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# Effects of Caffeine and Ryanodine on Low $pH_i$-Induced Changes in Gap Junction Conductance and Calcium Concentration in Crayfish Septate Axons

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**Summary.** Electrical uncoupling of crayfish septate axons with acidification has been shown to cause a substantial increase in $[Ca^{2+}]_i$ which closely matches in percent the increase in junctional resistance. To determine the origin of $[Ca^{2+}]_i$ increase, septate axons have been exposed either to drugs that influence $Ca^{2+}$ release from internal stores, caffeine and ryanodine, or to treatments that affect $Ca^{2+}$ entry. A large increase in junctional resistance and $[Ca^{2+}]_i$ maxima above controls resulted from addition of caffeine (10–30 mM) to acetate solutions, while a substantial decrease in both parameters was observed when exposure to acetate-caffeine was preceded by caffeine pretreatment. In contrast, ryanodine (1–10 $\mu$M) always caused a significant decrease in junctional resistance and $[Ca^{2+}]_i$ maxima when applied either together with acetate or both before and with acetate. Calcium channel blockers such as $La^{3+}$, $Cd^{2+}$ and nisoldipine had no effect, while an increase in the $[Ca^{2+}]$ of acetate solutions either decreased junctional resistance and $[Ca^{2+}]_i$ maxima or had no effect. The data suggest that cytoplasmic acidification causes an increase in $[Ca^{2+}]_i$ by releasing $Ca^{2+}$ from caffeine and ryanodine-sensitive $Ca^{2+}$ stores. The increase in $[Ca^{2+}]_i$ results in a decrease in gap junction conductance.

**Key Words** gap junctions · caffeine · ryanodine · calcium stores · intracellular $Ca^{2+}$ · Ca-sensitive microelectrodes

## Introduction

Gap junction channels provide the anatomical means for diffusion of small cytoplasmic molecules from cell to cell (Peracchia, 1980; Loewenstein, 1981). The permeability of these channels is finely regulated by mechanisms triggered by changes in the intracellular concentration of $Ca^{2+}$ (Loewenstein, 1966; Rose & Loewenstein, 1976) or $H^+$ (Turin & Warner, 1977, 1980; Spray, Harris & Bennett, 1981).

The decrease in channel conductance by lowered $pH_i$ is believed to be a direct effect of protons on the channel proteins (Spray et al., 1981), but recent data cannot be easily reconciled with this simple hypothesis and suggest a more complex mechanism (Ramon & Rivera, 1987; Peracchia, 1987*a*, 1990). In a recent study (Peracchia, 1990), we have measured simultaneously junctional resistance ($R_j$) and either $[Ca^{2+}]_i$ or $[H^+]_i$ in crayfish septate axons uncoupled by acidification. The data showed that the curve describing changes in $R_j$ differs significantly in shape and peak time from that of $[H^+]_i$, but it is very similar to that describing changes in $[Ca^{2+}]_i$; in addition, similar values of $pH_i$ obtained with different rates of acidification caused different increases in $R_j$, but changes in $[Ca^{2+}]_i$ were comparable in magnitude to the changes in $R_j$ (Peracchia, 1990). This indicated that the changes in gap junction conductance with low $pH_i$ are more closely related to acetate ($pH_i$)-induced changes in $[Ca^{2+}]_i$ than to changes in $pH_i$ itself and confirmed previous evidence for the capacity of $Ca^{2+}$ to close gap junction channels independently of $H^+$ (Rose & Rick, 1978).

In view of the fact that $Ca^{2+}$ plays a major role in the physiological regulation of gap junction-mediated communication (Loewenstein, 1966; Rose & Loewenstein, 1976), it seemed important to determine the mechanism of low $pH_i$-induced $[Ca^{2+}]_i$ increase. To do so, in the present study we have tested the effects of drugs that modify $Ca^{2+}$ release from internal stores, caffeine and ryanodine, and of treatments that affect $Ca^{2+}$ entry. Caffeine has been shown to potentiate $Ca^{2+}$ release from the sarcoplasmic reticulum (SR) of both skeletal (Stephenson, 1981; Nagasaki & Kasai, 1983; Delay, Ribalet & Vergara, 1986) and cardiac (Chapman & Miller, 1974; Meissner & Henderson, 1987) muscle fibers, while ryanodine appears to cause $Ca^{2+}$ release from SR of skeletal muscle (Fairhurst & Hasselbach, 1970), but to inhibit it in the heart (Sutko et al., 1979). Recently, both drugs have been tested on

$Ca^{2+}$-release channels incorporated into planar bilayers. In this system, caffeine increased frequency and duration of open channel events without affecting unit conductance (Rousseau & Meissner, 1989), while ryanodine greatly increased opening time but decrease unit conductance by more than 50% (Imagawa et al., 1987; Rousseau, Smith & Meissner, 1987; Smith et al., 1988).

Data from this study show that both $R_j$ and $[Ca^{2+}]_i$ changes with acidification are affected by caffeine and ryanodine but not by treatments that influence $Ca^{2+}$ entry, suggesting a participation of $Ca^{2+}$ stores in low $pH_i$-induced uncoupling of crayfish axons. A preliminary account of these data has been published (Peracchia, 1989).

## Materials and Methods

### Experimental Preparation

Crayfish (*Procambarus clarkii*), purchased from Carolina Biological Supply (Burlington, NC) were kept in a well-oxygenated aquarium at 22–24°C. The animals were anesthetized by cooling and sacrificed by decapitation. The ventral nerve cord was removed, and the sheath covering the dorsal side of the cord was cut away. A segment of the nerve cord comprising three ganglia was cut off and pinned dorsal side up to a plexiglass chamber. Either the third or forth abdominal ganglion was used.

The chamber was continuously perfused at a flow of 1 ml/min with a standard saline solution for crayfish (SES) (Johnston & Ramon, 1981) containing (in mM): NaCl, 205; KCl, 5.4; $CaCl_2$, 13.5 and Hepes, 5 (pH 7.5). The level of the solution in the chamber was maintained constant by continuous suction. Electrical uncoupling was obtained by superfusing the axons with a sodium acetate saline solution (Ac) containing (in mM): Na acetate, 205; KCl, 5.4 and $CaCl_2$, 13.5 (pH 6.3).

For testing the effects of drugs that modify the $Ca^{2+}$-release mechanism of internal stores, the axons were superfused with SES and/or Ac solutions containing 10–30 mM caffeine (Sigma, St. Louis, MO) or 1 nM to 10 $\mu$M ryanodine (a gift from Dr. S.S. Sheu, Department of Pharmacology, University of Rochester, Rochester, NY). Possible changes in $Ca^{2+}$ influx during acidification were tested by superfusing Ac solutions of different $[Ca^{2+}]$ (ranging from 5 to 40 mM) and both SES and Ac solutions containing either $LaCl_3$ (200 $\mu$M) or $CdCl_2$ (500 $\mu$M) or nisoldipine (1 $\mu$M; Miles Pharmaceuticals, West Haven, CT). For testing the effects of xanthines that, like caffeine, inhibit phosphadiesterases but, unlike caffeine, do not affect the $Ca^{2+}$ release mechanism, the axons were superfused with SES and Ac solutions containing 10 mM theophylline (Sigma).

