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Lowering of pH Does Not Directly Affect the Junctional Resistance of Crayfish Lateral Axons

Rogelio O. Arellano, Fidel Ramón, Amelia Rivera, and Guido A. Zampighi†
Departamento de Fisiología y Biofisica, 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. The effect of pH was tested on the junction between crayfish lateral axons. By means of a glass capillary inserted into one of the axons, one side of the junction was perfused with solutions of known pH while the junctional resistance, R_j, was monitored. Integrity of the gap junction was checked electron microscopically. R_j remained unchanged when the pH of the perfusate was lowered from 7.1 to 6.0. However, when the pH of the unperfused side of the junction was lowered by substituting acetate for chloride in the external solution, R_j rose, attesting to the integrity of the junction and its capacity to uncouple in the perfused state. We suggest that H^+ does not affect the junctional channels directly, but acts through an intermediary which is inactivated or removed by the perfusion.

Key Words gap junction channels · pH dependence

Introduction

An increase in the intracellular concentration of hydrogen ions has been implicated in closing gap junctional channels [8, 9, 11]. A related question is whether or not hydrogen ions act directly on the gap junctional channels. In blastomeres of amphibian and teleost embryos, Spray et al. [8] measured an apparent pK of 7.2 to 7.3 of the junctional resistance change with decreased pH_i. Based on these data, they assumed that H ions directly titrate the macromolecule that comprises the channel, perhaps in the imidazole groups of histidine residues, or the amino terminal of a polypeptide chain.

However, there are experimental results suggesting that hydrogen ions do not act directly on the junctional channels. Evidence of this comes from experiments with internally perfused coupled crayfish axons [3]. In these axons lowering the pH of the internal solution to 5.4 did not change the junctional resistance that increased significantly after addition of heptanol or octanol to the external medium. This result suggested the possibility of a required intermediary for the changes in junctional resistance and recent experimental evidence has suggested that calmodulin [5] or components of the phosphoinositide messenger route [14] may have such a role. Experiments performed on isolated junctional proteins from liver also indicate that the effect of hydrogen ions could be indirect, since no changes in the X-ray patterns of EGTA-treated junctions were detected using solutions with pH 6.0 to 8.0 [12, 13].

To further analyze if hydrogen ions can act directly on the junctional proteins to induce uncoupling, we have performed experiments on coupled crayfish axons, only one of which was internally perfused. Similarly, with respect to the bilateral perfusion, this unilateral perfusion does not produce changes in junctional resistance when the pH of the internal solution is lowered to 6.0. However, the resistance increases when the sodium chloride of the external solution is replaced by sodium acetate. Based on these results, we conclude that the effects of hydrogen ions on the junctional proteins is not exerted directly.

Materials and Methods

Experimental Preparation

Experiments were performed on lateral axons from the abdominal nerve cord of crayfish *Procambarus clarkii* obtained from a local supplier. Nerve cords were excised from crayfish, desheathed, and the second ganglion was placed in an experimental chamber containing a modified van Harreveld solution (*see below*).

Internal Profusion

Internal profusion of one axon of a coupled pair was performed in a manner similar to that previously described for perfusion of

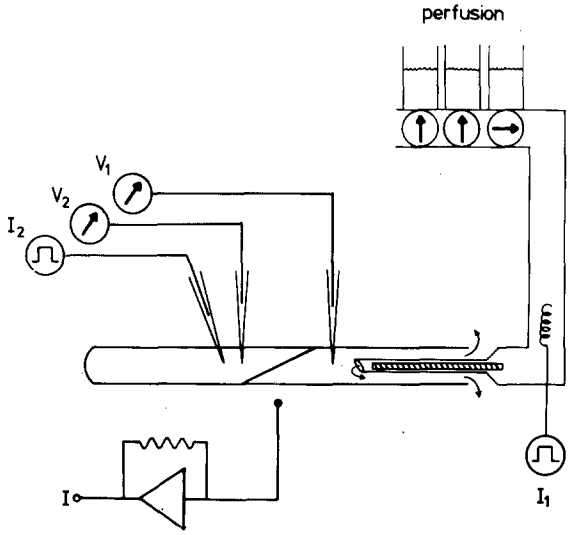

Fig. 1. Diagram of the experimental setup. An internal cannula and three intracellular microelectrodes provide the arrangement necessary to inject pulses of constant current and record the transmembrane potential at each side of the septum. The current injected into the axons is measured with an electrode in the bath solution

