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Intracellular pH, Intracellular free Ca, and Junctional Cell-Cell Coupling

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Summary. Intracellular pH (pH_i) and intracellular Ca^{2+} ($[Ca^{2+}]_i$) were determined in *Chironomus* salivary gland cells under various conditions of induced uncoupling. pH_i was measured with a *Thomas*-type microelectrode, changes in $[Ca^{2+}]_i$ and their spatial distribution inside the cell were determined with the aid of intracellularly injected aequorin and an image intensifier-TV system, and cell-to-cell coupling was measured electrically. Treatments with NaCN (5 mM), DNP (1.2 mM), or ionophore A23187 (2 μM) caused fall in junctional conductance (*uncoupling*) that was correlated with $[Ca^{2+}]_i$ elevation, as was shown before (Rose & Loewenstein, 1976, *J. Membrane Biol.* **28**:87) but not with changes in pH_i: during the uncoupling induced by CN, the pH_i (normally ≈ 7.5) decreased at most by 0.2 units; during the uncoupling induced by the ionophore, pH_i fell by 0.13 or rose by 0.3; and in any one of these three agents' uncouplings, the onset of uncoupling and recovery of coupling were out of phase with the changes in pH_i. Intracellular injection of Ca-citrate or Ca-EGTA solutions buffered to pH 7.2 or 7.5 produced uncoupling with little or no pH_i change when their free $[Ca^{2+}]_i$ was $> 10^{-5}$ M. On the other hand, such a solution at pH 4, buffered to $[Ca^{2+}] < 10^{-6}$ M, lowered pH_i to 6.8 but produced no uncoupling. Thus, a decrease in pH_i is not necessary for uncoupling in any of these conditions. In fact, uncoupling ensued also during increase in pH_i: exposure to NH_4HCO_3 or withdrawal of propionate following exposure to a propionate-containing medium caused pH_i to rise to 8.74, accompanied by $[Ca^{2+}]_i$ elevation and uncoupling at $pH_i > 7.8$.

Cell acidification itself can cause elevation of $[Ca^{2+}]_i$: injection (iontophoresis) of H^+ invariably caused $[Ca^{2+}]_i$ elevation and uncoupling. These effects were produced also by an application of H^+-transporting ionophore Nigericin at extracellular pH 6.5 which caused pH_i to fall to 6.8. Exposure to 100% CO_2 produced a fall in pH_i, associated in 10 out of 25 cases with $[Ca^{2+}]_i$ elevation and, invariably, with uncoupling. The absence of a demonstrable $[Ca^{2+}]_i$ elevation in a proportion of these trials is attributable to depression in Ca^{2+}-measuring sensitivity; in *in vivo* tests, detection sensitivity for $[Ca^{2+}]_i$ by aequorin was found to be depressed by the CO_2 treatment. Upon CO_2 washout, pH_i and coupling recovered, but onset of recoupling set in at pH_i as low as 6.32–6.88, generally lower than at the pH_i at which uncoupling had set in. Exposure to 5% CO_2 lowered pH_i on the average by 0.3 and depressed coupling (in initially poorly coupled cells). After CO_2-washout, pH_i and coupling recovered. During the recovery phase $[Ca^{2+}]_i$ was elevated, an elevation associated with renewed uncoupling or decrease in rate of recoupling. The results are discussed in connection with possible regulatory mechanisms of junctional permeability.

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Cell-to-cell coupling via junctional membrane channels is a feature common to all but a few tissues. Loewenstein (1966) advanced the hypothesis that the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) determines the permeability of the junctional membrane channels. Much evidence has accumulated in support of the hypothesis: junctional permeability of a variety of tissues was found to decrease in experimental conditions in which $[Ca^{2+}]_i$ was raised or expected to have risen. Among such conditions were intracellular Ca^{2+} injection (Loewenstein, Nakas & Socolar, 1967; DeMello, 1975; Délèze & Loewenstein, 1976; Loewenstein, Kanno & Socolar, 1978*a*), cell membrane injury in Ca^{2+}-containing medium (Loewenstein & Penn, 1967; Loewenstein *et al.*, 1967; Délèze, 1970; Oliveira-Castro & Loewenstein, 1971), exposure to Ca-transporting ionophores (Rose & Loewenstein, 1975, 1976; Gilula & Epstein, 1976), and inhibition of cell metabolism (Politoff, Socolar & Loewenstein, 1969; Politoff & Pappas, 1972; Peracchia & Dulhunty, 1976; Gilula & Epstein, 1976). The most direct evidence came from experiments in which $[Ca^{2+}]_i$ was monitored during junctional uncoupling with the aid of aequorin. These showed a correlation between $[Ca^{2+}]_i$ in the junctional locale and junctional coupling (Rose & Loewenstein, 1975, 1976).