### Electrical Measurements

Microelectrodes were pulled from borosilicate glass capillaries 1.2 mm (o.d.), 0.68 mm (i.d.) (Kwik fill, WP Instruments, New Haven, CT) and filled with a 2.5 M KCl solution buffered to pH 7 with 20 mM HEPES. The electrodes had an 8–10 MΩ resistance in SES. Four microelectrodes were inserted into a lateral giant axon, two on each side of the septum. The bath was grounded with a silver-silver chloride reference electrode connected to the chamber via an agar-SES bridge.

Hyperpolarizing square current pulses (150 nA, 300 msec) were generated by a Dell System 200 computer coupled to a D/A converter (DT 2801, Data Translation, Marlborough, MA) and a voltage-to-current converter (701 M, WPI). The pulses were injected every 10 sec alternatively into the posterior (C1) and anterior (C2) axon segment. The resulting electrotonic potentials $V_1$, $V_2$ (from current injection in $C_1$), $V_{1*}$, $V_{2*}$ (from injection in $C_2$) and the membrane potentials ($E_1$ and $E_2$) were recorded with two voltage microelectrodes through a voltage follower (AM-4, Biodine Electronics, Santa Monica, CA). The voltage signals were displayed on a storage oscilloscope and on a chart recorder and were digitized and stored both on the hard disc of the computer and on VCR tape (Peracchia, 1990).

### $Ca^{2+}$-Sensitive Microelectrodes

$[Ca^{2+}]_i$ was measured with $Ca^{2+}$-sensitive microelectrodes based on a neutral carrier sensor. The microelectrodes were prepared as previously described (Peracchia, 1990) using a recently developed calcium cocktail (ETH 129; Schefer et al., 1986) (Fluka Chemical, Ronkonkoma, NY). This cocktail contains the $Ca^{2+}$ ionophore N,N,N1,N1-tetracyclohexyl-3-oxapentanediamide, which forms an ideal coordination sphere of nine oxygen atoms for $Ca^{2+}$-uptake (Schefer et al., 1986). Ca-microelectrodes prepared with this cocktail have a logarithmic response down to $[Ca^{2+}]$ of $5 \times 10^{-10}$ M and are virtually insensitive to other ions (Ammann et al., 1987), including $H^+$ and acetate (Peracchia, 1990). The microelectrodes were backfilled with $Ca^{2+}$ solutions buffered with EGTA ($p$Ca 7; Alvarez-Leefmans, Rink & Tsien, 1981) or citrate (pH 7) and containing 220 mM KCl to match $[K^+]_i$.

The $Ca^{2+}$ sensitivity and response time of the microelectrodes were tested as previously described (Peracchia, 1990). The electrode response ranged 15–25 mV per $p$Ca unit. Microelectrodes typically had a 50% response time of 14 sec.

The $Ca^{2+}$ microelectrodes were inserted into either one of the two axon segments, as closely as possible to the septum. For testing possible differences in $[Ca^{2+}]_i$ between the two cells, in some experiments the $Ca^{2+}$ electrode was moved back and forth from one to the other axon segment.

### Analysis of Data

Both membrane ($Rm_1$, $Rm_2$) and junctional ($R_{j1}$, $R_{j2}$) resistances were calculated and plotted on-line on the computer monitor by means of a program written in ASYST language (Adaptable Laboratory Software, Rochester, NY). The resistances were calculated from current ($I_1$, $I_2$) and voltage ($V_1$, $V_2$, $V_{1*}$, $V_{2*}$) records using the $\pi - t$ transform (Bennett, 1966).

The voltage signal detected by the $Ca^{2+}$ microelectrode was passed through the high impedance amplifier, filtered (0.1 Hz) and displayed on the pen recorder after subtraction of membrane potential. At 10-sec intervals, the voltage of the $Ca^{2+}$ microelectrode was also sampled by the computer, 100 msec before each current pulse. The voltage signal was digitized, stored unfiltered on the hard disc and plotted on-line after subtraction of membrane potential (Peracchia, 1990).

## Results

### Effects of Caffeine on Changes in Junctional Resistance and Intracellular $Ca^{2+}$ Concentration with Acidification

Superfusion of crayfish septate axons with Ac causes a small bimodal change in membrane potential, an increase in $V_1$ and $V_{2*}$ and a decrease in $V_2$ and $V_{1*}$ (Fig. 1*A*). The voltage changes reflect a reversible increase in $R_j$ (Fig. 1*B*). The $R_j$ curve is fairly symmetrical, the recovery phase being only slightly slower (Fig. 1*B*), and the magnitude of the $R_j$ peak is quite consistent in a given preparation, although it might vary significantly in different axons and different seasons (Peracchia, 1990). With 2–4 min superfusion of Ac at a flow of 1 ml/min, $R_j$ increases from the basal values (70–130 kΩ) by 1.3–4.5 times.

Addition of 10–30 mM caffeine to Ac causes a greater increase in $V_1$ and $V_{2*}$, and a greater decrease in $V_2$ and $V_{1*}$ with respect to controls (Fig. 1*A*), reflecting a significant increase in $R_j$ maxima above control maxima (Figs. 1*B* and 2*D*). A greater deflection of membrane potential in the positive direction is also noticeable (Fig. 1*A*). The $R_j$ maxima with Ac-caffeine are 264 ± 111% (mean ± SD; $n$ = 11) of Ac-induced maxima, and there is no statistically significant difference between 10 and 30 mM caffeine treatments.

Exposure of the axons to caffeine either before or both before and during Ac treatment significantly reduces the $R_j$ maxima with respect to Ac-caffeine (Figs. 1*C* and 2*D*) or Ac alone (Fig. 2*B* and *D*). The $R_j$ maxima with Ac-caffeine, following caffeine-pretreatment, are 36 ± 22% (mean ± SD; $n$ = 5) of those induced by Ac-caffeine in the absence of pretreatment. There is no statistically significant differences among experiments with different pretreatment durations, ranging from 5 to 13 min, but pretreatments with 30 mM caffeine (Fig. 2*D*) cause greater inhibition than those with 10 mM caffeine (Fig. 1*C*).

