

Protein phosphorylation and hydrogen ions modulate calcium-induced closure of gap junction channels

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ABSTRACT The regulation of the cell-to-cell pathway formed by gap junctions seems to involve the interaction of the junctional channels with either calcium or hydrogen ions, as well as protein phosphorylation and calmodulin. These mechanisms of junctional regulation have been considered to act independently on specific sites of the gap junction protein; however, the possibility that they may be interrelated has not been adequately explored mainly

due to the difficulties involved in simultaneous measurement of intracellular cations and protein phosphorylation. To further understanding of mechanisms regulating gap junctions, we have internally perfused coupled lateral axons from crayfish with solutions containing different calcium and hydrogen concentrations under conditions favoring phosphorylation, while monitoring the junctional conductance. We found that calcium ions regulate cell commu-

nication probably through a direct interaction with the channel protein. Phosphorylation and low pH do not alter junctional conductance themselves, but appear only to modulate the effects of calcium, possibly by altering the affinity of the channel for calcium. We propose that a combination of free intracellular calcium and protein phosphorylation form an important physiological mechanism regulating intercellular communication.

INTRODUCTION

The crayfish nervous system possesses two giant lateral axons that course from the cerebroid ganglion to the sixth abdominal ganglion. They are formed by neurons that originate in each ganglion and are electrically connected to the axon of the posterior neuron by $\sim 4 \times 10^5$ gap junction channels (1). The regulation of these channels seems to include calcium (2) and hydrogen ions (3, 4), as well as protein phosphorylation (5–7) and calmodulin (8, 9). However, attempts to elucidate the relationships between these regulatory mechanisms have been hampered because the metabolic pathways in which they participate are interwoven (10). It seemed clear that the problem could be addressed by testing the effects of these putative regulators on junctional conductance in a controlled way, first alone and then in combination.

The large diameter of crayfish lateral axons permits both the control of their internal medium by perfusion and the simultaneous recording of the junctional conductance between cells, and we have used this preparation to test putative regulatory compounds for their effects on junctional conductance. Our results indicate that the channel protein changes to a closed state when calcium ions interact with a site that is exposed after phosphorylation or protonation.

METHODS

Preparation

Experiments were performed on the second abdominal ganglion of the crayfish. Fig. 1 shows a photograph of the preparation with the perfusion cannula and the recording arrangement. Injection of 150-ms rectangular current pulses and voltage recordings were made with the cannula and intracellular microelectrodes, and the junctional resistance was calculated as described previously (11); the data are presented as junctional conductance (g_j). In these experiments the internal cannula and one microelectrode in the nonperfused axon were used to stimulate, while the other two electrodes nearest the septum were used to record.

Internal perfusion

We perfused one axon of a coupled pair with a glass cannula 50 μm in diameter and short-circuited with an internal platinum wire 18 μm in diameter. The perfusion solution flowed into the cannula and axon with a pressure of 8 cm water, the axoplasm was almost totally removed, and the solution reached the junctional area in ~ 1 min (12, 13). Perfusion of the solution was maintained continuously, and fluid exited via the cut in the axon made to introduce the cannula (located ~ 7 mm from the septum). Experiments were performed at 18°C.

Solutions

In all experiments the preparation was bathed in a modified van Harreveld solution (SES) containing (in millimolar): NaCl 205, KCl 5.4, CaCl_2 13.5, Hepes 5 (Sigma Chemical Co., St. Louis, MO) at a pH of 7.4. The Standard Internal Solution (SIS) contained (in millimolar): NaCl 15, KF 33, K-glutamate 187, sucrose 12, and MOPS or MES 15 (Sigma Chemical Co.), for a pH of 7.0 or 6.0, respectively. The phosphorylating cocktail (SIS-P) contained, in addition to SIS (in

