J. Membrane Biol. 101, 119–131 (1988)

The Journal of
Membrane Biology
© Springer-Verlag New York Inc. 1988

Calmodulin Acts as an Intermediary for the Effects of Calcium on Gap Junctions from Crayfish Lateral Axons

Rogelio O. Arellano, Fidel Ramón, Amelia Rivera, and Guido A. Zampighi†

Departamento de Fisiología y Biofísica, Centro de Investigación del I.P.N., México, D.F. 07000, Mexico, and †Department of Anatomy, Jerry Lewis Neuromuscular Research Center, University of California at Los Angeles, Los Angeles, California 90024

Summary. Lateral axons from the abdominal nerve cord of crayfish were internally perfused with the calcium receptor calmodulin (CaM) in solutions with low (pCa > 7.0) or high (pCa 5.5) calcium concentrations and studied electrophysiologically and morphologically. Results from these experiments show that when the internal solution contains calcium-activated calmodulin (Ca^{2+}-CaM) the junctional resistance between the axons increases from control values of about 60 to 500–600 kΩ in 60 min. In contrast, axons perfused with calmodulin in low calcium solutions maintain their junctional resistance at control levels during the 60-min perfusion. Similar results are obtained when only one or both coupled axons are perfused.

The morphological study shows that in the perfused axons the axoplasmic organelles are replaced or grossly perturbed by the perfusion solution up to the region of the synapses. Additionally, in axons perfused with Ca^{2+}-CaM there are regions where the synaptic gap between the membranes decreases from a control 4–6 to 2–3 nm. Both electrophysiological and morphological results can be interpreted as indicating that calcium-activated calmodulin acts directly on the junctional channels to induce their closure.

Key Words calmodulin · gap junctions · axons · crayfish

Introduction

Gap junction channel regulation involves changes in junctional permeability in response to physiological or experimental stimuli. Two major hypotheses have been proposed for this regulation, whereby the junctional channels can be closed either by an increase in the intracellular concentration of calcium or of hydrogen ions. The calcium hypothesis was first proposed by Loewenstein (1966) and it has received wide experimental support from many cell systems in which junctional uncoupling has been obtained after increasing the intracellular calcium concentration. Junctional uncoupling has also been observed after a reduction of the intracellular pH, an observation that led Turin and Warner (1977) to propose the hydrogen hypothesis for channel regulation. Recent developments supporting these hypotheses have been reviewed by Loewenstein (1981), Spray and Bennett (1985) and Ramón and Rivera (1986).

Since the evidence in favor of either hypothesis was obtained in intact preparations, where the internal concentrations of calcium and hydrogen could at best be estimated, Johnston and Ramón (1981) internally perfused pairs of coupled crayfish axons with solutions containing high calcium or hydrogen. The results obtained were unexpected, since neither high calcium concentrations (up to 10^{-3} M) nor low pH (down to 5.4) produced an increase in the junctional resistance and uncoupling of the axons. These observations were interpreted as indicating that the perfusion solution had washed out a soluble intermediary necessary for the effects attributed to the ions. Taking this study one step further, Arellano et al. (1986) showed, using unilaterally perfused crayfish axons, that uncoupling due to reduced intracellular pH is only observed after acidifying an intact axon, thus demonstrating that hydrogen ions do not act directly on the junctional channels.

The mechanism of action of calcium could have been either a direct or an indirect one. Since the experiments of Johnston and Ramón (1981) ruled out a direct mechanism, the next logical step was to test for the possible involvement of calcium-binding intermediaries. Of these, calmodulin (CaM) was an obvious candidate, as it had already been shown to mediate many calcium-dependent processes in numerous cells (Cheung, 1980).

Peracchia and Bernardini (1984) used the CaM inhibitors trifluoperazine and calmidazolium on intact embryonic cells and reported that the inhibitors protected the cells from the uncoupling effect of 100% CO_2. Results from experiments conducted on crayfish axons also showed protection by an anti-calmodulin drug (W-7) against uncoupling produced by low pH_i (Peracchia, 1987). From these experi-

ments the suggestion that the closure of junctional channels is mediated by CaM was advanced (Peracchia & Girsch, 1985). However, this suggestion is based on indirect evidence, and the direct involvement of calmodulin on the junctional coupling mechanism was never tested.

In the present work CaM was directly delivered to the interior of crayfish lateral axons in solutions containing $<10^{-7}$ M and 3×10^{-6} M calcium. At $<10^{-7}$ M calcium the CaM-calcium binding sites are unoccupied and at 3 μM calcium the CaM-target protein complex is activated (Keller et al., 1982). Morphological studies of these axons were also performed to determine changes in gap junction structure resulting from calmodulin or the perfusion itself. We report here that calcium-activated calmodulin induced uncoupling concomitantly with changes in the structure of junctional membranes. Both these results strongly support the hypothesis that calcium-activated calmodulin is involved in the regulation of gap junctional conductance.

Materials and Methods

Preparation and Recording

Experiments were performed on lateral axons from the abdominal nerve cord of adult crayfish (*Procambarus clarkii*, obtained from a local supplier) in the intermolt period. Nerve cords were excised, desheathed, and the 2nd abdominal ganglion was placed in an experimental chamber containing a modified van Harreveld solution (for composition of all solutions *see* Table). The method for internally perfusing one or two coupled axons was similar to that described by Arellano et al. (1986). Briefly, a cannula 7–8 mm long is made from a glass capillary 50 μm o.d. and 35 μm i.d., and introduced into the axon through a small cut made about 5 mm from the septum, which also served as outflow for the perfusion solutions. The cannula contained a floating bare platinum wire (18 μm in diameter) that reduced its high frequency impedance when used as an electrode. When filled with SIS the DC resistance of the electrode was about 10 MΩ. Internal solutions were driven by a pressure head of about 8 cm of water, such that the axon volume was replaced every 2–3 min.

Rectangular pulses of constant current were injected in the axons alternately at each side of the septum, and resultant voltage changes were measured with intracellular microelectrodes connected to high input DC amplifiers. Microelectrodes were filled with 2.5 M KCl and had resistances of 5–10 MΩ. Total injected current was measured with an electrode in the bath solution connected to an amplifier in a currentometric configuration. The records were displayed on an oscilloscope and photographed for later measurement. The parameters of the equivalent circuit that describes two coupled axons (Watanabe & Grundfest, 1961) were calculated by solving the corresponding equations (Moreno et al., 1987*b*[1]). Experiments were performed at 18°C unless otherwise indicated.

[1] Moreno, A.P., Ramón, F., Spray, D.C., Zampighi, G.A., 1987*b*. Humoral modulation of gap junctional sensitivity. (*submitted*).

Solutions

Internal perfusion solutions contain a high fluoride concentration, which was shown by Tasaki, Singer and Takenaka (1965) to prolong the survival time of squid giant axons. However, as fluoride is a strong calcium precipitating agent, it interferes with those experiments where a high *p*Ca is required. A partial solution to this problem was presented by Johnston and Ramón (1981), who perfused the axons with solutions in which fluoride was substituted with flutamate or isethionate. In these conditions the input resistance of the axons decreased with time and experiments were limited to about 15 min.

In the present experiments axons could be perfused for more than 40 min with solutions containing high calcium. This was accomplished by first briefly perfusing the axon with an internal solution containing 109 mM KF, followed by a low fluoride solution (SIS, Table). After this, the Ca-containing fluoride-free solution was used (SIS-C, Table). Another variation of this protocol was to initially perfuse the axons with the low fluoride solution. This solution was then changed to one with low fluoride and either high or low calcium; in low fluoride the calcium concentration could be raised up to *p*Ca 5.5 (SIS-B). The input resistances under these conditions were more stable than when fluoride was totally omitted and perfusions could be maintained for an hour or more.

The preparation of the calcium-containing solution in low fluoride, SIS-B, was as follows: 10 μl of 0.1 M $CaCl_2$ was added to 1 ml SIS while measuring *p*Ca (*see below*). In the first 2–3 min *p*Ca increased to 4.0 and then decreased to 4.9, reaching 5.4 at 5 min and remaining at 5.5 or 5.6 for the next 40–50 min. At 70 min a steady value of *p*Ca 6.0 was reached. Perfusion of this solution was performed during the time when the *p*Ca value of 5.5 was stable for 40 min or longer. Since these changes in *p*Ca were due to the precipitating effect of fluoride, in all experiments the *p*Ca of the solution was measured immediately before adding CaM. A similar protocol was used to prepare SIS-C, using a 1 mM stock solution of $CaCl_2$.