both axons [3]. For this maneuver a long cannula is introduced into the axon through a small cut made as far as possible from the septum (about 5 mm). This cannula is a 50-μm o.d., 35-μm i.d. glass capillary containing a floating bare platinum wire (18 μm diameter) to reduce its high frequency impedance when used as an electrode. When filled with SIS the DC resistance of the electrode is about 10 MΩ (Fig. 1). The volume contained in the segment of the cannula inserted in the axon is 1/4-1/5 of the axonal volume left. Since solution flows driven by a pressure head of ~8 cm of water, the cannula fluid is replaced about twice per minute and the axon volume in 2-3 min.

The composition of the internal and external solutions are shown in the Table.

Electrophysiological

Injection of current pulses and measurement of membrane potentials were performed using internal microelectrodes (containing a glass fiber and with resistance of about 5-10 MΩ). The internal cannula was also used as an electrode, mostly for injection of current pulses. These pulses were delivered by a Howland current source and the current injected into the axons was recorded with a bath electrode connected to an operational amplifier. The basic relations used to calculate the septal (R_j) and input (R_m) resistances have been described elsewhere [6].

In these experiments we have used the replacement of sodium chloride in the SES for sodium acetate to produce a decrease in internal pH from a control value of about 7.1 to a minimum value of 6.2-6.3 as measured with pH-sensitive microelectrodes (A. Moreno, *personal communication*). As we have shown, this decrease in pH_i elicited by the acetate solution is always the same independently of the R_j values finally attained.

To test the extent to which a cation would diffuse into the perfused axons, we performed some experiments in which we added 1 mM $LaCl_3$ to a perfusion solution with potassium gluta-

Table. Composition of solutions (in mM)

	SIS	SIS-low pH	SES	SES-acetate
NaCl	15	15	205	—
Na-acetate	—	—	—	205
KCl	—	—	5.4	5.4
KF	109	109	—	—
K_3-citrate	37	37	—	—
$CaCl_2$	—	—	13.5	13.5
Sucrose	96	96	—	—
HEPES	5.0	—	5.0	5.0
MES	—	5.0	—	—
pH ± 0.1	7.1	6.0	7.5	7.4

SES, standard external solution; SIS, standard internal solution.

mate instead of potassium fluoride, since lanthanum precipitates in the presence of fluoride. These axons survive for only short periods of time (15-30 min), and we did not make electrical recordings under these conditions. The localization of lanthanum deposits (*see below*) is an indication of how far the perfusion solution reaches into the axon.

Morphological

The nerve cords containing the perfused axons were fixed with the cannula *in situ*, since we found that if the cannula is removed from the axon prior to fixation the septum is ruptured at the synaptic region. This rupture is due to suction produced by withdrawing the cannula, since the synaptic region consistently ballooned and broke towards the side of the perfused axon.

The fixative was a solution of 3% glutaraldehyde-hydrogen peroxide in 0.2 M cacodylate buffer containing 100 mM $NaHCO_3$, at a pH of 7.3. The addition of $NaHCO_3$ produced precipitation of the ionic lanthanum in the perfusion solution into large deposits easily visualized in the microscope. After 30 min, the fixative was replaced by 3% glutaraldehyde in 0.2 M cacodylate buffer with $NaHCO_3$ and the preparation left in this solution for 1 hr at room temperature. After fixation, the cords were washed three times in 0.1 M cacodylate buffer with 4% sucrose and $NaHCO_3$ and postfixed in 2% osmium tetroxide for 90 min. Dehydration was done in ethanol and infiltration and embedding in Epon 812 as described previously [15].

The perfused axon and the septal regions displaying the synapses were identified in thick sections cut perpendicular to the long axis of the axon. Thin sections were cut with a diamond knife, deposited on formar-coated single-hole grids and stained for 30 sec with lead citrate to help in the identification of the septum. Observations were made with a Zeiss EM10C electron microscope, using the reduced focal length specimen holder to increase the contrast of the specimen.