Following caffeine pretreatment, the $R_j$ maxima with Ac-caffeine or Ac are 65 ± 9% (mean ± SD; $n$ = 7) of those induced by Ac in the absence of pretreatment (Fig. 2*B*). Also in this case there is no statistically significant difference among experiments with different pretreatment durations, ranging from 8 to 40 min.

Experiments in which $Ca^{2+}$-sensitive microelectrodes are used show that $[Ca^{2+}]_i$ increases with Ac by one to one and one-half orders of magnitude from control values ranging from 80 to 200 nM. Caffeine causes changes in $[Ca^{2+}]_i$ maxima with Ac comparable to $R_j$ maxima (Fig. 2*B–D*). Caffeine pretreatment does not alter the basal $[Ca^{2+}]_i$ (Fig. 2*C* and *D*). As for $R_j$ $[Ca^{2+}]_i$ maxima with Ac-caffeine are much greater than with Ac alone, and no significant difference in the magnitude of the effect is observed with different caffeine concentrations, ranging from 10 to 30 mM. In contrast, pretreatments with 30 mM caffeine have a greater inhibitory effect on both $[Ca^{2+}]_i$ and $R_j$ maxima reached with Ac-caffeine (Fig. 2*D*) than pretreatments with either 10 mM (Fig. 2*A–C*) or 20 mM caffeine. In addition to the effects on $[Ca^{2+}]_i$ maxima, caffeine affects the rate of $[Ca^{2+}]_i$ increase with Ac. $[Ca^{2+}]_i$ increases at a maximum rate of ~0.8 $p$Ca unit/min with Ac alone and at a rate of ~1.3 $p$Ca unit/min with Ac-caffeine. In contrast, a substantial reduction of the rate of $[Ca^{2+}]_i$ increase is observed following caffeine pretreatment. In the absence of Ac, exposure to 10–30 mM caffeine for periods ranging from 5 to 40 min ($n$ = 12) does not affect $R_j$, $R_m$ and basal $[Ca^{2+}]_i$, the only apparent change being a transient depolarization of 2–6 mV (Fig. 2*A*).

### Effects of Ryanodine on Changes in Junctional Resistance and $Ca^{2+}$ Concentration with Acidification

The effects of ryanodine were tested by superfusing the axons with 1–10 μM ryanodine, either before and during Ac treatment or just with Ac. With Ac-ryanodine the increase in $V_1$ and $V_{2*}$, and the decrease in $V_2$ and $V_{1*}$ are smaller than those with Ac alone (Fig. 3*A*), reflecting a substantial decrease in $R_j$ maxima (Fig. 3*B*). The $R_j$ maxima with Ac-ryanodine are 34 ± 18% (mean ± SD; $n$ = 10) of Ac-induced maxima. There is no significant difference in percent reduction of $R_j$ maxima between experiments with Ac-ryanodine and experiments in which Ac-ryanodine is preceded by ryanodine pretreatments of various lengths, ranging from 1 to 15 min (Figs. 3*C* and 4*B*).

Experiments with $Ca^{2+}$-sensitive microelectrodes show that ryanodine affects the $[Ca^{2+}]_i$ maxima with Ac (Fig. 4*C*) similarly to $R_j$ maxima (Fig. 4*B*). In addition to a substantial decrease in $[Ca^{2+}]_i$ and $R_j$ maxima, ryanodine reduces the maximum rate of $[Ca^{2+}]_i$ increase with Ac by approximately 50%, as $[Ca^{2+}]_i$ increases at a rate of ~0.8 $p$Ca/min with Ac alone and at a rate of ~0.4 $p$Ca/min with Ac-ryanodine. The ryanodine effects are seen only with micromolar concentrations. Ryanodine, tested