It has long been known that H^+ competes with Ca^{2+} for binding sites at biological membranes (*see*, e.g., Carvalho, Sanui & Pace, 1963) and that mitochondria release H^+ in exchange for Ca^{2+} (Bartley & Amoore, 1958; Chappel, Greville & Bicknell, 1962; Chance, 1965). Therefore, an elevation of $[Ca^{2+}]_i$ might be expected to cause a fall in intracellular pH (pH_i). Indeed, Meech and Thomas (1977) showed this to be the case during injection of $CaCl_2$ into snail neuron. With regard to the above-mentioned Ca^{2+} hypothesis, this naturally raised the question whether the effect of $[Ca^{2+}]_i$ elevation on junctional permeability was mediated by H^+. Taking this lead, Turin and Warner (1977) showed that lowering pH_i of *Xenopus* embryo cells by CO_2 exposure caused junctional uncoupling. The question, however, remained: whether the lowering of pH_i elevated $[Ca^{2+}]_i$.

Here we attempt to clarify the issue. We simultaneously monitor pH_i, $[Ca^{2+}]_i$, and junctional coupling during various experimental conditions of uncoupling in *Chironomus* salivary gland cells: pH_i, with a pH-sensitive glass microelectrode (Thomas, 1974); changes of $[Ca^{2+}]_i$ and its spatial distribution, with the aid of aequorin luminescence and an image intensifier system (Rose & Loewenstein, 1976); and junctional coupling, with the conventional electrical arrangement. We address the following primary questions: (i) Is a decrease in pH_i necessary to effect junctional

uncoupling? (ii) Is it sufficient, i.e., does it cause uncoupling in the absence of $[Ca^{2+}]_i$ elevation?

We find that a decrease in pH_i is *not necessary* for junctional uncoupling. Furthermore, we find that both decrease and increase in pH_i can cause $[Ca^{2+}]_i$ elevation, an elevation associated with uncoupling.

Materials and Methods

Media. Salivary glands of mid-fourth instar *Chironomus thummi* larvae were dissected and kept in a physiological medium ("CHEER"). Basically, we used 2 families of media: one is based on the composition of CHEER (Politoff, Socolar & Loewenstein, 1969), a medium in which much of the related work on *Chironomus* has been done in this laboratory. This medium, designed to simulate *chironomous* hemolymph, contains several organic compounds. The other family is based on a simplified version of this medium ("Saline"), composed only of inorganic salts, a pH buffer, and trehalose for osmotic balance. These Salines were used in experiments in which it was desirable to avoid the extra buffering systems provided by the organic compounds. The glands kept well for many hours in either CHEER or Saline. Table 1 lists the composition of CHEER and the other media. 2.4-Dinitrophenol (DNP) was dissolved in the medium many hours before use to insure complete dissolution. Sodium cyanide (NaCN) was added to the medium immediately before application. Ionophores A23187 (E. Lilly) and Nigericin (a gift of Dr. B. Pressman) were first dissolved in dimethylsulfoxide (DMSO) in a stock solution and then added to the rapidly stirred medium, yielding a final concentration of DMSO of 0.02%, which by itself did not affect the cell parameters studied here.

Carbon dioxide (CO_2) exposure. CO_2/O_2 mixtures of 5–100% CO_2 were bubbled vigorously through the medium in a reservoir, from which the gas-saturated medium was pumped onto the preparation. In pCO_2 control measurements with a CO_2 probe (Microelectrodes, Inc.) the CO_2 concentrations in the preparation bath were $>80\%$ of the nominal value.

External pH *measurement.* The pH of the bathing medium (pH_e) was continuously monitored with a small pH probe (Microelectrodes, Inc.) in the petri dish close to the preparation.

Intracellular pH. For intracellular pH (pH_i) measurements, we manufactured recessed-tip pH-sensitive glass microelectrodes according to Thomas (1974). These electrodes respond specifically to H^+ ions without interference from other ions. The response of the electrodes was linear between pH 4 and pH 9 and generally lay between 57 and 60 mV/pH unit (Fig. 1). In most cases the electrode potential was stable for several days. The response time to changes in pH – to 90% of the maximum response – was 30–50 sec. The electrical response time was about 100 msec at an electrode resistance of 10^9–$10^{10}\Omega$. pH sensitivity and response time were determined immediately before and after each experiment; only experiments with unchanged readings were accepted.

The pH microelectrode potential, E_{total}, was recorded with a high impedance operational amplifier (Analog Devices, Model 311J; input impedance $>10^{14}\Omega$). The amplifier was part of an electrical circuit that permitted testing of the electrode resistance, recording of the intracellular reference potential (E_1) with a conventional KCl-filled microelectrode, and differential reading of the intracellular pH potential, $E_{pH_i}=E_{total}-E_1$.

