and Stokes, 1959). All of the test cations except Fe^{2+} were used as chlorides in this study; however, Fe^{2+} ions were added as sulfate salt. Although up to 20 mM sulfate anions were added with the Fe^{2+} ions, most of the anions in solution were Cl^- (>0.5 M); thus, it was concluded that, in terms of activity, the Fe^{2+} ion can be treated as accompanied by Cl^- and that the effects exerted by Fe^{2+} can be compared directly with those exerted by Co^{2+} and Ni^{2+}.

The effects exerted by divalent cations on the syllid calcium channel and the characteristics of these cations are shown in Table I. The sequence of ions shown from Ni to Ba is in order of increasing crystal ionic radii and decreasing energies of hydration. The transition-metal cations, from Ni to Mn, exhibit a smaller spectrum of crystal radii than do the alkaline-earth cations. Although the increase in hydration energy of the alkaline-earth cations can be related

to decreasing radii, the continuing increase in hydration energy of the transition-metal cations appears to be due in part to decreasing radii but also to the nonspherical density of the *d*-orbital electrons (for Fe, Co, and Ni), which is the crystal field stabilization energy (CFSE) exhibited by these ions (Ochiai, 1977); indeed, by contrasting the crystal radii of Mn and Zn, which are the values expected based on the progressive increase in atomic number across the period, it is possible to see that the radii of Fe, Co, and Ni are anomalously small, due to CFSE. Mn is spherical because each of its *d*-orbitals is half-filled (it has no CFSE) and its energy of hydration is the lowest of the four cations (Ochiai, 1977).

TABLE I

	Suppress spikes						Support spikes		
	IIB elements		Transition metals				Alkali earth metals (IIA)		
	Cd	Zn	Ni	Co	Fe	Mn	Ca	Sr	Ba
Ionic radius (pm)*	109	88	83	88.5	92	97	114 126**	132 140**	149 156**
Hydrated radius (pm)‡	237	233	232	233	234	235	238 262**	243 266**	248 271**
Hydration energy, $-\Delta H$ (kcal/mol)§	446	502	517	505	473	455	395	359	325

* Values are from Huheey (1978) for coordination numbers of 6 or, where indicated by **, 8.

‡ Computed according to Goldman and Bates (1972) using the ionic radii based on a coordination number of 6 or, where indicated by **, of 8 (Burgess, 1978).

§ Values are from Noyes (1962). The values for both enthalpy and free energy are consistent with the proposed hypothesis, regardless of the contribution of entropy. Because of the greater certainty of experimentally determined values of enthalpy (Noyes, 1962), enthalpy is reported here.

Energy of hydration is the common feature among both the alkaline-earth and the transition-metal cations that can most readily be associated with the trend from most effective blocker of spikes, to blocker, to ability to support spikes. Thus, specificity of the calcium channel would be explained by the channel's ability to distinguish among the hydration (solvation) energies of a population of similar ions. It is also of interest that Mn^{2+} ions, which resemble non-transition-metal ions because they are spherical, would be predicted to bond to oxygen in preference to nitrogen atoms (Basolo and Pearson, 1967). The effects of the transition-metal cations tested on the barnacle muscle fiber (Hagiwara and Takahashi, 1967) do not show the same trends as those found for the *Syllis* myoepithelial cells; all suppress the Ca spike, but in a different order of effectiveness from that seen in *Syllis*. It is difficult to compare the results from the two preparations because the experimental conditions were not similar.

Fukuda and Kawa (1977) reported that Cd^{2+} and Zn^{2+} could replace Ca^{2+} in the generation of spikes by the muscle fibers of larvae of the beetle *Xylotrupes*.

Both of these cations reversibly abolished the Ca spikes in the *Syllis* preparation. The concentration of $CdCl_2$ required to abolish the Ca spike was 10–20 mM; however, since Cd^{2+} forms complexes with chloride, $(CdCl_n)^{+2-n}$ (Sillén and Martel, 1971), the concentration of free Cd^{2+} ions in solution, would have been <1.0 mM in these experiments. It is of interest that Adams et al. (1980) suggested that $CdCl^{+1}$ carried current through the endplate channels of frog muscle fibers. Such a complex may have contributed to the support of the spikes observed by Fukuda and Kawa (1977). In the syllid preparation, Zn abolished the Ca spike at a concentration (0.5–1.0 mM) lower than would be predicted by its energy of hydration, and, unlike the transition-metal cations, Zn did not cause an increase in the duration of Ca spikes at below-blocking concentrations. It is important to note that certain chemical properties of Zn and Cd are different from both the transition-metal cations and the alkaline-earth metal cations: unlike the transition metals, Zn and Cd have completely filled *d*-subshells. Further, Zn and Cd have a greater effective nuclear charge than do ions to the left of their respective periods because of incomplete shielding of the nucleus by the *d*-electrons; thus, they tend to form stronger ionic bonds and more covalent bonds than do ions with fewer *d*-electrons (Basolo and Pearson, 1967). Finally, the configuration of Zn tends toward being tetrahedral instead of octahedral (Cotton and Wilkinson, 1980), and Cd tends to form chloride complexes in aqueous solutions (Sillén and Martell, 1971). Thus, it is difficult to compare the results obtained using Zn with those obtained using the first-row, transition-metal cations. It is also not possible to compare the results obtained using Cd with those obtained using Zn or the transition-metal cations, because in ASW essentially all of the Cd was complexed with Cl ions.