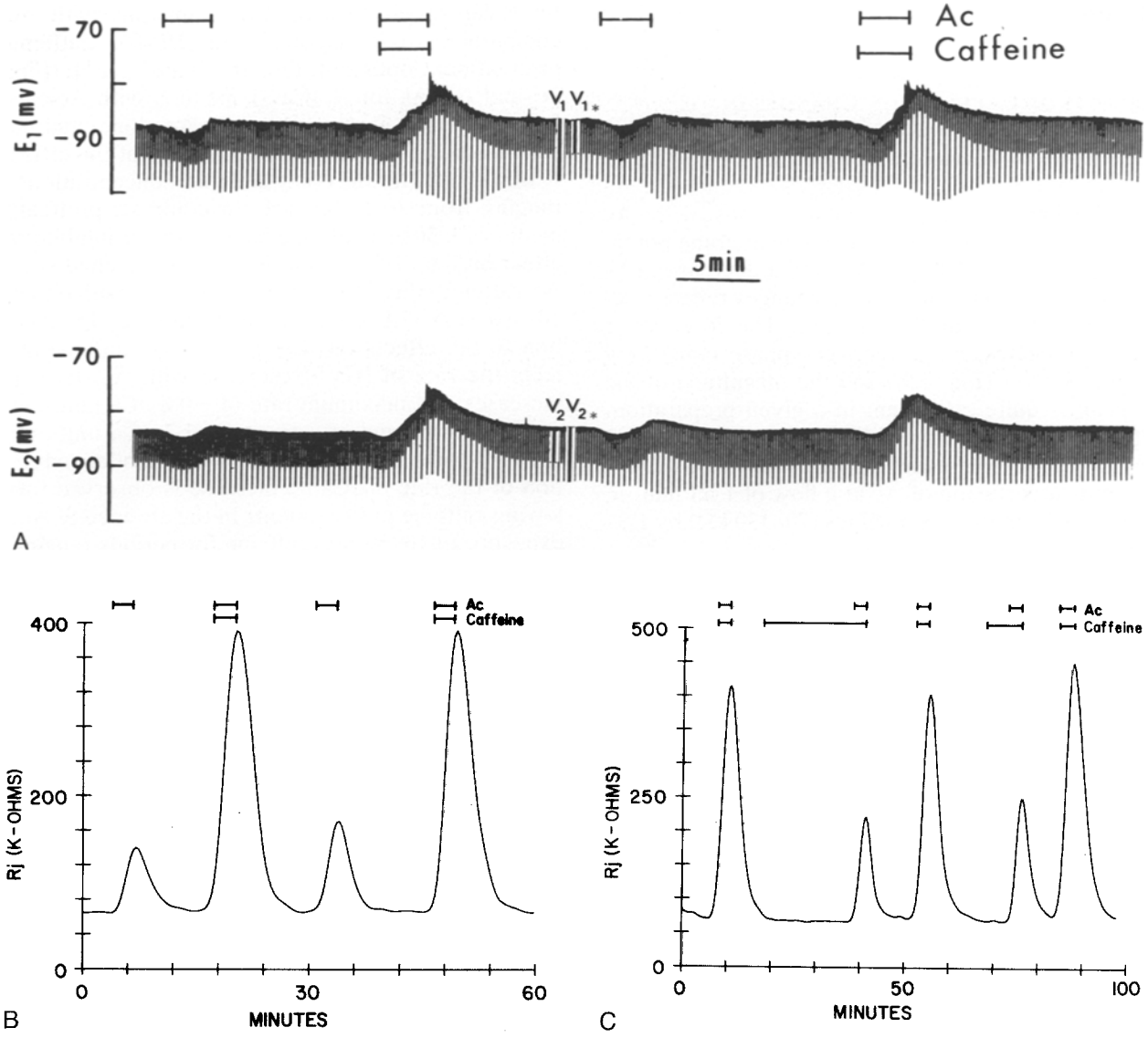


**Fig. 1.** Time course of changes in electrotonic potentials and $R_j$ in crayfish septate axons uncoupled with Ac in the presence and absence of 10 mM caffeine. (*A*) Low speed chart recording of membrane and electrotonic potentials in the posterior (upper trace) and anterior (lower trace) axon segment. Hyperpolarizing square current pulses (150 nA, 300 msec) are injected every 10 sec alternatively into the posterior ($C_1$) and anterior ($C_2$) axon segment. The resulting electronic potentials $V_1$ and $V_2$ (from current injection in $C_1$), and $V_{1^*}$ and $V_{2^*}$ (from current injection in $C_2$) as well as the membrane potentials ($E_1$ and $E_2$) are displayed on the chart recording and stored on VCR tape and on the computer's hard disc for calculating $R_j$ and $R_m$. With Ac, $V_1$ and $V_{2^*}$ increase and $V_{1^*}$ and $V_2$ decrease, due to an increase in $R_j$. With Ac-caffeine a larger change in electrotonic potentials takes place, indicating a larger increase in $R_j$, and membrane potential becomes more positive. (*B*) Computer-calculated changes in $R_j$ from the experiment shown in *A*. Note that $R_j$ increases with Ac-caffeine 3–4 times as much as with Ac alone. (*C*) Computer-calculated changes in $R_j$ from a different experiment, in which crayfish axons were uncoupled with Ac-caffeine, with and without caffeine pretreatment (10 mM caffeine). The $R_j$ maxima caused by Ac-caffeine after caffeine pretreatment are 50% smaller than those without pretreatment. Note that the inhibition induced by caffeine pretreatment disappears within a few minutes

at 1–10 nM concentrations does not have any appreciable effect on $R_j$ maxima when used either together with Ac (Fig. 5) or both before and together with Ac (*data not shown*). In the absence of Ac, exposure to 1–10 $\mu$M ryanodine for periods ranging from 1 to 15 min ($n = 5$) does not have any appreciable effect on $R_j$, $R_m$, $[Ca^{2+}]_i$ and membrane potential.

### Effects of Different External $Ca^{2+}$ Concentrations, $Ca^{2+}$ Channel Blockers and Theophylline on Changes in Junctional Resistance and Internal $Ca^{2+}$ Concentration with Acidification

To test the possible participation of $Ca^{2+}$ influx in the increase in $[Ca^{2+}]_i$ with acidification, some ax-