Table 1. Composition of media (mM)

	NaCl	LiCl	TRIS-HCl	NA-propionate	$NaHCO_3$	NH_4HCO_3	KCl	K_2SO_4	$CaCl_2$	$MgCl_2$	Mg-succinate	Na_2-succinate	Li_2-succinate	Na_2-fumarate	Glutamine	Trehalose	TES	pH adjusted to 7.4[b] with
CHEER[a]	28						2		5		7			28	80		5	NaOH
CHEER; Ca, Mg-free	38						2					7		28	80		5	NaOH
Li-CHEER; Ca, Mg-free		38					2						38		80		5	LiOH
$NaHCO_3$-CHEER					33		2		5		7			28	80			NaOH
Saline	70						2		5		7					80	5	NaOH
TRIS-Saline			70				2		5	7						80	5	TRIZMA base
$NaHCO_3$-Saline						75	2		5	7						80		[c]
Saline; Ca, Mg-free	83						2									80	5	NaOH
Propionate-Saline Ca, Mg-free				83				1								80	5	NaOH
NH_4HCO_3-Saline; Ca, Mg-free						83	2									80	5	[d]

[a] CHEER, 0.5 ... 2.5 mM Ca: CHEER media of lower Ca- (and Mg-)concentrations were prepared by appropriate mixing of CHEER and CHEER, Ca, Mg-free.

[b] In those experiments in which pH was adjusted to different levels, it is so stated in the text.

[c] pH 8.5 (not adjusted).

[d] pH 8.4 (not adjusted).

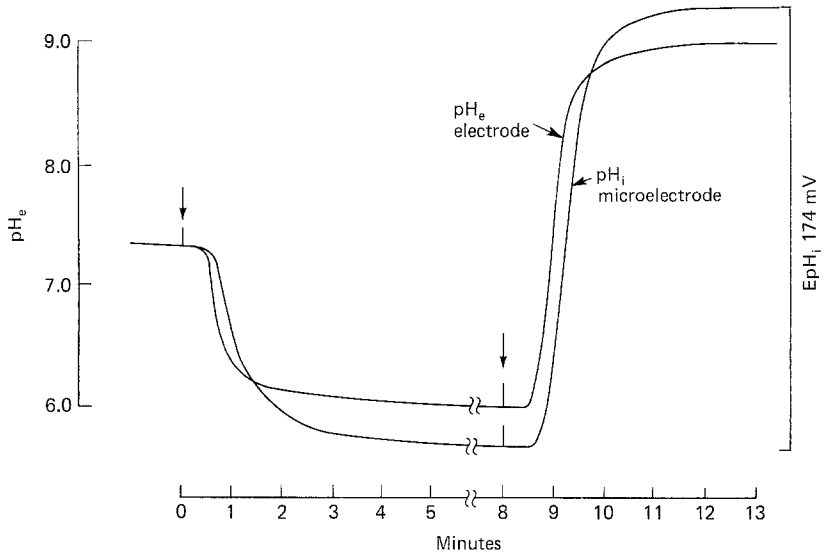


Fig. 1. Response of typical microelectrode used for measuring intracellular pH (pH_i). Calibration record. The electrode here is placed in the bathing solution. Solutions (pH buffers) are exchanged the same way (pumping) as in actual experiments in which the electrode is inside a cell. Arrows mark the onset of pumping of a different buffer. The pH-sensitivity of this particular microelectrode is 58 mV/pH unit ($E_{pH_i}=174$ mV/3 pH units). The time course of the bath exchange is given by the record of a pH "macro" electrode (pH_e) also placed in the bath

Junctional coupling. Three electrodes were used to monitor cell coupling (*see* Fig. 2): 2 adjacent cells in the gland were impaled each with a KCl electrode recording cell potentials (E_1; E_2). A third electrode was used to pass current test pulses (usually 0.1 sec^{-1}; 20–40 nA; 300 msec duration) between cell *1* and the grounded medium (i_1); and the resulting steady-state voltage deflections (V_1, V_2) in cells *1* and *2*, respectively, were monitored. The current injections, in addition, provided a check for proper impalement of the pH_i electrode: since the pH_i electrode and the reference electrode (in the same cell) see the same membrane potential, the current-induced voltage deflection from both E_{total} and from E_1 ($=V_1$) must be identical. $E_1(V_1)$, $E_2(V_2)$, i_1, E_{pH_i}, E_{pH_e}, and E_{total} were all recorded simultaneously on a 6-channel chart recorder (Soltec).