Drugs

Calmodulin from bovine brain (CaM, phosphodiesterase 3′-5′-cyclic nucleotide activator; mol wt 16,900, Watterson, Sharief & Vanaman, 1980) was obtained from Sigma (St. Louis, MO). The powder was dissolved in the pertinent SIS (Table) to obtain 10,000 U/ml, equivalent to a concentration of about 10 μM.

Calmidazolium (1-[bis-(*p*-chlorophenyl) methyl]-3-[2,4-dichloro-β-(2,4-dichlorobenzyloxyphenetyl] imidazolium chloride; R 24571, Janssen Pharmaceutica, Beerse, Belgium) was a generous gift from Dr. C. Peracchia. The drug was added to normal SIS to reach a final concentration of 1 μM.

Apyrase (adenosine 5′-triphosphate and an adenosine 5′-diphosphatase) was obtained from Sigma (St. Louis, MO) and diluted in SIS-B to a final concentration of 40 U/ml.

Calcium Electrode

The free calcium concentration of the internal solutions was measured with a calcium-sensitive electrode. This electrode was made with a 1:1 mixture of a cationic exchange resin (Fluka 9470) and polyvinyl chloride diluted with tetrahydrofurane. A pH meter (Corning) in its voltage measuring mode was used to calibrate the electrode and make the measurements. The electrodes have a Nernst potential of about 28 mV per tenfold concentration change, are linear in the range *p*Ca 7.0 to 3.0, and deviate some-

Table. Composition of the internal and external solutions[a]

Compound	SIS (normal) (mM)	SIS-A (low Ca) (mM)	SIS-B (high Ca) (mM)	SIS-C (high Ca no F) (mM)	SES (normal) (mM)
NaCl	15.0	15.0	15.0	15.0	205.0
KCl	—	—	—	—	5.4
KF	33.0	33.0	33.0	—	—
K-glutamate	187.0	187.0	187.0	220.0	—
$CaCl_2$	—	—	1.0	*	13.5
Sucrose	22.0	12.0	22.0	22.0	—
EGTA	0.1	10.0	0.1	0.1	—
MOPS	5.0	5.0	5.0	5.0	—
HEPES	—	—	—	—	5.0
pH (±0.1)	7.1	7.1	7.1	7.1	7.4
pCa (±0.1)	6.5	>7.0	5.5	4.0	1.8

[a] SIS, standard internal solution; SES, standard external solution.
* $CaCl_2$, 1 mM stock solution added up to *p*Ca 4.0.

what from linearity above *p*Ca 7.0. The standard solutions were prepared according to Tsien and Rink (1980).

Morphology

Nerve cords containing the perfused axons were fixed immediately after the electrical recordings. The cannula was kept *in situ* to prevent the rupture of the septa (Arellano et al., 1986). The cords were prepared for electron microscopy as described previously (Zampighi, Ramón & Duran, 1978). Fixation was performed in 3% glutaraldehyde-hydrogen peroxide in 0.2 M cacodylate buffer pH 7.3. After 30 min the fixative was replaced by 3% glutaraldehyde in 0.2 M cacodylate buffer pH 7.3 for 30 more min. A third fixation step was performed by replacing the solution with 3% glutaraldehyde in 0.2 M cacodylate pH 7.3 with 0.1–0.3% tannic acid for 1 hr at room temperature. After fixation the cords were washed with 0.2 M cacodylate buffer with 4% sucrose. Postfixation was performed in 2% osmium tetroxide in 0.2 M cacodylate buffer. Dehydration was done in graded concentrations of ethanol and infiltration and embedding in Epon 812.

The perfused axons and the septal regions were identified first in thick plastic sections cut perpendicularly to the long axis of the axon. The morphology of the unperfused axon was used as control for modifications resulting from the removal of the cords from the animal for the length of the experiment (3–4 hr). Thin sections were cut with diamond knives, deposited on formvar and carbon coated single hole grids and stained with uranyl acetate and lead acetate. Observations were made with a Zeiss EM 10C electron microscope.

Results

Perfusion with Solutions Containing High Calcium

Previous results have shown that bilateral internal perfusion of coupled lateral axons with solutions containing up to 1 mM of calcium have no effect on the junctional resistance (Johnston & Ramón, 1981). We confirmed these results for unilateral perfusion (Fig. 1). During the control period axons were perfused with solutions containing decreasing concentrations of fluoride (109 and 33 mM), after

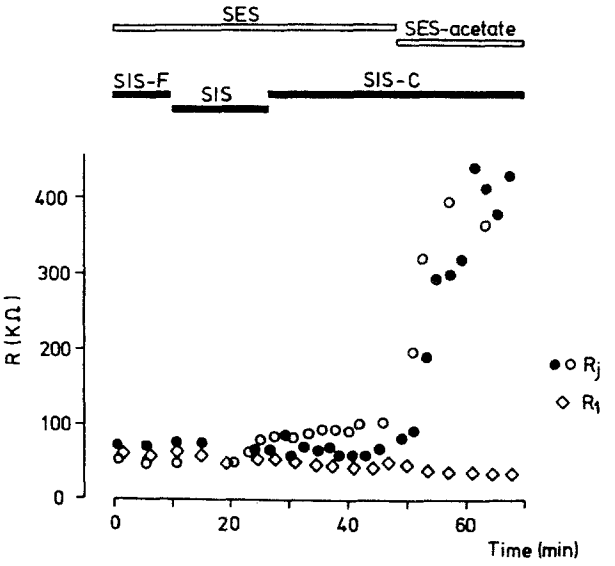

Fig. 1. Internal perfusion of a high calcium solution does not increase the junctional resistance of crayfish axons. One axon of a coupled pair was internally perfused while measuring the junctional resistance between both axons. Two experiments are shown (open and filled circles), each cannulated axon was initially perfused with SIS-F (containing 109 mM KF), then with SIS, and finally with a high calcium solution (SIS-C, *p*Ca 4.0). During 20 min of perfusion with SIS-C, no significant change in R_j was seen. After this time the external solution (SES) was changed to SES-acetate while maintaining internal perfusion with SIS-C. Under these conditions the junctional resistance increased to around 400 kΩ. The experiments were terminated after 1 hr because the input resistances of the perfused axons (diamonds) were falling due to lack of fluoride in the perfusion solution. Input resistances for both experiments fell within the size of the symbol

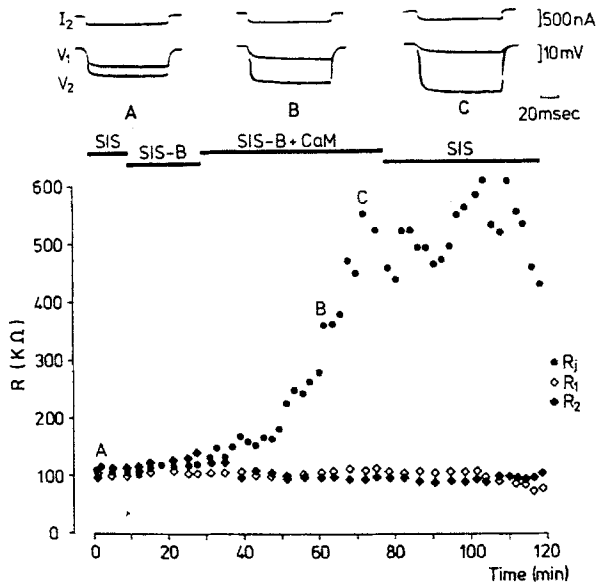

Fig. 2. Internal perfusion of high calcium and calmodulin increases the junctional resistance of crayfish axons. In this experiment one axon was initially perfused with SIS followed by a high calcium, low fluoride solution (SIS-B). No change in the junctional resistance (circles), nor in the input resistances (diamonds) was observed during 20 min perfusion. Calmodulin (10 μM) was then added to the perfusion solution (bar marked SIS-B + CaM). The junctional resistance then slowly increased and reached a maximum value around 500 kΩ 40 min after the start of the calmodulin perfusion. The calmodulin-induced uncoupling could not be reversed by washing out with SIS during 30 min (last bar marked SIS). The traces at the top show the voltage and current records corresponding to the R_j values labeled *A*, *B*, and *C* in the graph

which it was possible to perfuse them with fluoride-free SIS-C having a *p*Ca of 4.0. The filled and open circles in Fig. 1 show the results for two different experiments. During a 20-min period of perfusion with SIS-C no significant change in the junctional resistance was observed.