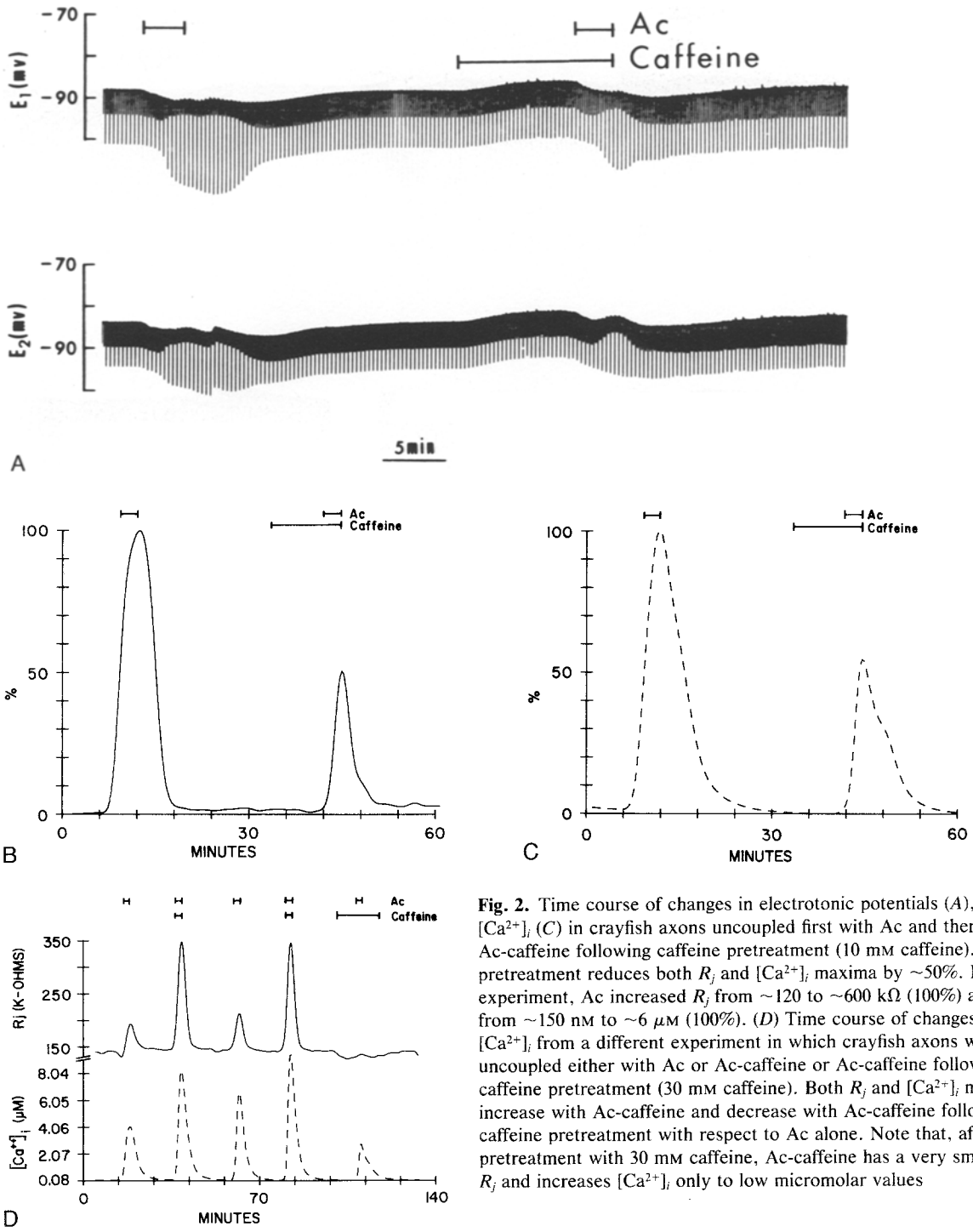


**Fig. 2.** Time course of changes in electrotonic potentials (*A*), $R_j$ (*B*) and $[Ca^{2+}]_i$ (*C*) in crayfish axons uncoupled first with Ac and then with Ac-caffeine following caffeine pretreatment (10 mM caffeine). Caffeine pretreatment reduces both $R_j$ and $[Ca^{2+}]_i$ maxima by ~50%. In this experiment, Ac increased $R_j$ from ~120 to ~600 kΩ (100%) and $[Ca^{2+}]_i$ from ~150 nM to ~6 μM (100%). (*D*) Time course of changes in $R_j$ and $[Ca^{2+}]_i$ from a different experiment in which crayfish axons were uncoupled either with Ac or Ac-caffeine or Ac-caffeine following caffeine pretreatment (30 mM caffeine). Both $R_j$ and $[Ca^{2+}]_i$ maxima increase with Ac-caffeine and decrease with Ac-caffeine following caffeine pretreatment with respect to Ac alone. Note that, after pretreatment with 30 mM caffeine, Ac-caffeine has a very small effect on $R_j$ and increases $[Ca^{2+}]_i$ only to low micromolar values

ons were superfused with SES and Ac solutions containing different $[Ca^{2+}]_i$ or $Ca^{2+}$ channel blockers. In most experiments a change of $[Ca^{2+}]_o$ from 5 to 40 mM did not significantly affect the $R_j$ and $[Ca^{2+}]_i$ maxima with Ac, but in a few cases a reduction of $[Ca^{2+}]_o$ caused a slight increase in both $R_j$ and $[Ca^{2+}]_i$ maxima (Fig. 6*A*). Addition of either 200 μM $La^{3+}$ (Fig. 6*B*) or 500 μM $Cd^{2+}$ (Fig. 6*C*) or 1 μM nisoldipine (*data not shown*) to Ac or both SES and Ac, does not change the magnitude of $R_j$ maxima with respect to controls. No effect on $R_j$ maxima is seen also with 10 mM theophylline, tested either before and during Ac (Fig. 6*C*) or just with Ac (*data not shown*).

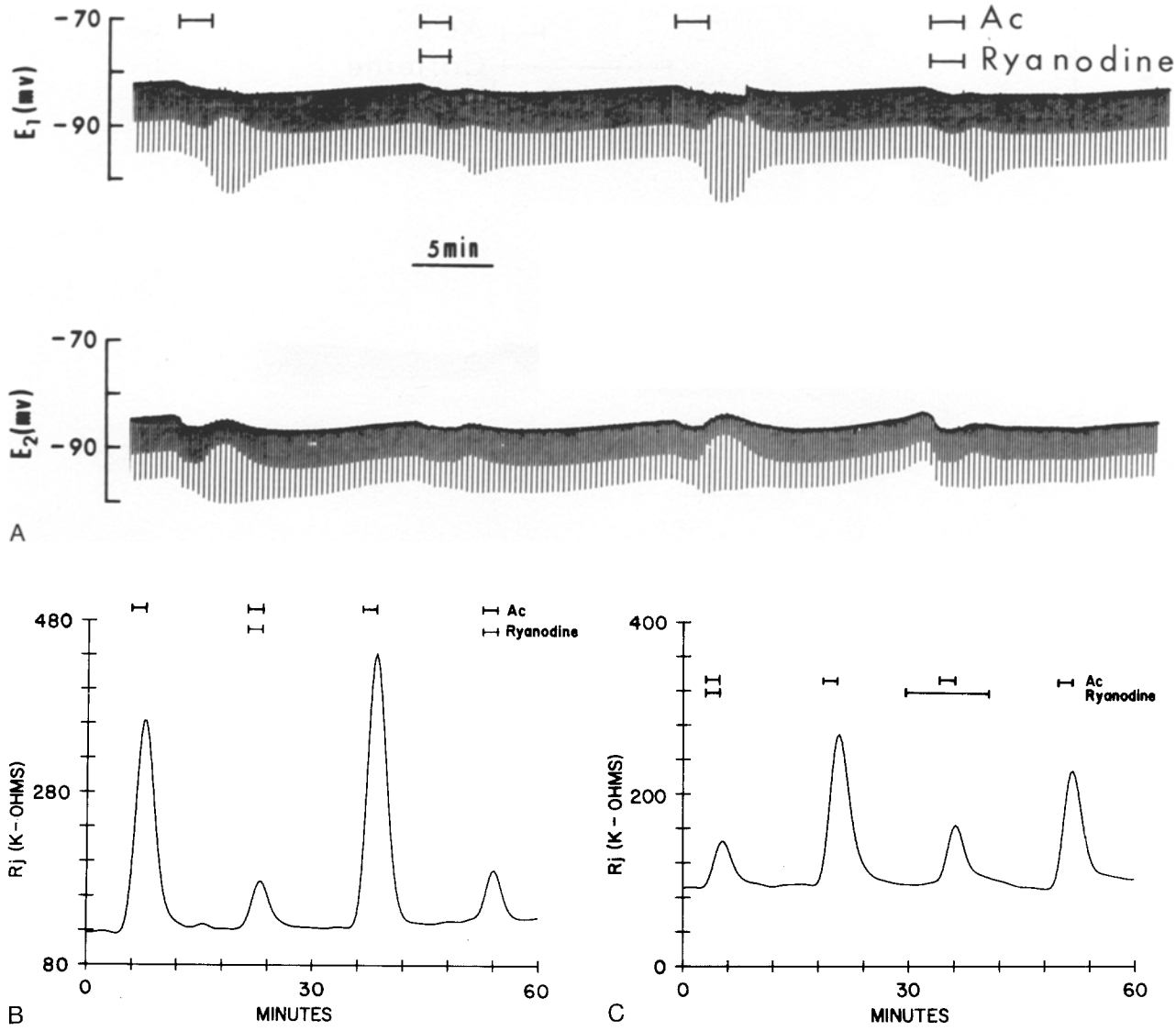


**Fig. 3.** Time course of changes in electrotonic potentials (*A*) and $R_j$ (*B*) in crayfish axons uncoupled with Ac in the presence and absence of 10 $\mu$M ryanodine. With Ac-ryanodine the increase in $V_1$ and $V_{2^*}$ and the decrease in $V_{1^*}$ and $V_2$ are not as large as with Ac alone (*A*), due to a large decrease in $R_j$ maxima (*B*). Note that the ryanodine-induced inhibition of the Ac effects on $R_j$ disappears within a few minutes. (*C*) Time course of changes in $R_j$ from another experiment in which crayfish axons were uncoupled with either Ac or Ac-ryanodine or Ac-ryanodine following ryanodine pretreatment (10 $\mu$M ryanodine). Ryanodine-induced inhibition of $R_j$ changes with Ac is expressed both with and without ryanodine pretreatment