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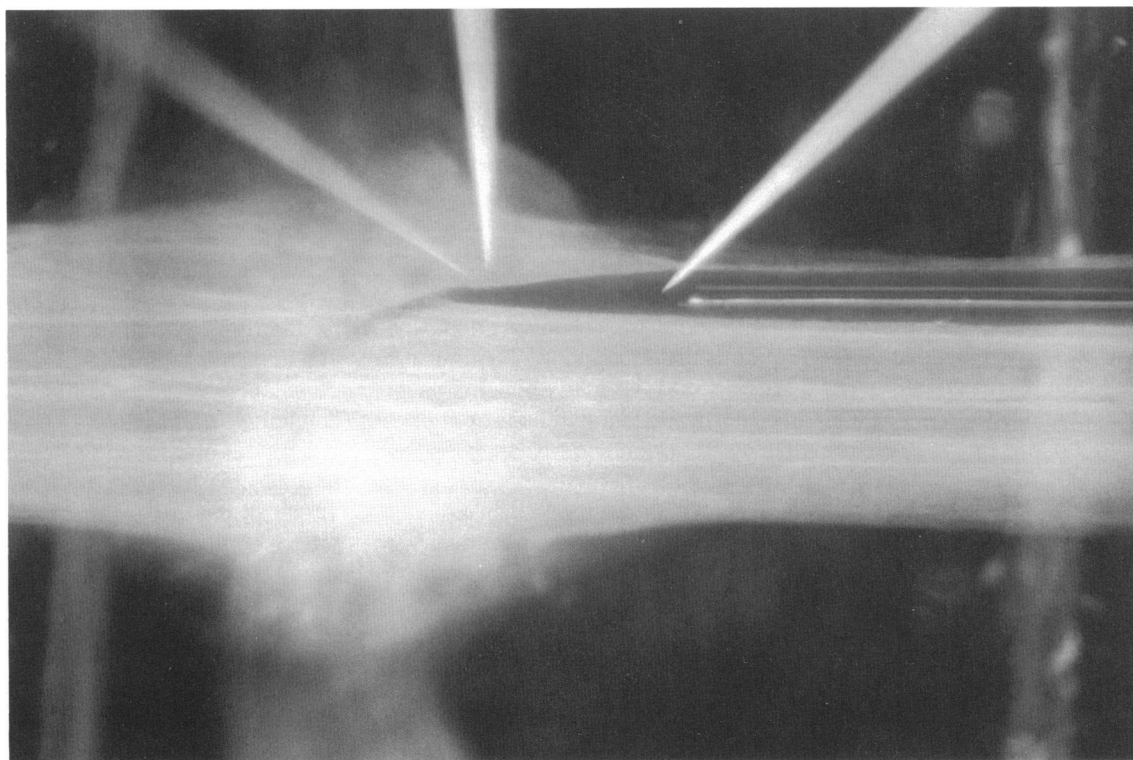


FIGURE 1 Second abdominal ganglion of the crayfish showing the internal cannula inserted into an anterior lateral axon and the electrodes used for voltage recording and current injection. The region of synaptic contact or septum is visible as a diagonal line dividing the lateral axons over the ganglion. In this photograph of an uncoupled preparation the perfusion solution contained Fast Green to aid visibility; the dye was not employed in the experiments. Experiments were performed at 18°C.

millimolar): MgCl_2 4, adenosine-5'-triphosphate 5 (ATP, 519987), cyclic adenosine monophosphate 0.01 (cAMP, 102300) both from Boehringer Mannheim GmbH, Mannheim, FRG, and cAMP-dependent protein kinase 100 $\mu\text{g}/\text{ml}$ (PK, P5511; Sigma Chemical Co.). Internal solutions containing calcium were made by titrating with 0.1 M CaCl_2 and measuring the calcium concentration with a calcium-sensitive electrode (9). EGTA (2–5 mM; Sigma Chemical Co.) was used as buffer substituting an equimolar quantity of sucrose. Solutions with low calcium ($\text{pCa} > 7.0$, labeled as 7.2) were prepared with 10 mM EGTA or 10 mM BAPTA (Sigma Chemical Co.) for pH 7.0 or 6.0, respectively. Other compounds used were adenylylmethylenediphosphate (AMP-PCP; 102555, Boehringer Mannheim GmbH), the catalytic subunit of the cAMP-dependent protein kinase (P6022, Sigma Chemical Co.), Walsh inhibitor (P5015, Sigma Chemical Co.), and guanosine-5'-triphosphate (GTP; 106372, Boehringer Mannheim GmbH).