A qualitative model similar to that proposed by Hille (1972, 1975*a*, *b*) for the sodium channel would explain the specific permeability of the calcium channel. The model assumes that cations of identical charge and similar hydrated radii (primary hydration sphere) enter the calcium channel. The channel may be thought of as an aqueous pore lined with sufficient charges, dipoles, and waters to permit stepwise dehydration and rehydration of ions that pass through it (Hille, 1975*c*). A given hydrated divalent cation would enter the channel and lose some waters to coordinate electrostatically at an anionic binding site located within the channel near the outer surface of the membrane. Attached to this site as part of a high-energy "activated complex," the cation would then be required to replace more of its waters with ligands lining the channel in order to traverse the narrowest dimension of the channel (the "selectivity filter" proposed by Hille [1975*b*] for the sodium channel). Those ions that exhibit low energies of hydration would succeed in substituting sufficient channel ligands for waters to cross the filter, after which they would detach, rehydrate, and proceed into the cell. Those ions that exhibit high energies of hydration would fail to dehydrate sufficiently to pass through the filter. Therefore, the more stable a cation's interaction with waters of its primary hydration sphere (e.g., Ni), the less likely it is to replace those waters with channel ligands, and the more effective that cation would be in prevent-

ing the passage of other, more permeant cations through the channel. Thus, the increasing energies of hydration shown from Fe to Co to Ni can explain the increasing effectiveness (on the basis of concentration) of these ions in suppressing spikes. The model does not require complete dehydration of the cation. The model also does not distinguish between a rate theory and an equilibrium theory, such as that of Eisenman (1962), to explain the selectivity patterns of pores. Although it is possible to suggest that those cations that pass through the selectivity filter (Ba, Sr, Ca, and Mn) make up an equilibrium sequence, the same sequence obtains for the characteristic rate constants of water substitution in the primary hydration spheres of these cations (Basolo and Pearson, 1967).

Finally, the results showing that Mn ions traverse the calcium channels of syllid myoepithelial cells suggests for this model that a channel ligand would have a crystal field strength somewhat weaker than that of a molecule of water. Although it is not possible to know the total distribution of energy between the ligands of the channel and the ions moving through it, it is reasonable to conclude that the ligands have weak field strengths. The Mn ion possesses symmetric distribution of charge (i.e., is spherical), and it has no crystal field stabilization energy and therefore would be expected to react preferentially with channel ligands that have a weaker field strength than water (Basolo and Pearson, 1967). The test cations Fe, Co, and Ni, however, have asymmetric distribution of charge and therefore exhibit crystal field stabilization energy; for each ion this energy increases with the electric field strength of the ligand (Cotton and Wilkinson, 1980). Such ions would be expected to react preferentially with channel ligands of strong electric fields (Basolo and Pearson, 1967). In the presence of channel ligands with electric fields weaker than that of a water molecule, Fe, Co, and Ni would tend to retain their waters of hydration, instead of exchanging them for channel ligands, and therefore not pass easily through the channel. The three test cations have increasing crystal field stabilization energies in the order Fe < Co < Ni (Basolo and Pearson, 1967), and this is the same order shown by the ions in effectiveness of blocking the channel. Thus, the assumption that the ligands of the calcium channel have weak electric fields leads to the predictions that Mn ions would be most likely to traverse the channel and that Ni ions would be least likely to traverse it and most likely to block it. The results are consistent with these predictions. If the channel ligand exerted a crystal field much weaker than that of a water molecule, however, Mn ions would not tend to dehydrate within the channel. Thus, the field of a channel ligand would have to be close enough to that of a water molecule so that Mn ions would tend to exchange a water molecule for a ligand, but weak enough so that Fe ions would not do so.

Using voltage-clamp techniques, Hagiwara et al. (1974) examined the membrane currents carried by the Ca-spiking barnacle muscle fiber. In the absence of divalent cations that block the calcium channel, they showed that Ba^{2+} carried more current than did Ca^{2+} or Sr^{2+}; the sequence was Ba > Sr = Ca. They interpreted this sequence to reflect mobility through the channel.