Results

Physiological

When all the sodium chloride of the external solution is replaced by sodium acetate, the junctional

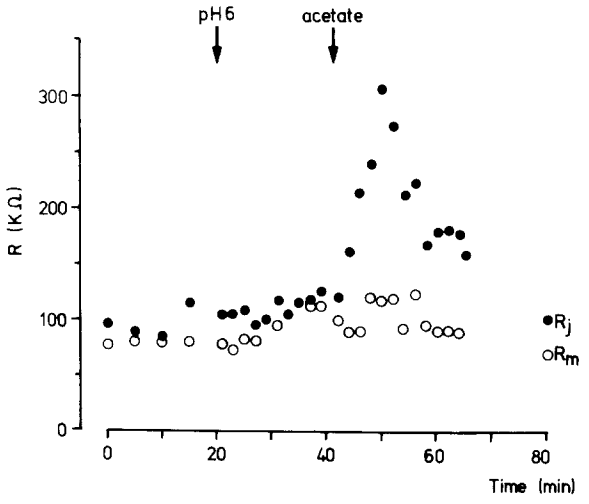

Fig. 2. Effect of pH_i on the junctional resistance of unilaterally perfused axons. The filled circles show the junctional resistance values which remain near the control values during the 20 min that SIS at pH 6.0 was flowing into the axon. However, as soon as SES-acetate is introduced in the external solution, R_j increases up to a value of about 300 kΩ. The open circles show that there is no appreciable change in the input resistance of the perfused axon during this time

resistance of control (unperfused) axons increases from a basal value of 50-100 to a maximum of about 1000 kΩ. Concomitantly, there is a decrease in the internal pH from 7.1 to about 6.2 (A. Moreno, *personal communication*).

To test the effect of internal pH on the coupling resistance we perfused only one axon of a coupled pair with SIS at different pH's. In all experiments we obtained data such as that illustrated in Fig. 2. Here the junctional resistance had a control value of about 80 kΩ and for 20 min after the perfusion solution was changed for another at pH 6.0, the resistance changed very little. However, when sodium acetate replaces the sodium chloride of the bath solution, R_j increases up to 300 kΩ and then drops to a steady value of about 150 kΩ. Throughout this time the input resistance (R_m) of the perfused axon remains at the control value. The increase in resistance produced by acetate is seen also when it is not preceded by the low pH internal solution.

The results from four different types of experiments on perfused and nonperfused axons are illustrated in Fig. 3. These results were obtained in three different occasions but we have plotted only one set of data. The stars show the response to acetate of control (nonperfused) axons where R_j increases from about 80 to the usual nearly 1000 kΩ. In the same figure we show (with open and filled circles) data from unilaterally perfused axons. The open circles correspond to an internal solution at a pH of 7.1, where the application of acetate solution produces an increase in R_j to about 500 kΩ. The filled circles show data from axons unilaterally perfused with SIS at a pH of 6.0 for 20 min, during which time there is no change in R_j. However, upon introducing the acetate solution in the bath, the junctional resistance increases to about 300 kΩ. For comparison, the X's show data obtained when both axons of a coupled pair are internally perfused (redrawn from Fig. 25 of ref. 2). In this later case, acetate has no effect on the junctional resistance, which remains at the control value.

Morphological

The septum connecting anterior and posterior lateral axons is a prominent structure about 4 μm thick. It is comprised of extracellular space (*ES*, Fig. 4) filled with a fibrillar material that forms electron-dense laminas with associated thin, overlapping glial flaps. The synapses (also called "windows") are seen as interruptions of the septum that allow both axoplasms to come in direct contact with each other.

In these experiments the two segments comprising the lateral axon synapse have a different appearance, even at the low magnification used to identify them in plastic thick sections. The perfused segment has a clear lumen because most of its axoplasm is removed by the perfusion solution, although some disorganized axoplasmic components are still seen. The nonperfused axonal segment retains its normal axoplasmic components (mitochondria, microtubules, synaptic vesicles, etc.). However, the axoplasm of the nonperfused axon shows the appearance of numerous 150-300 nm diameter vesicles. These vesicles are the result of perfusion because they were absent in the axoplasm of control axons [15].