RESULTS AND DISCUSSION

The perfusion of solutions containing high calcium alone (SIS, pCa 5.0) did not have an effect on the junctional conductance which, however, decreased markedly when a phosphorylating cocktail was added (SIS-P, pCa 5.0). Fig. 2 shows a plot of junctional conductance (solid circles) during perfusion of a high-calcium solution (bar labeled SIS pCa 5.0), which was substituted after 17 min

for one with high calcium and a phosphorylating cocktail (bar labeled SIS-P pCa 5.0). The conductance did not change from control values (in SIS) when high calcium was perfused, but decreased rapidly upon perfusion of high calcium and the phosphorylating cocktail. This effect was rapidly and completely reversed when a solution containing low calcium and no phosphorylating cocktail was perfused (SIS pCa 7.2). The input conductances (i.e., conductances to bath) of the axons did not change significantly during the experiment (inverted triangles).

This effect of the phosphorylating cocktail was further examined by using different calcium concentrations in the perfusion solutions, and the results of these experiments are shown in Fig. 3. The first 5 min show the control junctional conductance in SIS, which averaged 20 μS for all experiments. At 5 min, SIS was changed to a solution containing the phosphorylating cocktail and calcium at pCa 's of 7.2, 6.4, 6.0, 5.5, 5.0, or 4.0, all at pH 7.0 (bar labeled SIS-P). No change in junctional conductance was seen at pCa 7.2, an intermediate decrease at pCa 6.4 and 6.0, and a large decrease at pCa 5.5, 5.0, and 4.0. Most importantly, these effects were also completely reversed several minutes after washing out the internal solution with one containing low calcium (SIS pCa 7.2, not

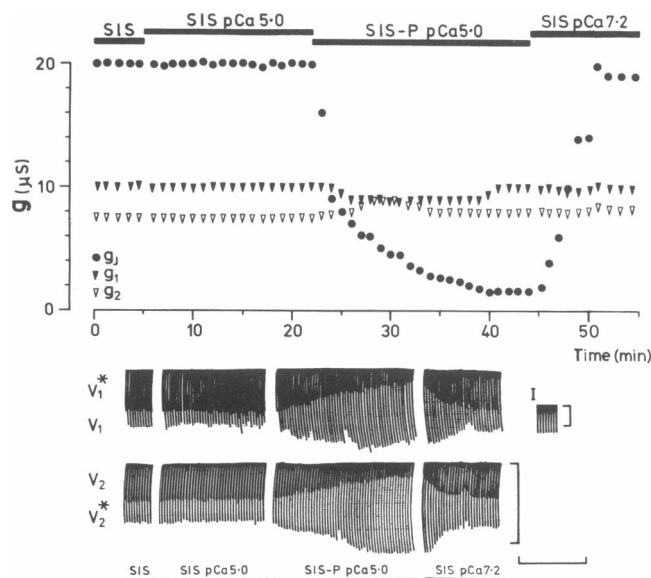


FIGURE 2 Internal perfusion of high calcium solutions induces a decrease in junctional conductance only in the presence of a phosphorylating cocktail. The figure shows a graph of g_j 's calculated from the original voltage traces from both axons shown at the bottom. The four voltage segments are labeled below with the corresponding solutions perfused during each. The asterisk on the V 's denotes the voltage responses to current injected in axon 2; no asterisk denotes the responses to current injected in axon 1. The applied current pulses are shown to the right (I). No change in junctional conductance was seen when the solution contained high calcium without the phosphorylating cocktail; however, a solution with the same calcium concentration and a phosphorylating cocktail decreased g_j . Upon perfusion with a solution containing low calcium and without the phosphorylating cocktail, the junctional conductance recovered. The low calcium solution contained 10 mM EGTA. All solutions were buffered at pH 7.0. Calibrations: vertical 5 mV and 50 nA; horizontal 10 min.