Nevertheless, the preparation was capable of uncoupling when the external solution was changed to one containing sodium acetate 205 mM instead of sodium chloride (SES-acetate). This solution decreases the internal pH of unperfused axons to about 6.2 (Moreno, Ramón & Spray, 1987*a*) and the junctional resistance increased to about 400 kΩ within 15 min. Therefore, calcium ions do not seem to be directly responsible for changes in junctional resistance.

Perfusion with Solutions Containing Calmodulin

Next we perfused one or both axons with CaM in solutions containing high and low calcium concentrations.

High Calcium

We first perfused unilaterally with SIS for 10 min, then changed to SIS-B containing 3.2 μM calcium (*p*Ca 5.5) and low fluoride (Fig. 2). As in the experiment above, the junctional resistance (filled circles) and the input resistance of both axons (filled and open diamonds) did not change during 20 min in these conditions. The perfusion solution was then changed to CaM-containing SIS-B. This caused an increase in the junctional resistance to about 500 kΩ in 40 min. This effect was not reversed by perfusion with CaM-free SIS containing low calcium. Except for a slight drop at the onset of the calmodulin perfusion, the input resistances remained stable throughout the experiment. A similar response was observed in five more experiments of this sort, in which no attempt was made to reverse the CaM-induced uncoupling.

Low Calcium

When an axon was perfused with CaM solutions containing a low calcium concentration (*p*Ca >7) the junctional resistance did not increase during more than an hour of perfusion. All five unilateral perfusion experiments gave similar results, and two of them are shown in Fig. 3.

Figure 3 summarizes the data obtained from four experiments with internal perfusion of calmodulin. Filled symbols represent two experiments in which an axon was first perfused with SIS-B and, after 20 min, the solution was changed to one containing 10 μM calmodulin in SIS-B (*p*Ca 5.5). In both cases the junctional resistance increased only during the period of calmodulin perfusion. The open symbols represent two experiments in which an axon was initially perfused with SIS and 20 min later calmodulin (10 μM) was added in low calcium (SIS-A, *p*Ca > 7.0). In these cases there was no change in the junctional resistance.

These results show that calmodulin produces an increase in the junctional resistance, from 50–100 kΩ to around 500 kΩ, only when perfused in the presence of high calcium.

Bilateral Perfusion

In the experiments described above only one axon of a coupled pair was internally perfused with calmodulin. Even though uncoupling occurred only when calmodulin was perfused with high calcium, thus suggesting a direct effect on the channels, we further tested this idea by perfusing both axons with SIS-B (*p*Ca 5.5) and calmodulin (10 μM in each axon). Results from these experiments show that

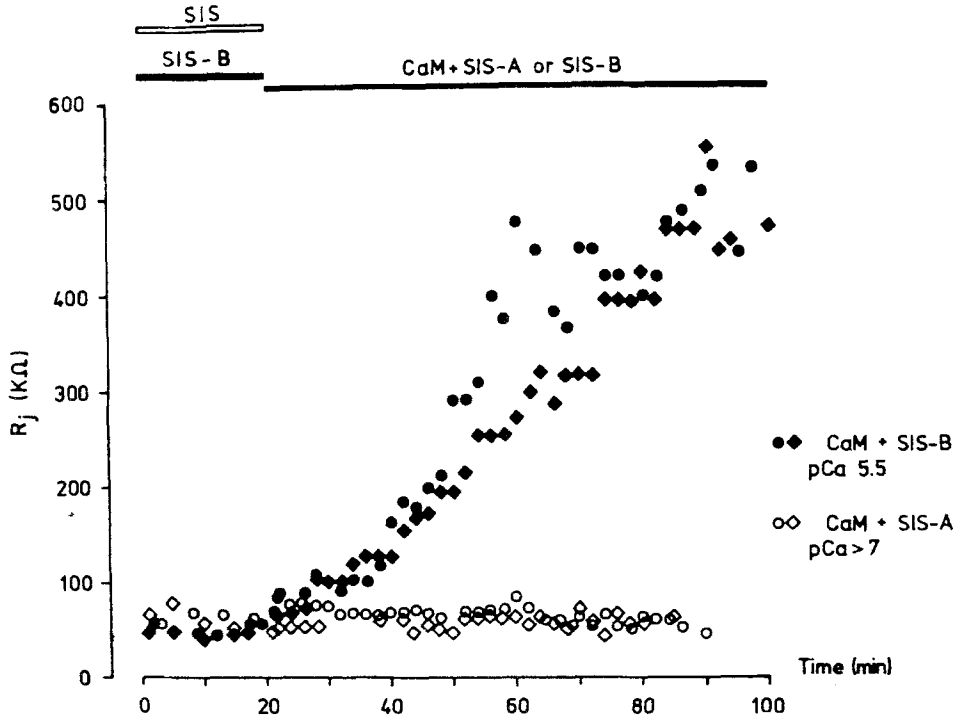

Fig. 3. Effect of unilateral perfusion of calmodulin in high or low calcium on the junctional resistance of lateral axons. Four experiments are shown in which one axon of a coupled pair was perfused with calmodulin (10 μM) in either a high calcium (pCa 5.5, filled symbols) or low calcium (pCa > 7.0, open symbols) solution. Each axon was initially perfused with a low (pCa 6.5, SIS) or high (pCa 5.5, SIS-B) calcium solution without calmodulin for 20 min. During this time no change was observed in the junctional resistance. At 20 min, the solution was changed to one containing calmodulin (bar marked *CaM + SIS-A or SIS-B*). The junctional resistance increased only when the perfusion solution contained calmodulin and high calcium, and remained constant in the presence of calmodulin and low calcium. The filled circles represent the data shown in Fig. 2, which was corrected for the distance between the microelectrode and the septum, thus shifting the data points by about 50 kΩ. Also, in this case, the calmodulin perfusion lasted approximately up to minute 60; subsequent data points were obtained in SIS, where no reversibility of the uncoupling was observed (*see* Fig. 2). All data points were included in this figure, since the trend illustrated was observed in five other experiments with prolonged perfusion of calmodulin and high calcium

the junctional resistance (R_j) increased and, similar to unilaterally perfused axons, reached a steady-state value (*not shown*) of about 500 kΩ in 60 min (Fig. 4). In this case the onset of the increase in R_j begins somewhat earlier than in the unilaterally perfused axons and the rate of increase is slightly greater. This could be due to Ca^{2+}-CaM reaching the junctional channels from both sides of the synapse.

Calmodulin and Temperature

The increase in junctional resistance seen with calmodulin and high calcium has a slow time course as compared to that produced by external application of acetate (*compare* Fig. 1 to Figs. 2 and 3). Figure 2 shows a delay of 15 min in the R_j response, while the maximum value of 500 kΩ was reached 25 min later. This delay and the slow rate at which the maximal uncoupling is reached could be due to several factors, such as slow diffusion of calmodulin to the junctional area, or slow kinetics of the uncoupling mechanism triggered by calmodulin. To distinguish between the possibilities of diffusional or energy-dependent delays, similar experiments were performed at 23°C.

Figure 5 shows the result of two experiments performed at 23°C, where calmodulin was perfused in a high calcium solution (SIS-B + CaM). For comparison, the results shown in Fig. 3, obtained at 18°C, are also presented. The junctional resistance also increased in this case, and at a faster rate, allowing for the calculation of a Q_{10} of about 4. This value suggests that the origin of the slow rate of increase of the junctional resistance in response to calmodulin is not due to diffusional factors but rather to an energy of activation-dependent step.