## Discussion

In a recent study (Peracchia, 1990) we have shown that in crayfish septate axons the increase in gap junction electrical resistance with acidification is more closely related to changes in $[Ca^{2+}]_i$ than $[H^+]_i$. In view of these findings and of previous evidence for a central role of $Ca^{2+}$ in gap junction regulation (Loewenstein, 1966; Rose & Loewenstein, 1976; Rose & Rick, 1978) it seemed crucial to determine the mechanism of low $pH_i$-induced increase in $[Ca^{2+}]_i$. For this purpose, the present study has monitored simultaneously $R_j$ and $[Ca^{2+}]_i$ in crayfish septate axons uncoupled by acidification both in the presence and absence of drugs that affect $Ca^{2+}$ release from internal stores, caffeine and ryanodine, and of treatments that affect $Ca^{2+}$ entry from the external medium. The data show that the magnitude of both $R_j$ and $[Ca^{2+}]_i$ maxima are significantly altered by caffeine and ryanodine, while $Ca^{2+}$-channel blockers and different $[Ca^{2+}]_o$ have no effect. This suggests a participation of $Ca^{2+}$ release from

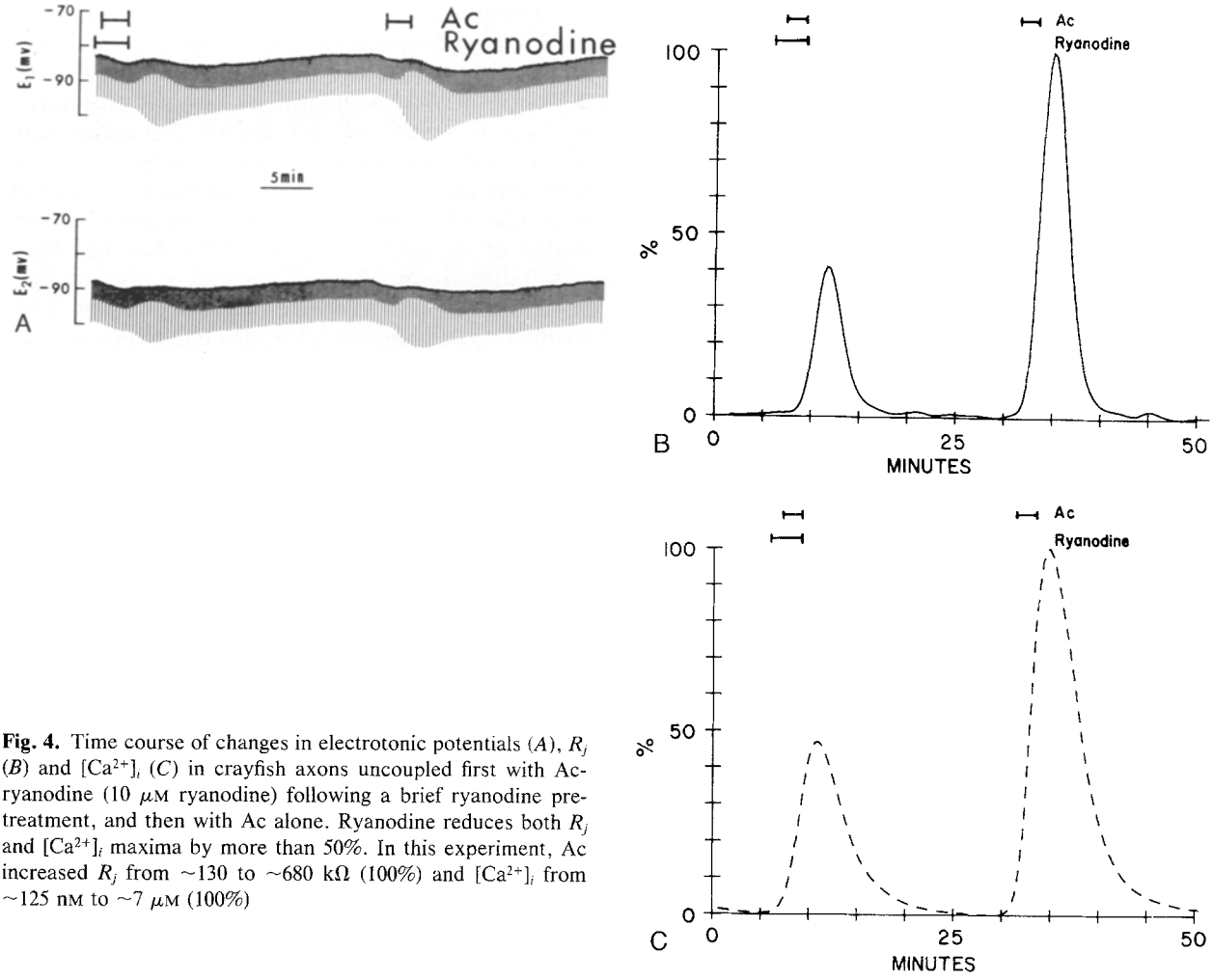


**Fig. 4.** Time course of changes in electrotonic potentials (*A*), $R_j$ (*B*) and $[Ca^{2+}]_i$ (*C*) in crayfish axons uncoupled first with Ac-ryanodine (10 $\mu$M ryanodine) following a brief ryanodine pretreatment, and then with Ac alone. Ryanodine reduces both $R_j$ and $[Ca^{2+}]_i$ maxima by more than 50%. In this experiment, Ac increased $R_j$ from ~130 to ~680 kΩ (100%) and $[Ca^{2+}]_i$ from ~125 nM to ~7 $\mu$M (100%)

intracellular stores in the mechanism of low $pH_i$-induced uncoupling of crayfish gap junctions.

Caffeine had a dual effect on $R_j$ and $[Ca^{2+}]_i$; when used in conjunction with Ac, it increased considerably $R_j$ and $[Ca^{2+}]$ maxima above controls, but when used both before and with Ac or just before Ac (as a pretreatment) it reduced substantially both parameters. This suggests that caffeine and Ac act synergistically on the $Ca^{2+}$ release mechanism of $Ca^{2+}$ stores such that, when applied together, a much larger amount of $Ca^{2+}$ diffuses into the cytosol and consequently a larger number of gap junction channels close. On the other hand, caffeine pretreatment reduces the $Ca^{2+}$ content of internal stores, such that, when $Ca^{2+}$ release is stimulated by either Ac alone or the combined action of Ac and caffeine, less $Ca^{2+}$ diffuses into the cytosol and fewer gap junction channels close. The increase in rate of $[Ca^{2+}]_i$ increase with Ac-caffeine *versus* Ac alone may result from an increase in open time and opening frequency of individual $Ca^{2+}$-release channels, rather than from an increase in channel size, because caffeine increases frequency and duration of open events but not unit conductance in cardiac $Ca^{2+}$-release channels incorporated in planar bilayers (Rousseau & Meissner, 1989).