shown). The time course of the decrease in conductance depended on the pCa, being faster for the solutions containing higher calcium concentrations. The changes on g_j were fitted to exponentials whose τ 's were 7.3 min for pCa 4.0, 8.8 min for pCa 5.0 and 5.5, 17 min for pCa 6.0, and 19.6 min for pCa 6.4.

To ascertain that the effect on junctional conductance was dependent on protein phosphorylation, we perfused the axons with either a solution of SIS-P where cAMP and cAMP-dependent PK were substituted by the catalytic subunit of the cAMP-dependent PK (100 U) at pCa 5.0, a solution of SIS-P where ATP was substituted by a nonhydrolyzable analogue (AMP-PCP; 5 mM) at pCa 5.0, or a solution of SIS-P to which we added Walsh inhibitor (WI; 1 mg/ml), a specific inhibitor for the cAMP-dependent protein kinase. The results are shown in the records labeled 5.0 CS, 5.0 AMP-PCP, and 5.0 WI of Fig. 3, where it can be seen that the AMP-PCP and WI solutions had no effect, whereas the CS solution produced

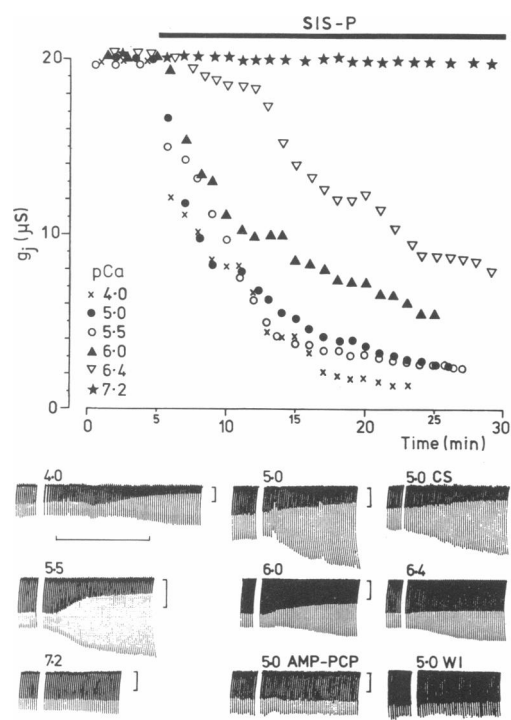


FIGURE 3 Effect of different calcium concentrations in the presence of a phosphorylating cocktail on the junctional conductance. The top shows a plot of g_j for each of the solutions tested. SIS-P containing low calcium (pCa 7.2, stars) had no effect on g_j , whereas high calcium (pCa 4.0, crosses) produced a large and fast decrease. The other pCa's (5.0, 5.5, 6.0, and 6.4) tested had intermediate effects. The lower part shows the original recordings of the voltage responses from which g_j was calculated. Only the responses from one axon are shown due to space limitations; both traces were similar. The traces are labeled with the pCa of the test solution, the CS, AMP-PCP, and WI records are explained in the text. The short segment at the left of each record shows the control response. At least two experiments were conducted at each pCa. Calibrations: vertical 5 mV; horizontal 10 min (bar below top left trace applies only to that trace).

a substantial decrease in the junctional conductance, which was 1 μ S at the end of the trace. The effects of the latter can be compared to its control solution which is SIS-P at pCa 5.0 and is labeled 5.0 in the figure. In other controls (not shown), the decrease in junctional conductance produced by the phosphorylating cocktail and calcium was largely inhibited (90%) by each of the following changes to the perfusion solution: elimination of $MgCl_2$; substitution of GTP (5 mM) for ATP, or substitution of the regulatory subunit of the PK (100 U) for PK and cAMP.