Other Controls

In another series of experiments we internally perfused coupled axons with compounds that might

have effects related to those of calmodulin. Of these, calmidazolium, a potent calmodulin blocker, did not elicit change in junctional or input resistance at a concentration of 1 μM for about 40 min. We also perfused apyrase and 10 μM calmodulin at pCa 5.5. In these conditions there was an increase in the junctional resistance of the axons similar to that shown in Fig. 2 (*data not shown*).

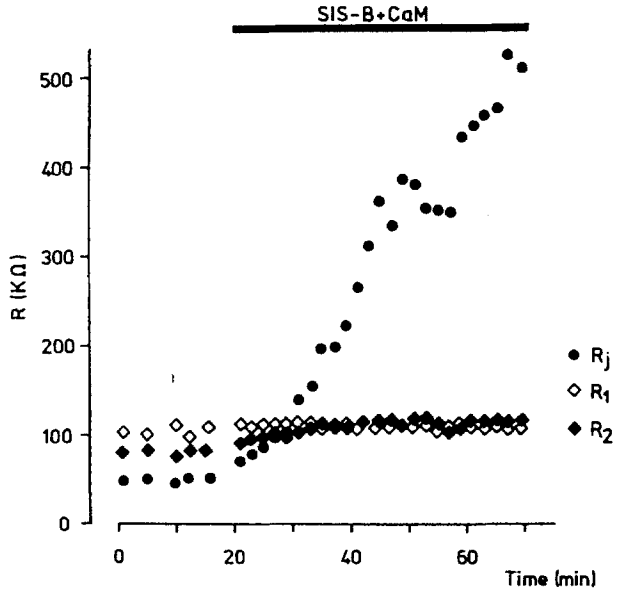

Fig. 4. Effect of calmodulin and high calcium on the junctional resistance of bilaterally perfused crayfish axons. In this experiment both coupled axons were cannulated and perfused, initially with SIS for about 20 min, and then with calmodulin and high calcium (SIS-B + CaM). The junctional resistance (filled circles) began to increase after the introduction of calmodulin and reached a value of around 500 kΩ in 60 min. The input resistances of both axons (open and filled diamonds) remained nearly constant at control values

Ultrastructure of the Junctional Region

Unperfused Septa

The morphology of septa and gap junctions between intact lateral axons has been published previously (Hama, 1961; Pappas, Asada & Bennett, 1971; Peracchia, 1973*a,b*; Zampighi et al., 1978). Crayfish lateral giant axons establish contact at specific regions of the nerve cord called *septa*, located at the rostral third of each abdominal ganglion and extending from anterior to posterior and from lateral to medial directions. They are constructed from extracellular laminae made of fibers and ground substance covered by glial cells. The septa contain interruptions (the synapses or "windows"; arrows in Figs. 6*A*, 7*A*, 8) where axolemmae establish gap junctional contacts (Fig. 6*B*).

The fine structural organization of crayfish gap junctions differs from that of mammalian gap junctions (Zampighi et al., 1978). In thin sections they appear as two closely apposed plasma membranes 20–22 nm in overall thickness separated by an unstained extracellular gap 4–6 nm wide (Fig. 6*C*). Densities spaced about 20 nm apart traverse the axolemmae, the extracellular gap, and protrude in the axoplasm for 2–3 nm (Fig. 6*C*). They form two-dimensional hexagonal arrays in the gap junction plane (Fig. 6*D*). In addition, crayfish gap junctions contain sheets of vesicles 50–70 nm in diameter attached to the gap junctional areas.

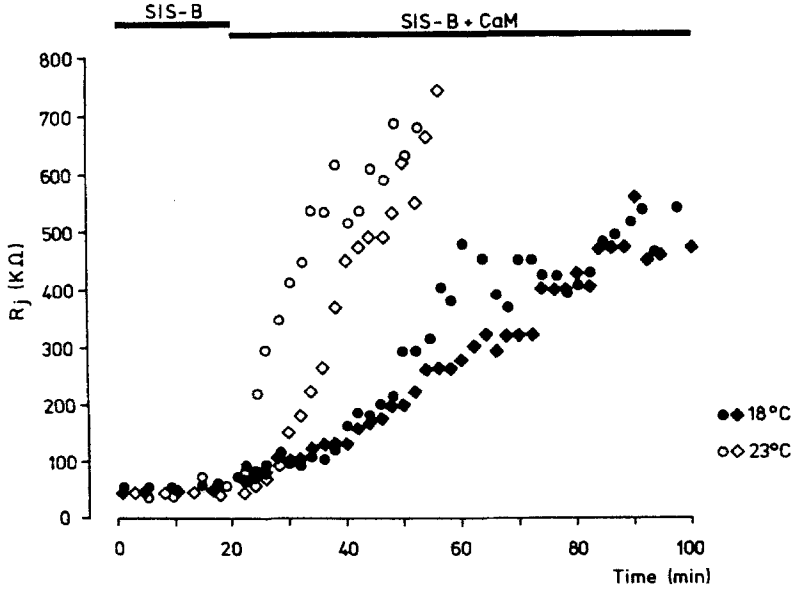

Fig. 5. Effect of temperature on the rate of calmodulin-induced uncoupling of crayfish axons. The open symbols represent two experiments in which calmodulin (10 μM) and high calcium was perfused in one axon of a coupled pair while maintaining the bath solution temperature at 23°C. The junctional resistance increased at a faster rate than that observed in similar experiments performed at 18°C (filled symbols, data from Fig. 3 replotted here for comparison). The slopes of the tangents that fit the data points at 50% of maximal response were used to calculate a Q_{10} of around 4.0. All axons were initially perfused for 20 min with a high calcium solution (SIS-B)