Intracellular Ca^{2+}. Intracellular Ca^{2+} changes were visualized with the aid of the Ca^{2+}-sensitive photoprotein aequorin (Shimomura, Johnson & Saiga, 1962) and an image intensifier-television system (Fig. 2). This technique has been described elsewhere (Rose & Loewenstein, 1976). Aequorin was injected into one or two neighboring cells, bathed in Ca, Mg-free CHEER. After injection and withdrawal of the aequorin pipette, the pH microelectrode was inserted, followed by electrodes E_1, E_2, and i_1—generally in that order. The medium then was changed to one containing 0.5 to 1 mM Ca, or to CHEER. The $[Ca^{2+}]_i$ detection threshold of the method (in a normal cell) was $1\text{–}2\times10^{-6}$ M, and the response time ≈ 100 msec.

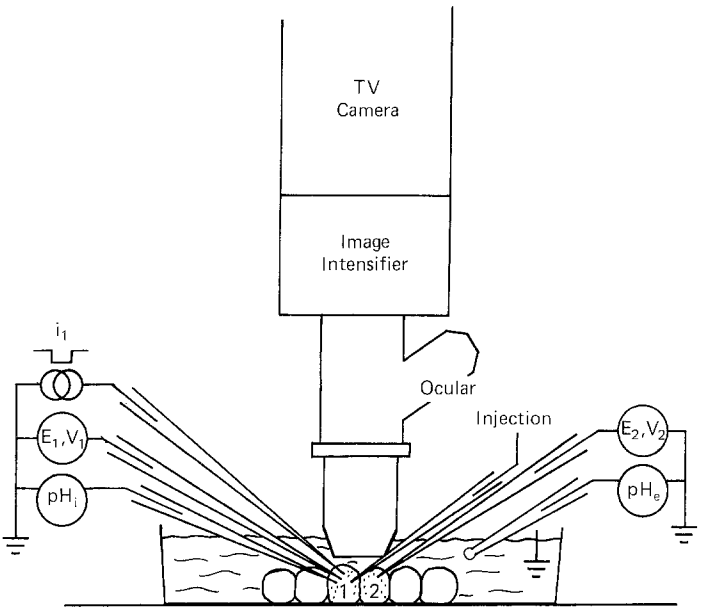


Fig. 2. Experimental setup. One or two cells (dotted) are injected with aequorin. An image intensifier coupled to a TV camera views the aequorin luminescence through a microscope. Electrical coupling is measured by pulsing current (i_1) from an electrode in cell *1* to the grounded bath, and measuring the resulting steady-state changes (V) in membrane potential (E) in cells *1* and *2*. (The electrical coupling parameters are displayed on an oscilloscope onto which a second TV camera is focussed. The two camera outputs are displayed and videotaped simultaneously.) Intracellular pH (pH_i) is measured with a pH-sensitive microelectrode in cell *1*. Intracellular injections are made with an additional micropipette. Extracellular bath pH (pH_e) is measured with a commercial pH electrode placed close to the preparation

Intracellular injections.

1) H^+: H^+ was injected iontophoretically either (i) by passing an outward current from an electrode filled with 1 N HCl; or (ii) by passing a current between the 2 barrels of a double-barrelled electrode filled with 1 N HCl and 1 M KCl, respectively; or (iii) by simultaneously passing an inward current from a 3-M KCl-filled electrode (i_1) and an outward current from a 1-N HCl-filled electrode. *ii* and *iii* allowed H^+ injection without imposing a potential change onto the cell.

2) Ca^{2+}: Ca^{2+} was injected (i) iontophoretically, by passing an outward current from an electrode filled with 0.4 M $CaCl_2$ and 0.2 M ethylene-glycol-bis-(*b*-aminoethylether)-N,N′-tetra acetic acid (EGTA); or (ii) by pressure (Rose & Loewenstein, 1976), using the following solutions: $Ca(OH)_2$, 0.06 M; K_3-citrate, 0.1 M, pH 7.5; or $CaCl_2$, 9.5 mM; EGTA, 10 mM, in PIPES buffer, 0.2 M, pH 7.2; or $CaCl_2$, 2 mM; EGTA, 10 mM; pH 6.4 (with KOH).

Temperature. All experiments were done at room temperature (20–23 °C). Although CO_2-bubbling caused a substantial drop in temperature of the medium in the reservoir, the temperature of the medium reaching the preparation was only about 2 °C below room temperature.