In the barnacle preparation, the ion exhibiting the least energy of hydration appears to pass most readily through the calcium channel. In the presence of Co^{2+}, the sequence of current-carrying capability was Ca > Sr > Ba; the authors interpreted this sequence to reflect an increasing affinity for channel sites. Although comparable experiments have not been performed using the *Syllis* myoepithelial cells, it is of interest that Ba^{2+} in Ca-free solutions can sustain spikes in the myoepithelial cells at a concentration of only 1 mM, whereas Sr^{2+}, Mn^{2+} (in Ca-free solutions), or Ca^{2+} must be present at greater concentrations to sustain spikes (Anderson, 1979*a*).

Ba, Sr, and Mn spikes are all of longer duration than Ca spikes, so the simple explanation that Ba supports spikes at a low concentration because it slows repolarization (see below) does not seem to hold in this case; an alternative explanation is that Ba^{2+} ions may pass more easily through the calcium channel than do Ca^{2+}, Sr^{2+}, or Mn^{2+} ions, as observed in barnacle muscle. This explanation would fit a model in which the ease of dehydration of a divalent cation determines its permeation through the calcium channel.

An additional observation made in the course of the study is that the three transition-metal test cations lengthen the durations of the Ca spikes and shorten those of the Mn spikes. Previous experiments showed that spikes elicited in the presence of Mn^{2+} ions, either when Ca^{2+} ions were present or not, were consistently of longer durations than Ca spikes, increased in duration with increasing $[Mn]_o$, and, at a constant $[Mn]_o$, decreased in duration with increasing $[Ca]_o$ (Anderson, 1979*a*).

These results suggest that Ca^{2+} ions contribute in some way to repolarization and that Mn^{2+} ions cannot replace and/or may compete with Ca^{2+} ions in this function. The transition-metal test cations used in this study appear to prevent the repolarizing action of Ca^{2+}, and they also appear to interrupt the prolonging action of Mn^{2+}. Several explanations for the observations made so far are possible, and one or more of them may apply to the myoepithelial cells of *Syllis*. Potassium channels of both nerve (Hermann and Gorman, 1979; Gorman and Hermann, 1979; Eaton and Brodwick, 1980) and muscle (Werman and Grundfest, 1961; Sperelakis et al., 1967; Hagiwara et al., 1974; Standen and Stanfield, 1978) cells are blocked by Ba^{2+}; it is possible that the divalent cations tested on the *Syllis* proventriculus may also block some population of K channels and thereby slow repolarization. Indeed, Ba spikes elicited from syllid myoepithelial cells in Ca-free solutions are of longer duration than Ca spikes (Anderson, 1979*a*). Furthermore, several different preparations exhibit a Ca-dependent potassium conductance (Meech, 1978). The repolarization of the myoepithelial cells of *Syllis spongiphila* may depend on such a mechanism, and other divalent cations may interfere with the action of Ca^{2+}. Finally, the calcium channels of *Paramecium* are reported to undergo a Ca-dependent inactivation (Brehm and Eckert, 1978), and it is possible that the *Syllis* calcium channels undergo a similar inactivation, which is interfered with by other divalent cations. Further experiments are required to clarify the mechanism(s) involved in repolarization, to characterize the actions of the cations that affect repolarization, and to show whether, and how, such cations differ from one another in their effectiveness.

Finally, these experiments show that Fe^{2+}, if maintained in deoxygenated solutions, can be useful in testing the physiological effects of a series of divalent cations. Under the appropriate experimental conditions, the effects of ferrous ions appear to resemble more those of Co^{2+} than those of Ni^{2+} ions, as would be expected from their physicochemical properties. If ferrous ions are used in solutions containing even small amounts of oxygen, they are oxidized and the pH of the solution is reduced. Under such conditions it would not be possible to distinguish the effects of the test ions from the effects of an increased concentration of hydrogen ions. The animals used in this study were collected from the harbor of San Juan and from mangrove swamps in Puerto Rico; both of these environments would be expected to have only low levels of oxygen, and it seems possible that *Syllis spongiphila* may be able to survive in waters of low oxygen tension. It is of interest in this regard that the sea anemone *Bunodosoma cavernata*, which can survive long periods of anoxic conditions, exhibits neuromuscular responses that appear the same whether or not oxygen is present (Mangum, 1980). Thus, organisms or tissues that can undergo periods of anoxia would be suitable for experiments in which Fe^{2+} ions were used.

It is a pleasure to thank Ms. P. Lessie for invaluable technical assistance, Mr. F. McKenzie for providing animals, Dr. R. F. Olivo for critically reading the manuscript, and Drs. S. W. Kirtley and R. Linck for helpful discussions.
Supported by National Institutes of Health grant NS12196.

Received for publication 31 July 1981 and in revised form 9 September 1982.

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