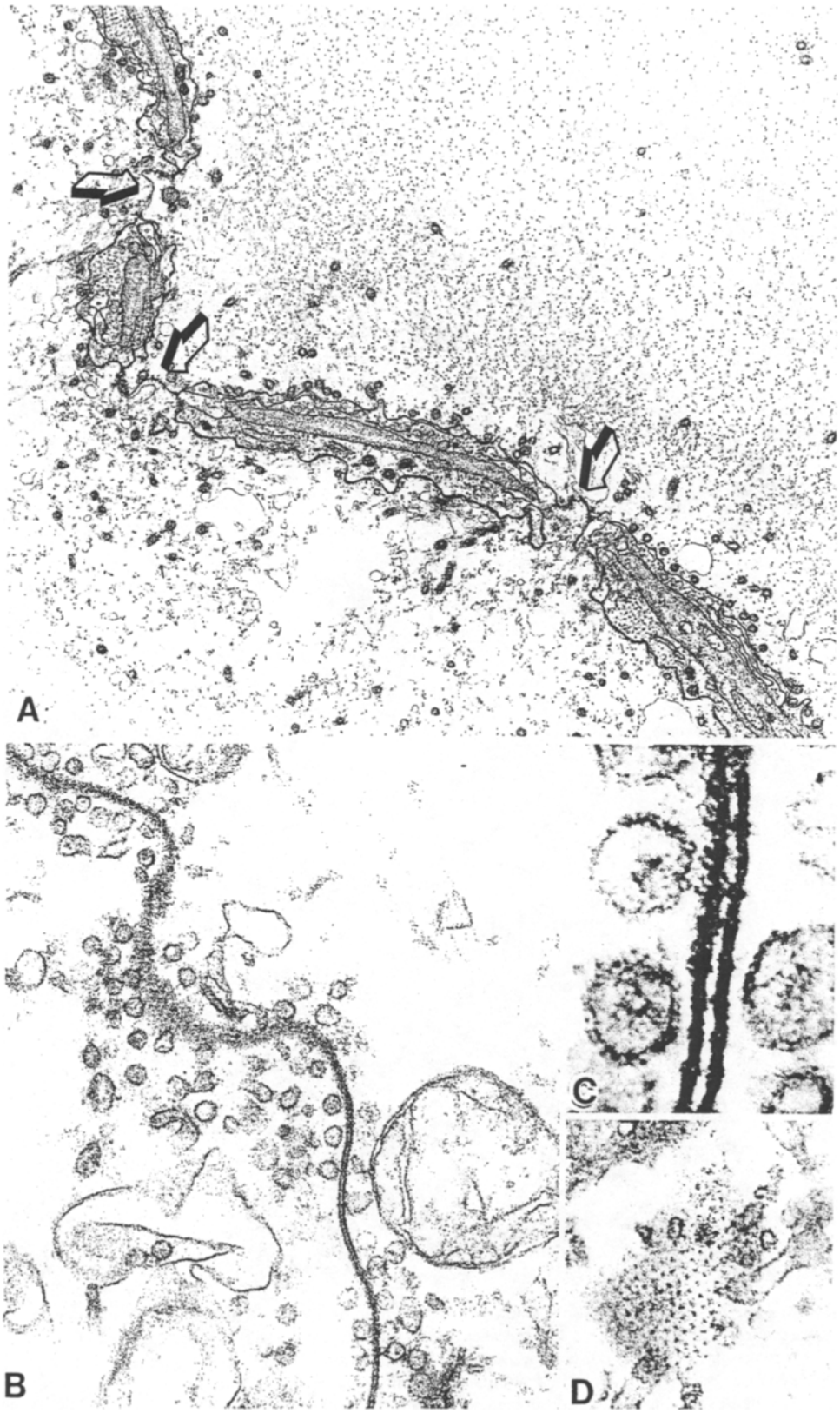

Fig. 6. Morphology of the septal region in unperfused crayfish axons. (*A*) A low magnification of the septum between the axonal segments forming the lateral giants (anterior axon is above). The arrows point to the synapses ("windows"). Magnification: ×6,250. (*B*) High magnification of a synaptic region to demonstrate that the axolemmae are in close contact along most of their surface. Vesicles, 50–70 nm in diameter, are seen associated with the axolemmae, or forming loose aggregates in the vicinity. Magnification: ×50,000. (*C*) At higher magnification, the two membranes are separated by an unstained extracellular gap and the transverse densities spaced 20 nm apart span the entire junctional complex. Magnification: ×250,000. (*D*) *En face* view of a gap junction to show that the densities seen in transverse sections (Panel *C*) form arrays with 20 nm center-to-center spacing between particles. Magnification: ×100,000

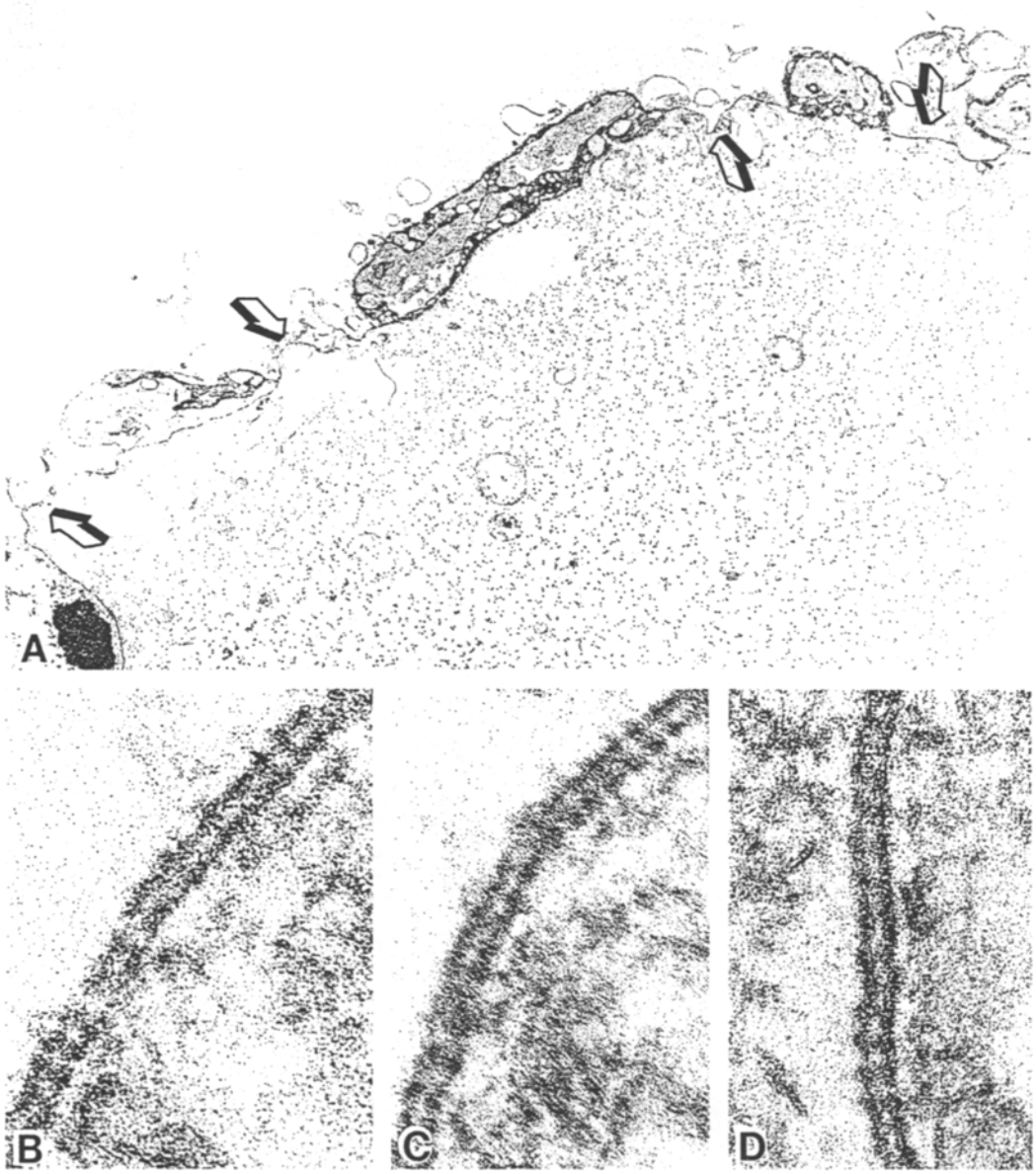

Fig. 7. Electron micrograph of the septal region after unilateral perfusion with calmodulin and low calcium. (*A*) Low magnification view of the septal region. The perfused axon (above) displays a lumen essentially free of normal axoplasmic organelles. The large arrows point to the synaptic regions where the gap junctional contacts are located. Magnification: ×9,000. (*B–D*). Higher magnification views of gap junctions from the same synapses pointed in panel *A* but obtained from different sections. Note that the junctions retain their overall thickness, width of the unstained extracellular gap, and presence of transverse densities. Only a few 50–70 nm diameter vesicles remain associated with the synapses. Magnification: ×250,000

Unilaterally Perfused Septa

The morphology of the axoplasm of unilaterally perfused axons depends upon the length of the perfusion time, and not on the composition of the perfusion solutions. With short perfusion times (30 min or less, *see* Arellano et al., 1986) the changes were restricted mostly to the axoplasmic organelles on

the perfused side; microtubules and microfilaments were disorganized and mitochondria appeared distended. Large vacuoles also appeared on the unperfused side. The fine structure of the gap junctions (overall thickness, width of the gap, and presence and spacing of transverse densities) remained unaltered, but the sheets of 50–70 nm diameter vesicles lost their attachment to the junctional region. With perfusion times longer than 1 hr (this paper), most microtubules and microfilaments were washed away on the perfused side. However, a few distended mitochondria and some vacuoles are still observed associated with the surface membrane. Also, the mitochondria of the unperfused axon were enlarged and showed distended cristae.

Unilaterally Perfused Septa with Calmodulin and Calcium

The septa of six axons unilaterally perfused with solutions containing calmodulin and low calcium or calmodulin activated with calcium were examined in this study. All the septa were first characterized electrically to determine the effect of perfusion on junctional permeability. Due to the length of time required to perform electrophysiological experiments, all preparations studied morphologically were perfused for more than one and a half hours.

Figure 7*A* shows a low magnification view of the septal region where one of the axons (upper) was perfused with a solution (SIS-A) of low calcium (pCa > 7.0), EGTA (10 mM) and calmodulin (10 μM). The perfused axon was easily recognizable because of the absence of most axoplasmic organelles. The septum in Fig. 7*A* shows four synapses ("windows") where the axolemmae come in direct contact to form gap junctions (arrows). Note that the structural integrity of the region of contact depicted here was maintained even after these long perfusions (*compare* Figs. 6*A* and 7*A*). Figure 7*B–D* shows selected regions of the synapses shown at low magnification in panel *A* to demonstrate that most structural characteristics, such as the overall thickness of the junction, the width of the unstained extracellular gap, and the presence of transverse densities spaced about 20 nm apart, were maintained. Even after extensive perfusion, a few 50–70 nm diameter vesicles persisted in association with the gap junctions.