Results

Criterion for Uncoupling

We shall use the term *uncoupling* throughout this paper to denote a decrease in junctional conductance. Our general criterion for uncoupling thus defined is a rise in V_1 concomitant with a fall in V_2/V_1. A simultaneous change in opposite direction of these parameters unambiguously indicates a fall in junctional conductance (Loewenstein *et al.*, 1967). This criterion is stringent, but without simultaneous measurement of yet another parameter we could not reliably assess the absolute conductance change in those experiments in which the uncoupling agents were applied to the outside of the gland and thus could affect the junctional (g_j) and nonjunctional (g_n) membrane conductances of all cells. This was so in most of the present experiments. Only in the experiments of intracellular Ca^{2+}-injection may we reasonably assume that g_j and g_n of only the injected cell are affected, because Ca^{2+} does not significantly diffuse through the junctions (Rose & Loewenstein, 1976). The reduction factor (α) in junctional conductance therefore may readily be calculated, since $(V_1-V_2)/V_2=g_n/g_j$ of cell *2*, thus $((V_1-V_2)/V_2)$ (control) $=\alpha\ ((V_1-V_2)/V_2)$ (injection). In all other cases we use the qualitative, though more stringent, criterion defined above. In the presentation of our results, the coupling coefficient V_2/V_1 is always plotted (or listed in the Tables), because it reflects quite faithfully at least the onset of the junctional conductance change. We also present the plots of V_1 (and V_2) or, when this could conveniently be done, the actual continuous or sample voltage records, or mention V_1 in the figure legends.

"Normal" Intracellular pH

Following insertion of the pH- and other microelectrodes into the cell, the measured pH_i usually showed an increase of 0.1 to 0.2 units, for about 2–10 min. During this period the cell membrane probably

Table 2. Normal intracellular pH_i and coupling coefficient

Medium	$pH_i \pm$ SD	$V_2/V_1 \pm$ SD	*n*
CHEER	7.51 ± 0.17	0.93 ± 0.06	20(6)
CHEER-Ca, Mg	7.55 ± 0.16	0.86 ± 0.10	9(4)
CHEER-1 mM Ca	7.47 ± 0.25	0.86 ± 0.20	7(7)

n=number of cells; in parentheses, number of cells injected with aequorin.