The effects of caffeine on $[Ca^{2+}]_i$ and $R_j$ in crayfish axons are consistent with published reports on caffeine effects in muscles, as caffeine has been found to exert a positive inotropic effect on heart and skeletal muscle (Chapman & Miller, 1974) by affecting the $Ca^{2+}$-release pathway without appreciably altering the electrical properties of the surface membrane (Delay et al., 1986). This has been clearly demonstrated also by reports of a caffeine-induced rapid efflux of $Ca^{2+}$ from skinned fibers of skeletal muscle (Stephenson, 1981) and from SR vesicles isolated from skeletal muscle (Nagasaki & Kasai, 1983) and heart (Meissner & Henderson, 1987). Recently, a caffeine-induced $Ca^{2+}$ release in mammalian neurons has also been reported (Lipscombe et al., 1988).

In addition to the effects on $Ca^{2+}$-release channels, caffeine is known to inhibit phosphodies-

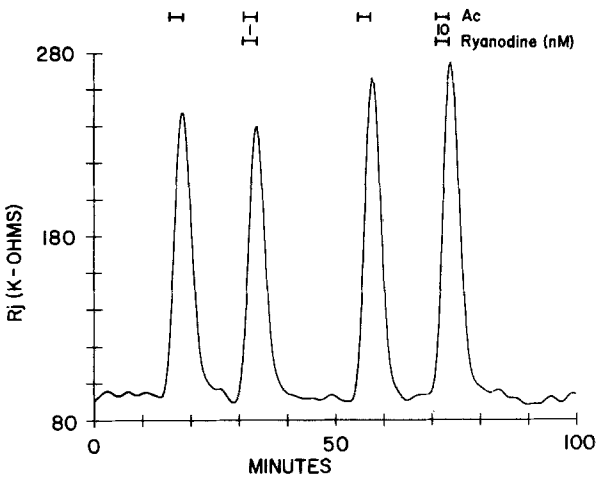


**Fig. 5.** Time course of changes in $R_j$ in crayfish axons uncoupled with Ac in the presence or absence of either 1 or 10 nM ryanodine. Note the absence of an effect of nanomolar concentrations of ryanodine on $R_j$ maxima

terases causing an increase in cAMP (Butcher & Sutherland, 1962). This, however, is unlikely to play a role in the effects of caffeine on $[Ca^{2+}]_i$ and $R_j$ for several reasons. First of all, theophylline, another xanthine which inhibits phosphodiesterases even more effectively than caffeine (Butcher & Sutherland, 1962), but has no significant effect on $Ca^{2+}$-release mechanisms, did not have any effect on $R_j$ and $[Ca^{2+}]_i$ when tested either before and during Ac or just with Ac. Secondly, the dual effect of caffeine would be hard to explain on the basis of an increase in cAMP because if one were to interpret the large increase in $R_j$ maxima with Ac-caffeine as being the result of an increase in cAMP, one would expect an even greater increase in $R_j$ maxima with Ac-caffeine after caffeine pretreatment, while in fact the opposite is true. In addition, we have previously reported that an increase in the intracellular cAMP concentration by exposure to db-cAMP influences neither the basic values of $R_j$ nor their changes with Ac in these very axons (Peracchia, 1987*b*).

The possibility that caffeine had a significant effect on $[H^+]_i$ maxima is unlikely because no effect of caffeine on $pH_i$ was detected in skeletal muscle in the absence of a large release of $Ca^{2+}$ (Connett, 1978). When $[Ca^{2+}]_i$ increased, a small increase in $pH_i$ was observed, and this was found to depend entirely on the increase in $[Ca^{2+}]_i$ because when caffeine was used in conjunction with drugs that inhibit the increase in $[Ca^{2+}]_i$ alkalinization was not observed. Moreover, other drugs that induce Ca-release from the SR also caused alkalinization.

The effects of ryanodine suggest an inhibition of $Ca^{2+}$ release from stores. The action of ryanodine in muscle is still not fully understood. While in skeletal muscle ryanodine treatment causes a strong contraction, indicative of $Ca^{2+}$ release and consequential depletion of the $Ca^{2+}$ stores, in heart muscle ryanodine does not cause contraction and does not alter $[Ca^{2+}]_i$ but has just a negative ionotropic effect (Sutko et al., 1979). Recently ryanodine has been tested directly on channels formed in planar bilayers by reconstitution of $Ca^{2+}$ release channels with purified ryanodine-receptor protein (Rousseau et al., 1987). In single channel recording, nanomolar concentrations of ryanodine greatly increased the duration of open events, driving the channel into a virtually permanent open state and decreased channel conductance to approximately half the normal conductance (Rousseau et al., 1987; Smith et al., 1988). On the basis of these data, it has been proposed that both in skeletal muscle and in heart ryanodine opens the $Ca^{2+}$-release channels and depletes the stores, and the absence of $[Ca^{2+}]_i$ increase and contraction in the heart has been attributed to rapid $Ca^{2+}$ extrusion via $Na^+/Ca^{2+}$ exchange (Fill & Coronado, 1988). However, recently McGrew et al. (1989) have shown that ryanodine is capable of both opening and closing the $Ca^{2+}$-release channels depending on concentration. This study has determined the presence of both high affinity ($K_D$ = 5–10 nM) and low affinity ($K_D$ = ~3 $\mu$M) ryanodine sites in both heart and skeletal muscle; nanomolar concentrations of ryanodine were found to open the channels, while micromolar concentrations blocked the channels in a closed state ($K_m$ = 1.11 $\mu$M in heart and 3.67 $\mu$M in skeletal muscle). Since in our study ryanodine inhibited the Ac-induced increase in $[Ca^{2+}]_i$ and $R_j$ at concentrations ranging from 1 to 10 $\mu$M, the data are best explained on the basis of a ryanodine-induced block of $Ca^{2+}$-release channels.