Figure 8 shows a low magnification view of a septum unilaterally perfused with solutions containing calmodulin activated with calcium (CaM + SIS-B). The axoplasm of the perfused side (upper) lacked membranous organelles such as mitochondria and large vacuoles, but still contained some microtubules and microfilaments. Note that because of the long perfusion the mitochondria of the unperfused axon were broken and their cristae had largely swelled.

A special characteristic of the synapses of axons perfused with calcium-activated calmodulin was the presence of junctional profiles of reduced overall thickness intermingled with "normal" gap junctions. Figure 9*A* shows the two types of junctions coexisting in the same synapse and continuous with each other. The area inside the corners depicts the region of transition between the two types of junctional profiles. The overall thickness of the gap junction decreases from about 20 to 16–18 nm. The 50–70 nm diameter vesicles, which are characteristic of the crayfish gap junctions even after perfusion, were absent from the thin junctions. Figure 9*B* shows the transitional region at higher magnification to demonstrate that the decrease in overall thickness of the thinner junction was associated with a decrease in the width of the extracellular gap (from 5–6 to about 2–3 nm). Figure 9*C* shows a higher magnification of the collapsed region below the corners in *A*. Figure 9*D* shows a higher magnification of the *en face* profile below the corners in Fig. 9*A* to demonstrate the presence of particles spaced about 20 nm center-to-center. The thinner junctional profiles were not observed in synapses from unperfused axons nor in synapses of axons perfused with calmodulin and low calcium.

Discussion

The main result reported here is that the junctional resistance between crayfish lateral axons increased when calmodulin was internally perfused unilaterally or bilaterally in solutions with pCa 5.5 (3.2 μM), whereas there was no change in resistance when the pCa was >7.0 (<0.1 μM). In addition, as shown before (Johnston & Ramón, 1981), internal perfusion of high calcium solutions did not affect the junctional resistance of coupled axons. These results fully support the notion advanced by Johnston and Ramón (1981) that internal perfusion removes a soluble intermediary required for uncoupling by calcium, and they show that the intermediary could be calmodulin. Altogether, our results demonstrate that intracellular calcium via activated CaM can reduce junctional conductance in support of the calcium hypothesis originally proposed by Loewenstein (1966).

Since perfusion of both coupled axons with calmodulin and high calcium produced junctional uncoupling, the simplest explanation for the action of calmodulin is that it binds directly to the junctional proteins and induces closure of the channel. This idea is also supported by the results of *in vitro* experiments where calmodulin has been shown to bind directly to mammalian lens and liver (Hertz-

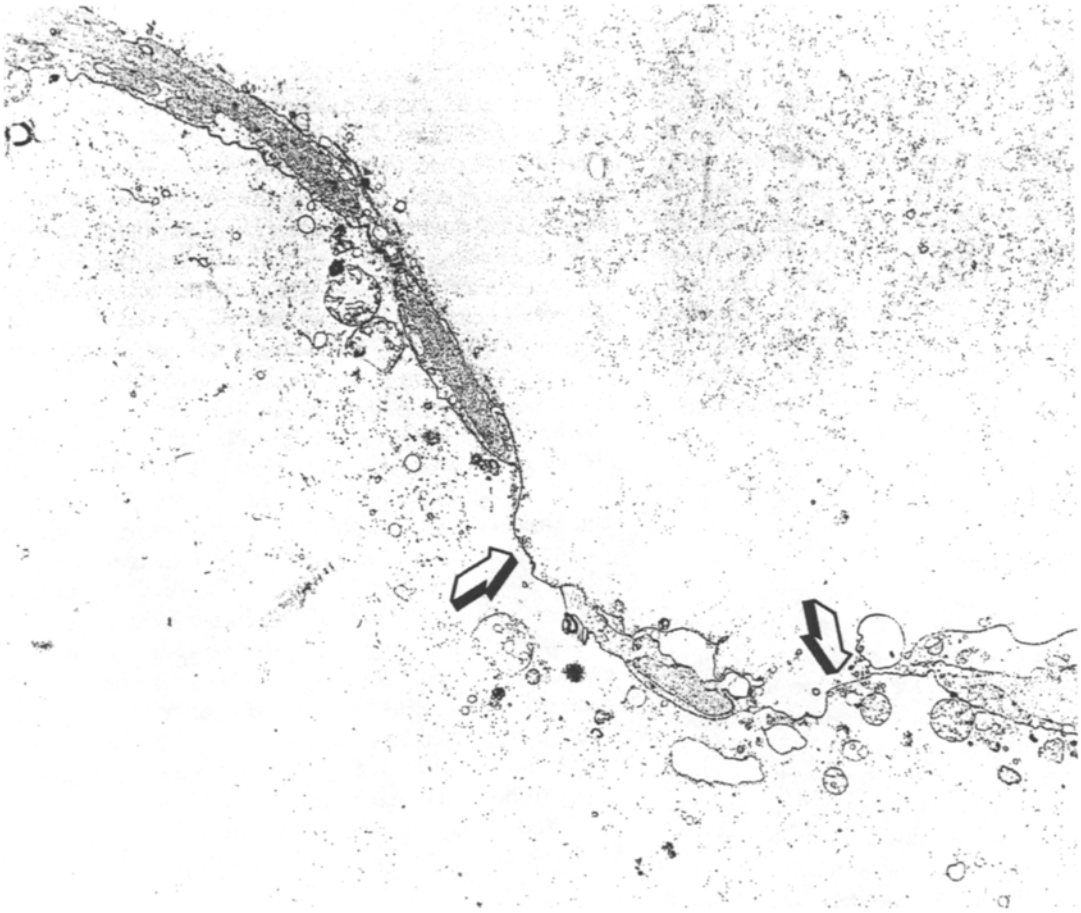

Fig. 8. Electron micrograph of a septum where one of the axons (upper) was perfused with solution containing calmodulin (10 μM) and high calcium (pCa 5.5, SIS-B). The axoplasm of the perfused axon still contains some microfilaments, but it is devoid of mitochondria and membranous vacuoles. The large arrows point to the synapses where the gap junctions are located. Magnification: ×6,000

berg & Gilula, 1981; Welsh et al., 1982), arthropod gap junction proteins (Van Eldik et al., 1985), and with the effect of calcium-activated calmodulin on junctional proteins incorporated into liposomes (Girsch & Peracchia, 1985). Our results with unilateral perfusion of calcium-activated calmodulin and apyrase, in which uncoupling was not affected by the drug, and the bilateral perfusion of Ca^{2+}CaM suggest that the actions of calmodulin are not dependent on other molecules (ATP) traversing the junction from the intact axon.

Two observations made in this study are of interest for a possible physiological role of the uncoupling produced by the perfusion of calcium-activated CaM. As can be seen from Figs. 2 and 3, the time course of uncoupling by calcium-activated CaM is slow when compared to that observed by decreasing the internal pH (Campos de Carvalho, Spray & Bennett, 1984; Moreno et al., 1987*b*[2]; Fig. 1, this paper). However, this rate of uncoupling is similar to that of externally applied heptanol (Johnston, Simon & Ramón, 1980). Increasing the temperature markedly accelerates the rate of the CaM-induced uncoupling (Fig. 5); however, even at the high temperature, maximal steady values of junctional resistance were still reached slowly (about 30 min). A possible explanation of the slow time course of uncoupling could be found in the dependence of the calcium-calmodulin complex on the ionic strength of the solution and requirement of the activated calmodulin-target protein complex for magnesium (Brostrom & Wolff, 1976; Haiech, Klee & Demaille, 1981; Keller et al., 1982; Steiner, Lam-

[2] *See* footnote 1, p. 120.