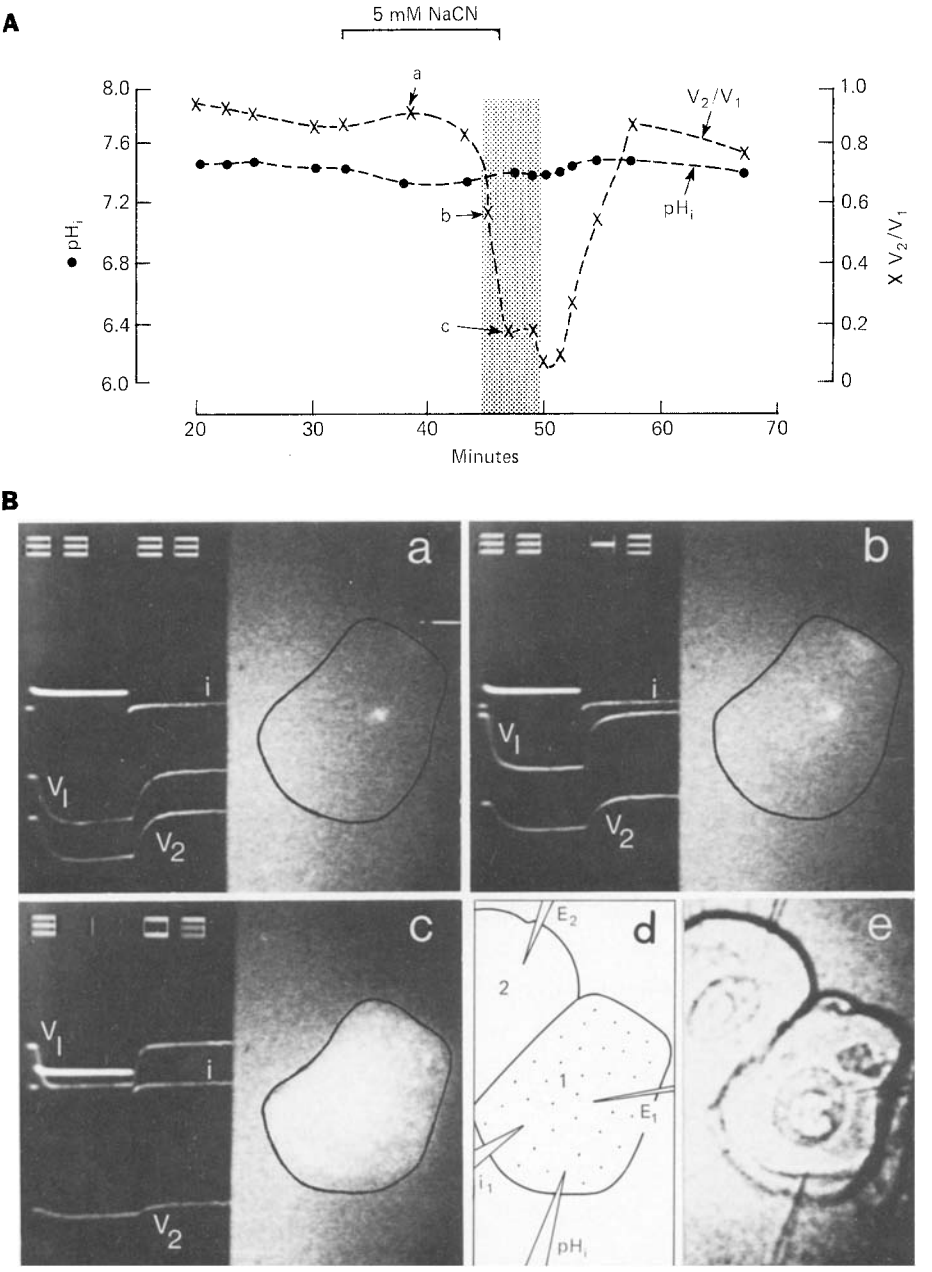


Fig. 3. Uncoupling correlates with $[Ca^{2+}]_i$ rise, not with pH_i change during treatment with CN. (*A*): Plot of pH_i and coupling coefficient V_2/V_1. Preparation in CHEER medium, 0.5 mM Ca. Bar marks period of exposure to NaCN, and shaded area the period of $[Ca^{2+}]_i$ elevation as seen by the image-intensifier system in the aequorin-injected cell. (*B*, *a–c*): Photographs of video display, taken at the times indicated in *A*. Right halves of the photos display the image intensifier view of the cells in darkfield, outline of the injected cell drawn in. Left halves display the simultaneous storage oscilloscope records of i_1 (20 nA; 300 ms; 0.1 sec^{-1}), V_1 and V_2. (*a*): V_1, V_2 are nearly equal in these well-coupled cells;

sealed around the electrodes. Thereafter, pH_i remained stable in the otherwise undisturbed cell. This stable value (at 10 min after impalements) is listed in Table 2 as the "normal" pH_i for three different bathing media, together with the corresponding coupling coefficients V_2/V_1. Mean pH_i values ranged 7.47 to 7.55. The pH_i values of cells injected with aequorin did not differ from those of uninjected cells.

pH_i during Junctional Uncoupling Produced by Treatments Elevating $[Ca^{2+}]_i$

The following experiments were undertaken to determine the temporal relation of changes in coupling, pH_i, and $[Ca^{2+}]_i$ under various experimental conditions known to cause uncoupling associated with an elevation of $[Ca^{2+}]_i$ (*c.f.* Loewenstein & Rose, 1978).

a) NaCN

Exposure to NaCN (5 mM) caused lowering of pH_i by 0.2 units (*see also* Boron & DeWeer, 1976). As in earlier experiments (Rose & Loewenstein, 1976), the onset of uncoupling—as signalled by a rise in V_1 with a concomitant fall in V_2/V_1—coincided with an elevation of $[Ca^{2+}]_i$ (Fig. 3*B*). However, there was no correlation with pH_i: uncoupling set in much later than the small change in pH_i (Fig. 3*A* and *B*), and in 1 case, in which the pH_i change was transient, the uncoupling set in during the return phase of pH_i, close to the original level.

the aequorin-injected cell (1) is dark, i.e., $[Ca^{2+}]_i \lesssim 10^{-6}$ M, except for a tiny spot of strong luminescence around the E_1 electrode, caused by Ca^{2+} entering the cell through a membrane leak at the E_1 electrode. (The brightness in the left lower portion of the darkfield stems from background "white" noise of the TV system ("blooming"), not from the cell. This "blooming" is also present in most of the following illustrations.) (*b*): Junctional conductance has decreased as reflected by the rise of V_1 and fall of V_2 ("uncoupling"). Luminescence now appears faintly throughout the cell, indicating diffuse $[Ca^{2+}]_i$ rise. (*c*): V_2 now $\ll V_1$; cell *1* has depolarized strongly (upward shift of V_1-trace), and its nonjunctional membrane resistance has fallen (V_1 now smaller than in *a*), associated with the high $[Ca^{2+}]_i$ elevation). (*d*): Diagram of cells and electrode arrangement. Dotted cell contains aequorin. (*e*): brightfield TV photo of cells. Several of the electrodes are visible