These results strongly suggest that the observed changes in conductance are dependent on protein phosphorylation. While it is not practicable to demonstrate junctional channel phosphorylation in a single axon, it is encouraging to note that some junctional proteins have

been shown to be phosphorylated by the cAMP-dependent protein kinase (5, 14). Thus, it is possible that phosphorylation of the protein channel, or of some associated protein, is involved in closure. An attractive mechanism that may explain the effects of phosphorylating solutions and high calcium is that protein phosphorylation modulates the calcium-induced closure of the channels (see below).

We also explored the effects of low pH on the uncoupling produced by the phosphorylating cocktail and different calcium concentrations. For these experiments (not shown) all solutions were buffered at pH 6.0. The results were qualitatively similar to those described for pH 7.0, i.e., solutions with pCa 7.2 induced only a very slight decrease in the junctional conductance, whereas those with pCa's less than 7 produced marked decreases, to 1–2 μ S, whose time constants were again dependent on the pCa of the solution. However, the time constants at each pCa were faster than those observed for the equivalent solutions buffered at pH 7.0. The time constants ranged from 5.2 ± 2.0 (mean \pm SE) min for pCa 5.0 to 7.0 ± 1.1 (mean \pm SE) min for pCa 6.8. In another series of experiments (not shown), solutions of different calcium concentrations but without the phosphorylating cocktail were tested at pH 6.0. Those solutions having pCa 6.0 or less induced a decrease in the junctional conductance, whereas those of pCa 7.2 had negligible effect. Final conductance decreases were $\sim 2 \mu$ S. The time constants of the conductance changes also varied with pCa, with values ranging from 12.1 ± 1.1 (mean \pm SE) min for pCa 6.0 to 9.1 ± 1.5 (mean \pm SE) min for pCa 5.2.

These results indicate that low pH and phosphorylation, either alone (13) or in combination, do not induce significant changes in the junctional conductance. In addition, calcium and low pH induce a conductance decrease similar to that seen with the phosphorylating cocktail and calcium (see below); and low pH accelerates the decrease produced by phosphorylation and calcium. It is therefore possible that, analogous to the proposed role for phosphorylation, protonation may also modulate the calcium-induced closure of the channels (see below).

The data reported here has been summarized in Fig. 4, which shows a plot of the normalized time constants of the decrease in junctional conductance against the pCa's of the solutions tested. Time constants, as opposed to steady-state values, were used because for some solutions, particularly those with low calcium, steady state was reached after ~ 2 h of perfusion, and the viability of the preparation would have been compromised during the time required for control, steady-state, and recovery measurements. The curve for solutions containing calcium and the phosphorylating cocktail at pH 7.0 has a $K_{0.5}$ of 0.94 μ M, the same value as that obtained for solutions containing calcium at pH 6.0. A significantly different value ($K_{0.5}$