Figure 4 shows a thin section through the septal region of a preparation where the anterior axon was perfused. Note in this axon (left) the swelled mitochondria and large vacuola. Note also, toward the septum, small deposits of a dense material associated with the plasma membranes (arrows). These deposits are due to the lanthanum chloride perfused in this experiment. The septum in Fig. 4 shows a synaptic region at the upper end (region enclosed by the square).

Figure 5 shows the synapse enclosed in the square in Fig. 4. Figure 5*A* shows the entire length of contact between the two axons and *B* a higher magnification of the synaptic region. The following several features shown in this plate further demonstrate that the perfusion solution washed the synapse area: (i) electron-dense deposits produced by precipitation of ionic lanthanum are seen only asso-

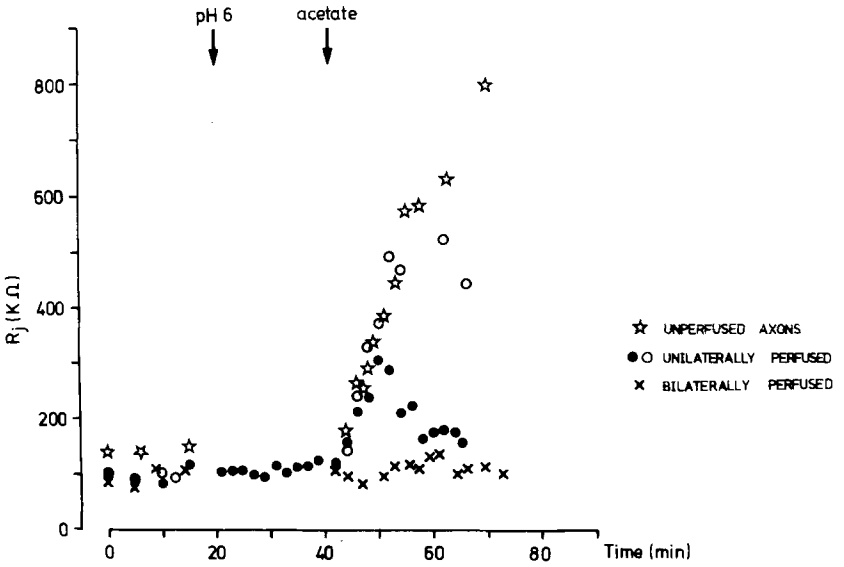

Fig. 3. Comparison of the effects of pH_i on the junctional resistance of crayfish septated axons under different conditions. The stars show the effect of SES-acetate on control (nonperfused) axons where R_j increases up to about 800 kΩ. Acetate also produces an increase in axons unilaterally perfused with SIS at a pH of 7.1 (open circles) or 6.0 (filled circles). This last case is plotted in Fig. 2. In contrast, acetate does not increase R_j when both axons of a coupled pair are internally perfused (×'s; redrawn from Fig. 25 of ref. 2)

ciated with the cytoplasmic surface of the perfused axon (side arrows, Fig. 5*A*) and not to the other axon or the extracellular space; (ii) similar deposits are also associated with the cytoplasmic surface of the synapse on the perfused side (middle arrow, Fig. 5*A*); (iii) the vesicles that have been described associated with both sides of the synapse [4, 15] are clearly present on the normal side, but absent on the perfused side (arrows, Fig 5*B*).

Discussion

In this paper we have shown data obtained when one axon of a coupled pair is internally perfused. This experimental arrangement is ideally suited to studying the effects of compounds applied to an internally perfused axon, as the nonperfused axon serves as a control.

A primary concern when attempting the internal perfusion of coupled axons is that the perfusing maneuvers do not damage the coupling junctions (e.g., the perforation of the septum that has been suggested by others [8]). The absence of damage was demonstrated in the bilaterally perfused preparation by the increase in junctional resistance produced with the external application of the alcohols, heptanol and octanol [3]. The experiments presented in this paper provide further proof of the intactness of the gap junctions during internal perfusion as the external substitution of chloride by acetate succeeds in increasing the junctional resistance. It is difficult to see how this can happen if the gap junctions were mechanically disrupted during perfusion. However, we explored further this possibility by performing a parallel ultrastructural characterization of the unilaterally perfused axons. This study shows that a large amount of axoplasm can be removed from unilaterally perfused axons while keeping the integrity of the gap junctions. Therefore, internal perfusion as described in this, as well as in a previous publication [3], does not perforate the septal area as has been suggested by others [7].