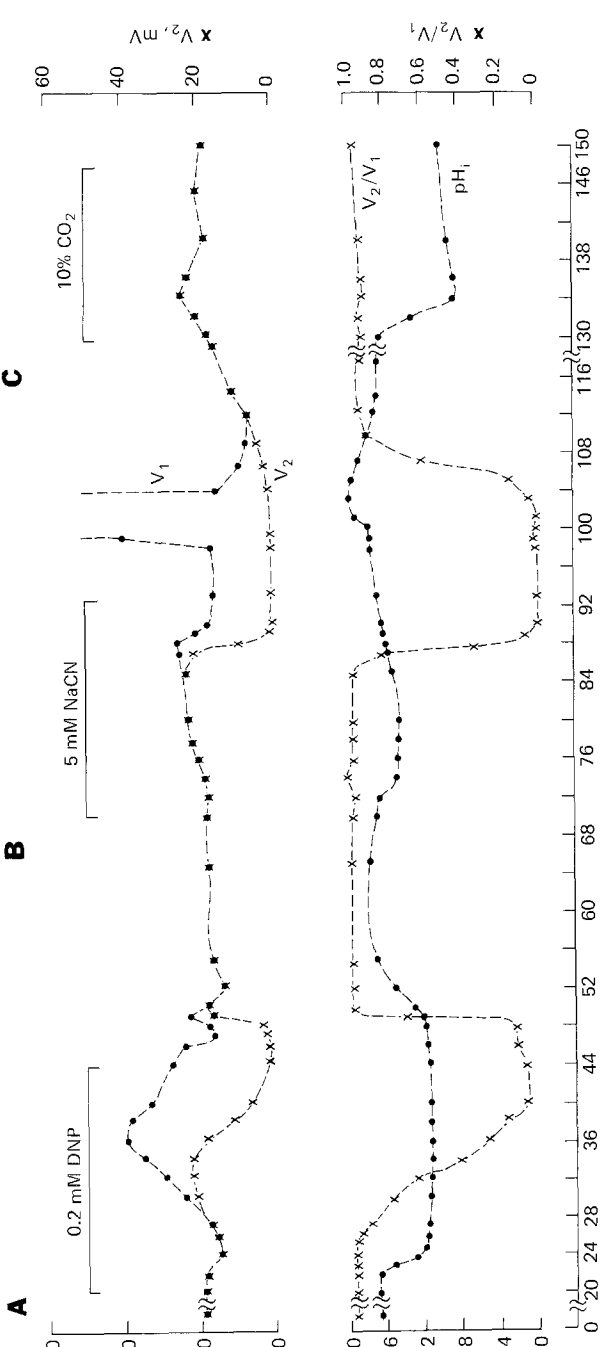


Fig. 4. Lack of correlation between pH_i and coupling changes. In this experiment cells are exposed in sequence to (*A*) 0.2 mM DNP; (*B*) 5 mM NaCN; and (*C*) 10% CO_2 (CHEER throughout). *Top:* plot of V_1 and V_2; *bottom:* pH_i and V_2/V_1. DNP and NaCN produce uncoupling and pH_i depression: uncouplings set in later than the pH_i decreases (in *B*, 15 min later); recoupling upon DNP washout is nearly complete at the time pH_i only begins to recover. A sustained decrease in pH_i to 6.87 (even more pronounced than in *A* and *B*) by exposure to 10% CO_2 in *C*, does not uncouple (coupling coefficient actually increases slightly). The sudden transient increase of V_1 in *B* after washout of CN reflects the return to normal of nonjunctional membrane conductance, which—perhaps due to $[Ca^{2+}]_i$ elevation—had increased during CN treatment. Because cells initially are still uncoupled during this recovery phase, V_1 increases dramatically

b) DNP

In medium containing DNP (0.2 mM), pH_i fell rapidly (5 min) by about 0.5 units, similar to snail neuron (Thomas, 1974) and squid axon (Boron & DeWeer, 1976). Uncoupling occurred later, at a time when the pH_i change was already complete. Moreover, upon washout of the poison, cell coupling was almost fully restored when pH_i only began to recover (Fig. 4*A*). (Note that in the same experiment, application of 10% CO_2 caused a drop in pH_i by 0.9 units without diminishing coupling in the least. Fig. 4*C*).

c) Ca^{2+}-Transporting Ionophore A23187

Two experiments of pH_i measurements during exposure to 2 μM A23187 were performed. In one experiment (Fig. 5), addition of the ionophore produced a rise in pH_i by 0.3 units. In the other experiment, the ionophore caused pH_i to fall by 0.13 units. In both cases the cells uncoupled as in earlier experiments (Rose & Loewenstein, 1976), in which this uncoupling was shown to be associated with $[Ca^{2+}]_i$ elevation. The lack of correlation between pH_i and uncoupling is further underlined by the fact that in the experiment in which pH_i fell, coupling recovered partially as pH_i continued to decline (to 7.4) during ionophore washout.