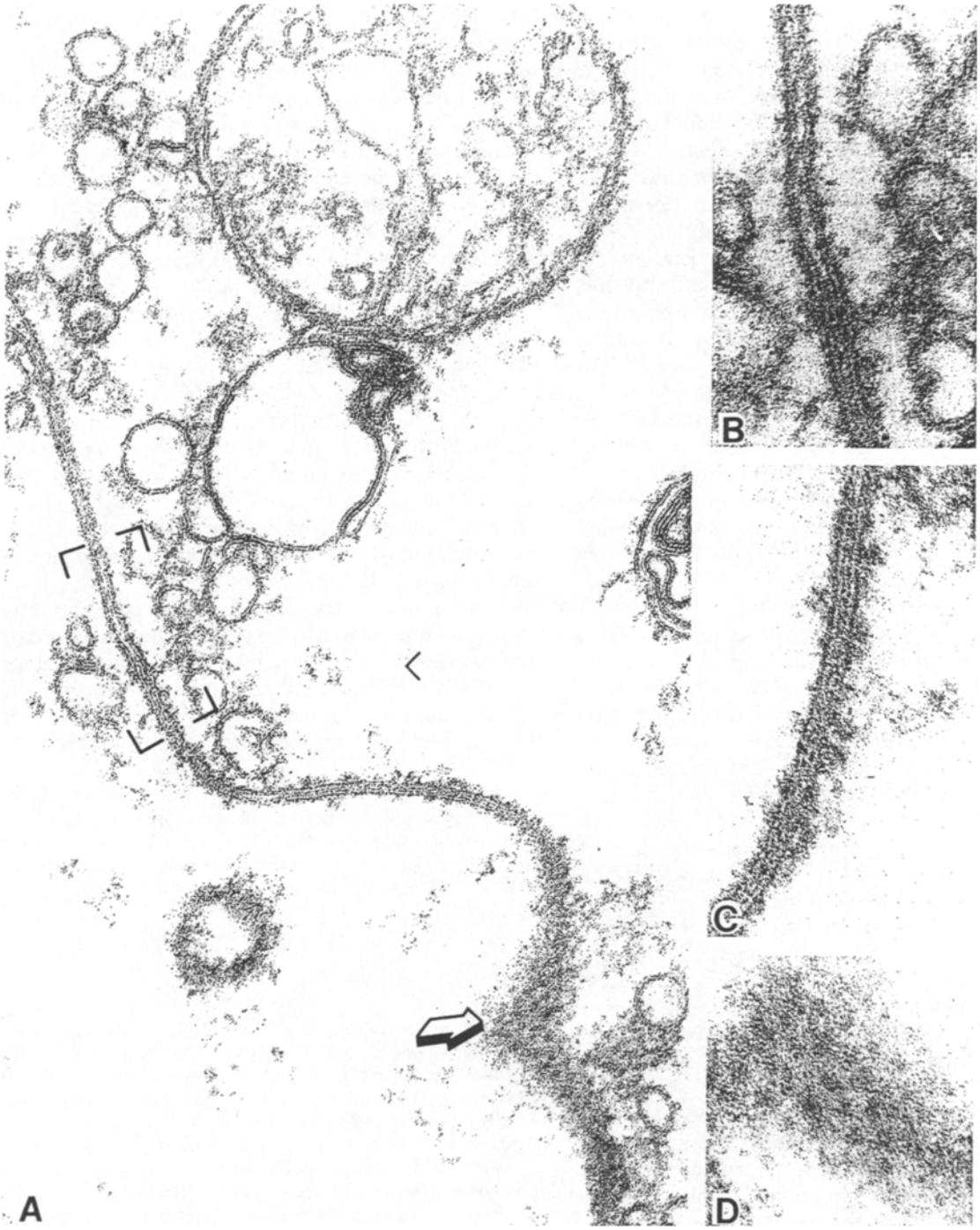

Fig. 9. (*A*) High magnification view of the synapse marked by the right-hand arrow in Fig. 8*A*. The upper left-hand region of the synapse has most of the characteristics of the gap junctions described previously, and the portion at the bottom (arrow) shows an *en face* view displaying the array of particles. However, the junctional profile in the center differs in the width of the extracellular gap, which appears narrower than the one observed in unperfused axons or in unilaterally perfused ones with calmodulin and low calcium (*see* Fig. 7). Magnification: ×50,000. (*B*) Higher magnification view of the region enclosed within the corners in panel *A*. This area shows the transition between the thin and thick junctional profiles. The gap junction at the top has a 5–6 nm wide extracellular gap, whereas the gap of the junctional profile immediately below is only 2–3 nm (bottom part). (*C*) Higher magnification view of a portion of the thin junctional profile shown in panel *A*. (*D*) Higher magnification view of the *en face* region shown in panel *A*. Magnification *B–D*: ×250,000

booy & Sternberg, 1983). Thus, it is possible that the perfusion solution was not completely adequate and calmodulin was not fully activated, or that the calmodulin-channel protein binding was not optimal under these conditions. Both factors could account for the slow rate of uncoupling observed.

Another point relevant to a possible physiological role of calcium-activated CaM in junctional regulation is the irreversibility of the uncoupling effect. It is known that some calcium binding proteins release calcium very slowly if no higher affinity sites are available (for example, the case of troponin C and parvalbumin; Haiech et al., 1979). A similar situation could have occurred here, such that calmodulin did not release calcium in the 40 min in which the low calcium solution was perfused. On the other hand, prolonged perfusions with calcium-activated CaM may uncouple the junctions irreversibly due to some other still poorly understood mechanism. Shorter perfusion periods are being tested currently in our laboratory to answer this question.

The morphological study of the perfused axons demonstrated that the perfusion maneuvers were effective in disrupting cyto-skeletal elements and thus replaced most soluble compounds present in the axoplasm. This replacement was accomplished without substantial modifications of normal characteristics of the gap junctions such as overall thickness and presence of transverse densities. Additionally, the synapses of axons perfused with calcium-activated CaM showed, in addition to normal gap junctions, thinner junctions where the width of the extracellular gap was reduced to about 3 nm. It seems unlikely that such an observation could correspond to a fixation artifact because neither unperfused axons nor synapses perfused with CaM and low calcium demonstrated these thinner junctions. Also, transitional regions were observed where the normal gap junctions were continuous with the thinner junctional profiles. Similarly, Peracchia and Dulhunty (1976) have reported that the width of the extracellular gap of crayfish gap junctions decreased in nerve cords treated with van Harreveld solutions containing dinitrophenol. Thus, the collapsing of the extracellular space resulting in a thinner junctional profile in axons perfused with calcium-activated calmodulin might be characteristic of uncoupled gap junctions. Although a detailed interpretation of the structure of the thinner junctional profiles is not possible, this observation suggests that the actions of CaM may involve both closure and removal or rearrangement of particles from the junctional plaques. This idea is congruent with the observations on axons uncoupled by axoplasmic acidification, where the number of channels in plaques decreases while the number of single channel-like particles increases (Zampighi et al., 1987). It is therefore possible that channel removal, perhaps by dispersal and concomitant with uncoupling via at least two mechanisms, may be a common feature of junctional regulation.

There is increasing evidence suggesting that a variety of hormones and neurotransmitters can alter the intracellular concentration of cAMP (Hax, van Venrooij & Vossenberg, 1974; Flagg-Newton, Dahl & Loewenstein, 1981; De Mello, 1984; Piccolino, Neyton & Gerschenfeld, 1984; Lasater & Dowling, 1985; Saez et al., 1986), and that these changes affect junctional coupling. The results reported here that junctional resistance is affected by the cytoplasmic calcium receptor calmodulin are not inconsistent with these views. As the metabolic pathways in which both second messengers cAMP and Ca^{2+} act are tightly interwoven, it is possible that effects in intact cells will not be clearly separable and, in fact, there may even be instances of parallel mechanisms of modulation.

In summary, the results described here show that calcium, via activated calmodulin, can increase the resistance of gap junction in crayfish lateral axons. This mechanism may also be at work in regulating junctional conductance in intact axons when the internal calcium concentration is increased.

We are grateful to Dr. J. Vergara for help with the calcium electrodes and useful comments throughout this work. This work was partially supported by NIH Grant EY04110 and MDA through the Center Grant. R.O.A. was supported by a scholarship from CONACyT (Mexico).