The nature of caffeine- and ryanodine-sensitive $Ca^{2+}$ stores in nonmuscle cells in still uncertain. Recently, vesicles (0.1 $\mu$m in diameter) located near the plasma membrane in various cells have been found to contain calsequestrin-like proteins and have been interpreted as a type of $Ca^{2+}$-storing organelle (calciosome) (Meldolesi, Volpe & Pozzan, 1988). Whether crayfish axons contain calciosomes is still unknown, but it is interesting that in septate axons 50–80 nm vesicles containing a fine granular material cover both gap junction surfaces (Peracchia, 1973). These vesicles have been interpreted as $Ca^{2+}$-sequestring organelles, anchored to the gap junctions for maintaining the channels in a low $Ca^{2+}$ environment (Peracchia & Dulhunty, 1976). However, these vesicles have been seen so far only at

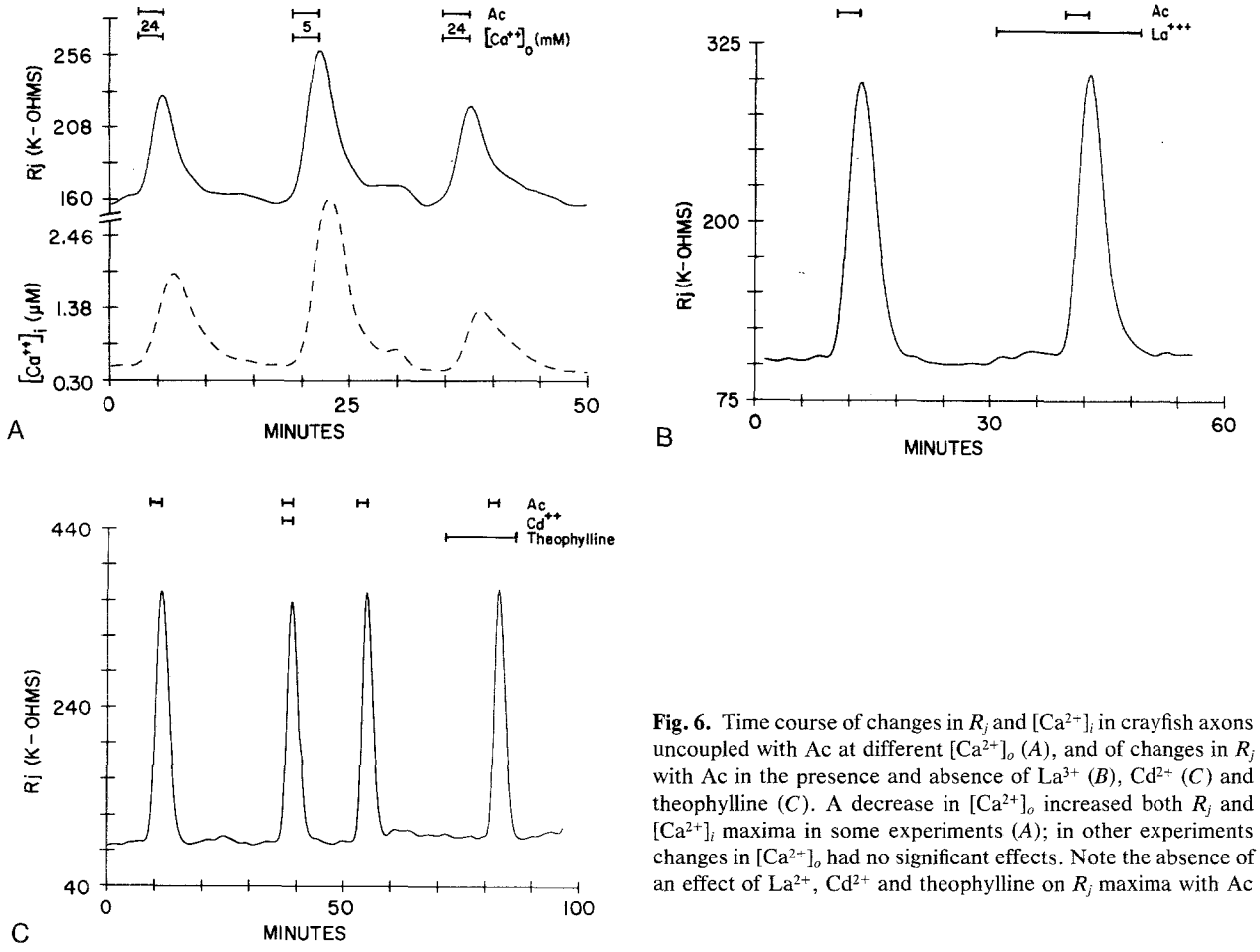


**Fig. 6.** Time course of changes in $R_j$ and $[Ca^{2+}]_i$ in crayfish axons uncoupled with Ac at different $[Ca^{2+}]_o$ (*A*), and of changes in $R_j$ with Ac in the presence and absence of $La^{3+}$ (*B*), $Cd^{2+}$ (*C*) and theophylline (*C*). A decrease in $[Ca^{2+}]_o$ increased both $R_j$ and $[Ca^{2+}]_i$ maxima in some experiments (*A*); in other experiments changes in $[Ca^{2+}]_o$ had no significant effects. Note the absence of an effect of $La^{2+}$, $Cd^{2+}$ and theophylline on $R_j$ maxima with Ac

junctional regions and thus could hardly be the only source of $Ca^{2+}$ release. Therefore, it seems likely that the endoplasmic reticulum, which in fact is very abundant in septate axons and much more so than in median giant axons (Peracchia & Robertson, 1971), may play a major role in low $pH_i$-induced $Ca^{2+}$ release.

While our data are consistent with the idea of a low $pH_i$-induced $Ca^{2+}$ release from stores, no data on possible mechanisms have been obtained. A $H^+$-induced $Ca^{2+}$ release from SR of barnacle muscles has been reported (Lea & Ashley, 1981). In this case, $Ca^{2+}$ release was observed with acidification induced by $CO_2$ or weak acids and depended more on the capacity of the acid to diffuse into the SR cisterns than on the actual pH of the cytosol. On this basis, acetate, a diffusable acid, may cause $Ca^{2+}$ release by lowering the pH of $Ca^{2+}$-storing organelles. Consistent with this hypothesis is evidence for the capacity of weak (diffusable) acids to uncouple cells more efficiently than intracellularly injected, poorly permeable, acids (strong acids) (Bennett et al., 1988).

The lowest $[Ca^{2+}]_i$ effective on coupling was found to range from high nanomolar to low micromolar. A more precise value could not be stated at present because the effects of possible changes in phosphorylation state and/or protonation of the channels, that could alter their $Ca^{2+}$ sensitivity, have not been determined.

In conclusion, this study shows an effect of caffeine and ryanodine on changes in $[Ca^{2+}]_i$ and $R_j$ induced by cytoplasmic acidification in crayfish septate axons. The data are consistent with the hypothesis that low $pH_i$ uncouples crayfish gap junctions by releasing $Ca^{2+}$ from caffeine- and ryanodine-sensitive stores and confirm previous data showing that calmodulin inhibitors block uncoupling by low $pH_i$ in crayfish axons (Peracchia, 1987*b*) and in other systems (Peracchia, 1988).

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