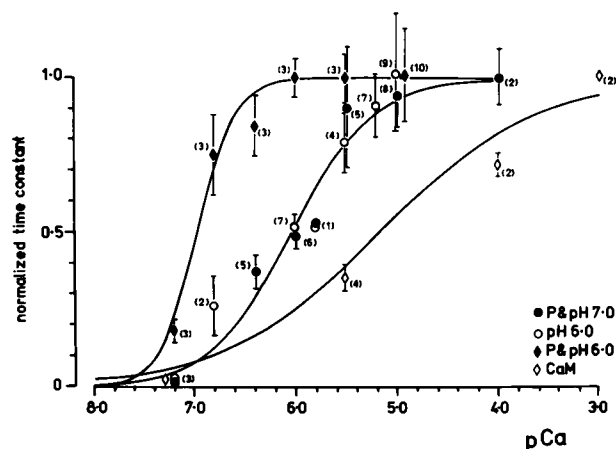


FIGURE 4 Normalized time constants of the decrease in junctional conductance elicited by perfusion solutions of different calcium concentrations. In each of the three series of experiments, each experiment at one pCa value was fitted to an exponential curve, and the time constants calculated and averaged. These were normalized with respect to the maximal rate of decrease in conductance (smallest time constant) within the series. The reciprocal of this value was then plotted against the calcium concentration. Calmodulin (CaM) data was analyzed similarly. Data points are plotted as mean \pm SE. Solid circles represent the effect of the phosphorylating cocktail buffered at pH 7.0, whereas open circles show the effect of pH 6. Both sets of data points fell on the same curve, with a $K_{0.5}$ of 0.94 μ M. Solid diamonds show the effect of the phosphorylating cocktail buffered at pH 6.0 and open diamonds show data obtained with CaM (10 μ M) in the perfusing solution (15). The $K_{0.5}$'s are 0.1 and 7.4 μ M, respectively. The number of experiments performed at each pCa is shown between parenthesis. The data points have been fitted to a curve of the type $y = 1/(1 + \exp[(pCa - pK_{0.5})k])$, where $pK_{0.5}$ is the half maximal pCa and k is the slope at half maximal response. The value of k for the curves that fit the data points for the phosphorylating cocktail at pH 7.0, and low pH, is ~ 0.5 .

0.1 μ M) is seen, however, in the curve for solutions containing the phosphorylating cocktail and calcium at pH 6.0, which is shifted to the left. For comparison, Fig. 4 also includes a curve calculated from the effect of perfusion of calcium-activated camodulin and reported elsewhere (15).

The data presented above show that calcium, in the presence of a phosphorylating cocktail or low pH, can produce junctional uncoupling. We favor the idea that calcium interacts directly with the junctional protein to induce closure and that protein phosphorylation and protonation modulate this effect of calcium. In support of this hypothesis our results show that neither phosphorylation nor low pH, alone or in combination, can produce uncoupling. In Fig. 4 we have presented the four possible ways by which we can induce channel closure in this preparation; all require calcium ions to exert their effects. These considerations lead us to propose a modulating role for phosphorylation and protonation, and one possibility is that both processes change the affinity of the channel

protein for calcium ions. In this respect their $K_{0.5}$'s are very similar, $\sim 0.94 \mu\text{M}$ for phosphorylation and calcium, and for low pH and calcium. The maximal rate of decrease in junctional conductance was observed, however, when both calcium and the phosphorylating cocktail were perfused at pH 6.0. This effect could be due to a summation of the contributions of phosphorylation and low pH, or to an increase in the activity of the protein kinase at pH 6.0 (16). While the cAMP-dependent PK used in this study yielded unequivocal results, we are currently testing the effects of other protein kinases, particularly protein kinase C, in similar experiments.

Our hypothesis of a direct action of calcium on the junctional proteins is in agreement with the calcium hypothesis advanced by Loewenstein (1966) and structural data (17). In addition, recent work (18) has also shown that calcium plays a major role in junctional regulation of cardiac cells. Indeed, the value for the threshold concentration of calcium, pCa 6.7 for cells dialyzed in the presence of ATP, is almost identical to our value of pCa 6.8, and the $K_{0.5}$ values are also very similar.

Whereas other explanations are consistent with the data, we believe that the proposed roles of phosphorylation and pH as modulators of calcium-induced channel closure are an attractive possibility that provides a new ground on which to examine the mechanisms of junctional regulation. This hypothesis, if shown to apply to other preparations, may prove useful in reconciling many controversial reports on the effects of calcium, hydrogen, and other compounds on cellular uncoupling as well as explaining recent results on the modulation of gap junction channels by gene products (19, 20).

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