The morphological characterization of the unilaterally perfused axons shows that portions of the axoplasm remain in the lumen of the perfused axons. This result raised the question of whether solutions perfused through the cannula reached the septal regions containing the gap junctions. To test this possibility, axons were perfused with solutions containing 1 mM $LaCl_3$ that was precipitated with $NaHCO_3$ at the same time that the tissue was fixed. The direct observation of lanthanum deposits associated with the axolemmae and gap junctions indicates that the perfusion solution reaches the synapses. Therefore, the fact that internal solutions at pH 6.0 do not uncouple both unilaterally (this study) or bilaterally [3] perfused axons cannot be interpreted as rupture of the gap junctions or to failure of hydrogen ions to reach the gap-junctional proteins. Similar lack of effect of acid solutions has been observed in single channels from lens gap junctions reconstituted in planar lipid films [16]. However, liver gap junctions reconstituted in lipid films by Spray et al. [10] seem to close at acid pHs.

In the unilateral perfusion experiments reported here the internal pH of the nonperfused axon was not measured directly upon replacement of the external chloride for acetate. However, we have shown (A. Moreno, *personal communication*) that every time chloride was replaced by acetate, the internal pH of the axoplasm decreased from 7.1 to

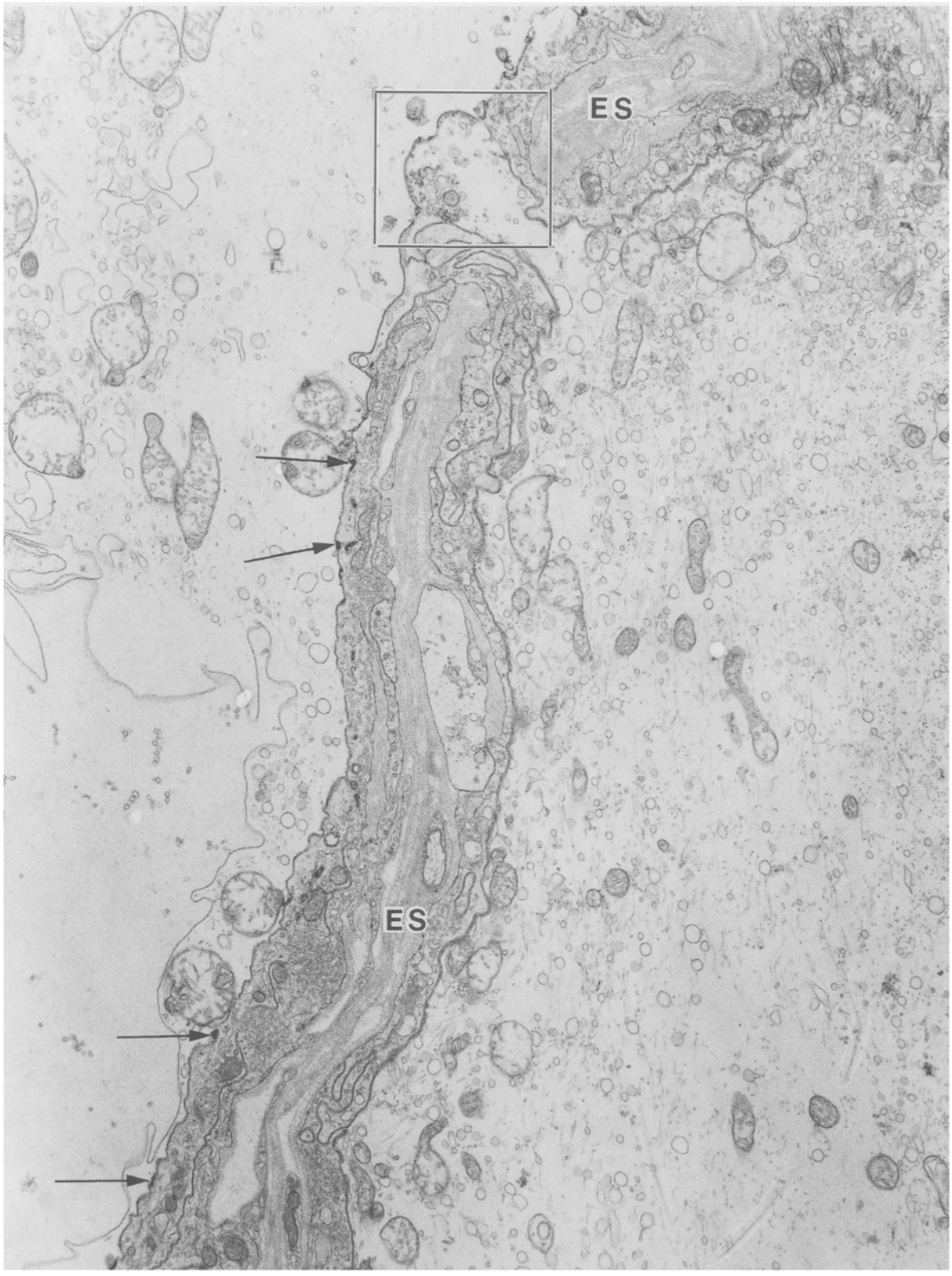

Fig. 4. Low magnification view of the region of the septum between two crayfish lateral axons. The anterior axon (left side) was internally perfused. The square encloses a gap junction. The arrows point to small dense lanthanum deposits located exclusively on the perfused side. Magnification: ×8,460