References

Arellano, R.O., Ramón, F., Rivera, A., Zampighi, G.A. 1986. Lowering of pH does not directly affect the junctional resistance of crayfish lateral axons. *J. Membrane Biol.* **94**:293–299

Brostrom, C.O., Wolff, D.J. 1976. Calcium-dependent cyclic nucleotide phosphodiesterase from brain: Comparison of adenosine 3',5'-monophosphate and guanosine 3'-5'-monophosphate as sustrates. *Arch. Biochem. Biophys.* **172**:301–311

Campos de Carvalho, A., Spray, D.C., Bennett, M.V.L. 1984. pH dependence of transmission at electronic synapses of the crayfish septate axons. *Brain Res.* **321**:279–286

Cheung, W.Y. 1980. Calmodulin plays a pivotal role in cellular regulation. *Science* **207**:19–27.

DeMello, W.C. 1984. Effect of intracellular injection of cAMP on the electrical coupling of mammalian cardiac cells. *Biophys. Res. Commun.* **119**:1001–1007

Flagg-Newton, J.L., Dahl, G., Loewenstein, W.R. 1981. Cell junction and cyclic AMP: I. Upregulation of junctional membrane permeability and junctional membrane particles by administration of cyclic nucleotide or phosphodiesterase inhibitor. *J. Membrane Biol.* **63**:105–121.

Girsch, S.J., Peracchia, C. 1985. Lens cell-to-cell channel pro-

tein. I. Self-assembly into liposomes and permeability regulation by calmodulin. *J. Membrane Biol.* **83**:217–225

Haiech, J., Deracourt, J., Pechère, J.F., Demaille, J.G. 1979. Magnesium and calcium binding to parvalbumins: Evidence for differences between parvalbumin and an explanation of their relaxing function. *Biochemistry* **18**:2752–2758

Haiech, J., Klee, C.B., Demaille, J.G. 1981. Effects of cations on affinity of calmodulin for calcium: Ordered binding of calcium ions allows the specific activation of calmodulin-stimulated enzymes. *Biochemistry* **20**:3890–3897

Hama, K. 1961. Some observations on the fine structure of the crayfishes (*Cambarus virilis* and *Cambarus clarkii*) with special reference to the submicroscopic organization of the synapse. *Anat. Rec.* **141**:275–293

Hax, W.M.A., Venrooij, G.E.P.M. van, Vossenberg, J.B.J. 1974. Cell communication: A cyclic-AMP mediated phenomenon. *J. Membrane Biol.* **19**:253–266

Hertzberg, E.L., Gilula, N.B. 1981. Liver gap junctions and lens fiber junctions: Comparative analysis and calmodulin interactions. *Cold Spring Harbor Symp. Quant. Biol.* **46**:639–645

Johnston, M.F., Ramón, F. 1981. Electrotonic coupling in internally perfused crayfish segmented axons. *J. Physiol. (London)* **317**:509–518

Johnston, M.F., Simon, S.A., Ramón, F. 1980. Interaction of anesthetics with electrical synapses. *Nature (London)* **286**:498–500

Keller, H.C., Bradley, B.O., LaPorte, D.C., Storm, D.R. 1982. Determination of the free-energy coupling for binding of calcium ions and troponin I to calmodulin. *Biochemistry* **21**:156–162

Lasater, E.M., Dowling, J.E. 1985. Electrical coupling between pairs of isolated fish horizontal cells is modulated by dopamine and cAMP. *In:* Gap Junctions. M.V.L. Bennett and D.C. Spray, editors. pp. 393–404. Cold Spring Harbor Lab., New York

Loewenstein, W.R. 1966. Permeability of membrane junctions. *Ann. N.Y. Acad. Sci.* **137**:441–472

Loewenstein, W.R. 1981. Junctional intercellular communication. The cell-to-cell membrane channel. *Physiol. Rev.* **61**:829–913

Moreno, A.P., Ramón, F., Spray, D.C. 1987*a*. Variation of gap junction sensitivity to H-ions with time of day. *Brain Res.* **400**:181–184

Pappas, G.D., Asada, Y., Bennett, M.V.L. 1971. Morphological correlates of increased coupling resistance at an electrotonic synapse. *J. Cell Biol.* **49**:173–188

Peracchia, C. 1973*a*. Low resistance junctions in crayfish: I. Two arrays of globules in junctional membranes. *J. Cell Biol.* **57**:54–65

Peracchia, C. 1973*b*. Low resistance junctions in crayfish: II. Structural details and further evidence of intercellular channels by freeze-fracture and negative staining. *J. Cell Biol.* **57**, 66–76

Peracchia, C. 1987. Calmodulin-like proteins and communicating junctions: Electrical uncoupling of crayfish septate axons is inhibited by the calmodulin inhibitor W7 and is not affected by cyclic nucleotides. *Pfleugers Arch.* **408**:379–385

Peracchia, C., Bernardini, G. 1984. Gap junction structure and cell-to-cell coupling regulation. Is there a calmodulin involvement? *Fed. Proc.* **43**:2681–2691

Peracchia, C., Dulhunty, A. 1976. Low resistance junctions in crayfish. Structural changes with functional uncoupling. *J. Cell Biol.* **70**:419–439

Peracchia, C., Girsch, S.J. 1985. Functional modulation of cell coupling: Evidence for a calmodulin-driven channel gate. *Am. J. Physiol.* **248**:*H765–H782*

Piccolino, M., Neyton, J., Gerschenfeld, H.M. 1984. Decrease of gap junction permeability induced by dopamine and cyclic adenosine 3′-5′-monophosphate in horizontal cells of turtle retina. *J. Neurophysiol.* **4**:2477–2488

Ramón, F., Rivera, A. 1986. Gap junction channel modulation. A physiological viewpoint. *Prog. Biophys. Molec. Biol.* **48**:127–153

Saez, J.C., Spray, D.C., Nairn, A.C., Hertzberg, E., Greengard, P., Bennett, M.V.L. 1986. cAMP increases junctional conductance and stimulates phosphorylation of the 27-kDa principal gap junction polypeptide. *Proc. Natl. Acad. Sci. USA* **83**:2473–2477

Spray, D.C., Bennett, M.V.L. 1985. Physiology and pharmacology of gap junctions: *Annu. Rev. Physiol.* **47**:281–303

Steiner, R.F., Lambooy, P.K., Sternberg, H. 1983. The dependence of the molecular dynamics of calmodulin upon pH and ionic strength. *Arch. Biochem. Biophys.* **222**:158–169

Tasaki, I., Singer, I., Takenaka, T. 1965. Effects of internal and external ionic environment on excitability of squid giant axons. A macromolecular approach. *J. Gen. Physiol.* **48**:1095–1123

Tsien, R., Rink, T.J. 1980. Neutral carrier ion-selective microelectrodes for measurement of intracellular free calcium. *Biochim. Biophys. Acta* **599**:623–638

Turin, L., Warner, A. 1977. Carbon dioxide reversibly abolishes ionic communication between cells of early amphibian embryo. *Nature (London)* **270**:56–57

Van Eldik, L.J., Hertzberg, E.L., Berdan, R.C., Gilula, N.B. 1985. Interaction of calmodulin and other calcium-modulated proteins with mammalian and arthropod junctional membrane proteins. *Biochem. Biophys. Res. Commun.* **126**:825–832

Watanabe, A., Grundfest, H. 1961. Impulse propagation at the septal and commisural junctions of the crayfish lateral giant axons. *J. Gen. Physiol.* **45**:267–308

Watterson, D.M., Sharief, F.S., Vanaman, T.C. 1980. The complete amino acid sequence of the Ca^{++}-dependent modulator (calmodulin) of bovine brain. *J. Biol. Chem.* **255**:962–975

Welsh, M.J., Aster, J.C., Ireland, M., Alcalá, J., Maisel, J. 1982. Calmodulin binds to chick lens gap junction protein in a calcium-independent manner. *Science* **216**:642–644

Zampighi, G.A., Kreman, M., Ramón, F., Moreno, A., Simon, S.A. 1987. Structural characteristics of gap junctions: I. Channel number in coupled and uncoupled conditions. *J. Cell Biol. (in press)*

Zampighi, G.A., Ramón, F., Durán, W. 1978. Fine structure of the electrotonic synapse of the lateral giant axons in a crayfish (*Procambarus clarkii*). *Tissue Cell* **10**:413–426

Received 22 June 1987; revised 16 November 1987