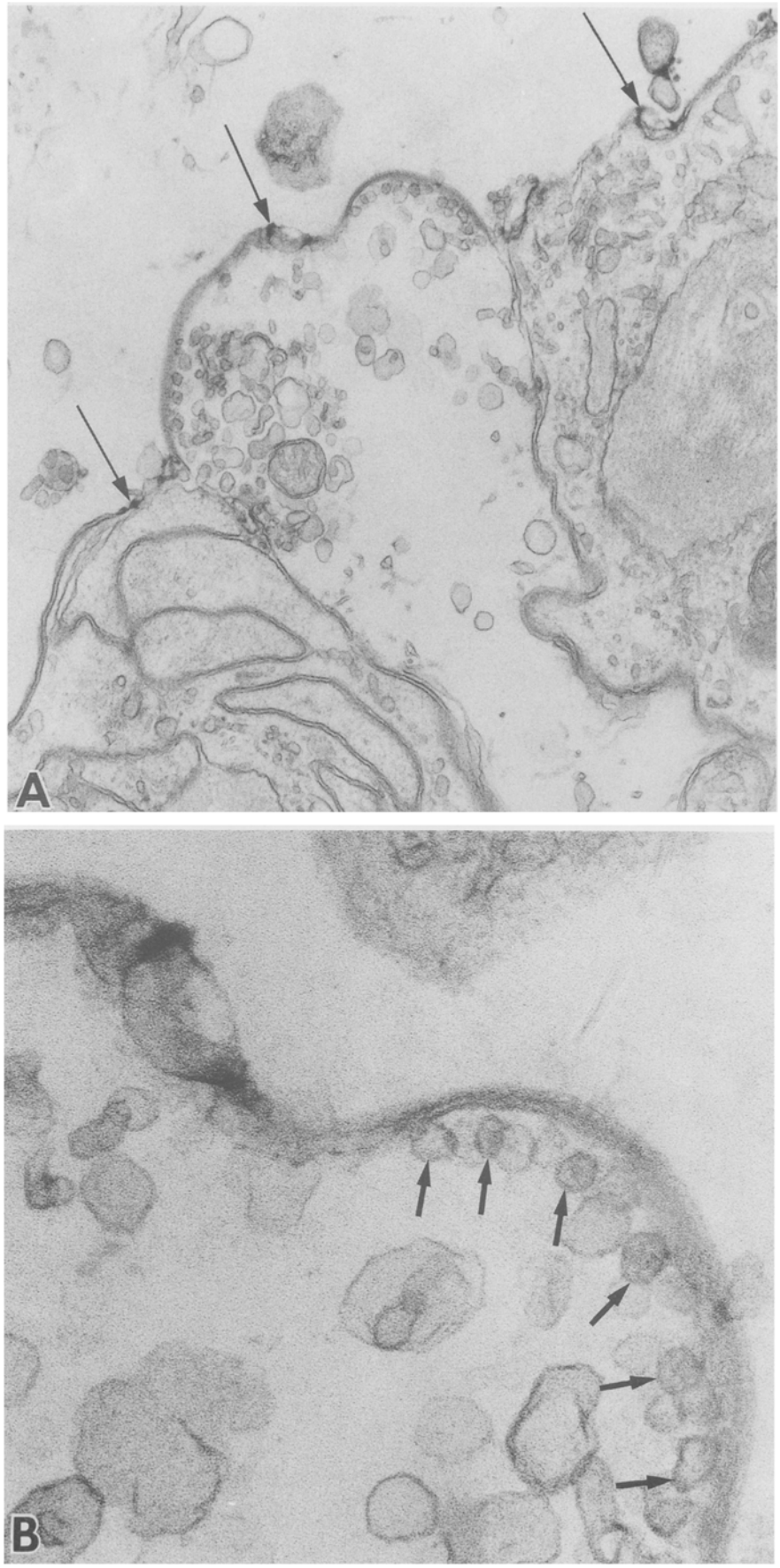

Fig. 5. (*A*) Shows the area enclosed in the square of Fig. 4. Note the presence of dark deposits associated not only with the axolemmae, but also with the gap junction membrane of the perfused axon. Note also the lack of synaptic vesicles in the perfused axon. Magnification: ×68,250. (*B*) Higher magnification of the gap junction, close to the region labeled by the middle arrow in *A*. The arrows point to the asymmetric distribution of synaptic vesicles, which are absent in the perfused side. Magnification: ×109,200

about 6.3 independently of the value of R_j finally attained. Therefore, there is little doubt that the internal pH of the nonperfused axons must also decrease, whereas the perfused axon should remain at the value fixed by the perfusing solution. The same effect is observed whether the anterior or posterior axon was perfused, which is an indication of the symmetry of the system [13].

Acidification of the axoplasms by acetate results in an increase in R_j of both intact and unilaterally perfused axons (Fig. 3). However, the maximum value of R_j is always larger in intact than in unilaterally perfused axons. A possible explanation for this observation could be that the perfusion contributes a washing effect which could, among other things, remove small molecules from the nonperfused side. In this respect we note that the axoplasm of the nonperfused axon contains numerous 150-300 nm diameter vesicles which are not present in the controls. Therefore, the unilaterally perfused axon can be considered the intermediate case between the intact axons (large uncoupling by acetate) and the bilaterally perfused axons (no uncoupling by acetate).

The most important observation reported here is that perfusion of a solution at pH 6.0 has no effect on R_j, but acidification of the axoplasm at pH 6.3 by addition of acetate to the *external* solution always uncouples the axons. The simplest interpretation of this observation is that hydrogen ions do not uncouple the crayfish lateral axons by direct action, but that they necessitate some still unidentified axoplasmic component(s) which is (are) washed away or inactivated by the perfusion. This interpretation contrasts with the one proposed for *Ambystoma* and *Fundulus* blastomeres [8], where a direct interaction between protons and channels was proposed. On the other hand, this interpretation supports the original suggestion of an intermediary necessary for closing the channels advanced by Johnston and Ramón [3]. This interpretation also supports recent observations that second-messenger molecules are involved in gap-junction regulation [14].

In summary, our experimental results indicate that hydrogen ions do not act directly on the gap junction channels of crayfish lateral axons to produce changes in coupling resistance. Therefore, the present results suggest that the apparent pK of 6.7 and the Hill coefficient of about 2.7 previously reported for this gap junction [1] should be interpreted to indicate the behavior of the whole system (the gap junctions and the surrounding axoplasm), rather than that of the communicating channels.

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