Table 3 summarizes the results obtained with NaCN, DNP and A23187.

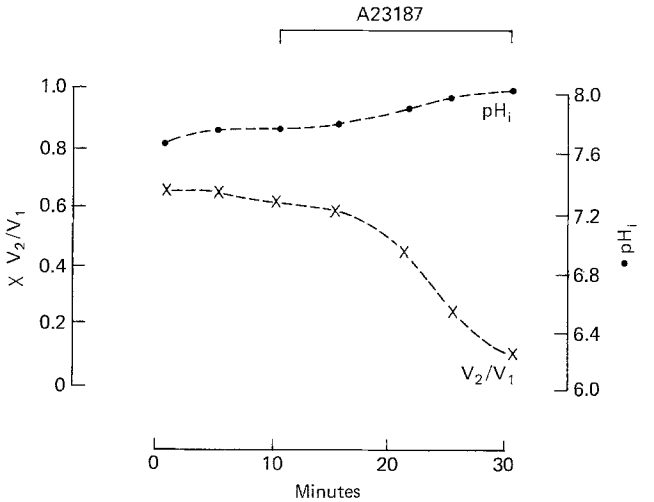


Fig. 5. pH_i rises during uncoupling by Ca-transporting ionophore A23187 (2 μM) in 2.5 mM Ca-CHEER

Table 3. Effect of NaCN, DNP, and A23187 on pH_i and coupling

Treatment	Medium	Exposure time (min)	pH_i control	pH_i during treatment		V_2/V_1		Remarks
				At onset of uncoupling	max ΔpH_i	Control	Treatment	
A: NaCN (5 mM)	CHEER	23	7.70	7.58	−0.23	0.97	<0.01	Illustrated in Fig. 4
	CHEER	25	7.72	7.52	−0.22	0.89	0.04	
	CHEER, 0.5 mM Ca	14	7.48	7.35	−0.13	0.87	0.07	In this experiment $[Ca^{2+}]_i$ wa monitored and seen to rise at time of uncoupling onset. *See* Fig. 3
B: DNP (0.2 mM)	CHEER	20	7.68	7.18	−0.55	0.97	0.07	Illustrated in Fig. 4
	CHEER; Ca, Mg-free + 10^{-5} M EGTA	15	7.65	7.35	−0.30	0.67	0.42	Uncoupling remained partial
(0.4 mM)	CHEER	10		6.80	−0.56		0.11	Continuation of experiment listed immediately above
C: A23187 (2 μM)	CHEER 2.5 mM Ca	20	7.75	7.87	+0.3	0.64	0.14	Illustrated in Fig. 6
	CHEER; Ca, Mg-free + 10^{-5} M EGTA	7	7.79	7.71	−0.13	0.71	0.04	pH_e here was 6.(

d) Ca^{2+} Injections

We injected (by pressure) Ca^{2+}-solutions buffered in $[H^+]$. In one solution, the buffer was calcium citrate, pH 7.5, with a free $[Ca^{2+}] > 10^{-4}$ M; in the other, $[H^+]$ was buffered by 0.2 M PIPES, pH 7.2 in a Ca/EGTA mixture yielding free $[Ca^{2+}] > 10^{-5}$ M (*see Methods* for composition of injection solutions). The various injections depressed pH_i only by 0.05–0.28 units, yet junctional conductance, g_j, dropped substantially (Table 4).

In striking contrast is the result of an injection of a solution buffered to $[Ca^{2+}] < 10^{-6}$ M, and of pH 6.4. This caused no change in junctional

Table 4. Effect of Ca^{2+} injection on pH_i and coupling

Injection solution				pH_i			V_2/V_1		αg_j[e]
Cell pair[a]		$[Ca^{2+}]$	pH	Control	Injection	ΔpH_i	Control	Injection	
A *a)*	Ca/citrate[b]	$\gtrsim 10^{-4}$ M	7.5	7.54	7.47	−0.07	0.90	0.82	1.98
b)	Ca/citrate	$\gtrsim 10^{-4}$ M	7.5	7.58	7.44	−0.14	0.92	0.56	9.04
c)	Ca/citrate	$\gtrsim 10^{-4}$ M	7.5	7.70	7.47	−0.23	0.89	0.48	8.76
d)	Ca/citrate	$\gtrsim 10^{-4}$ M	7.5	7.70	7.44	−0.26	0.84	0.47	5.92
e)	Ca/EGTA[c]	$\lesssim 10^{-6}$ M	6.4	7.58	6.76	−0.82	0.90	0.90	No change
B *a)*	Ca/EGTA[d]	$\gtrsim 10^{-5}$ M	7.2	7.40	7.12	−0.28	0.72	0.24	8.14
b)	Ca/EGTA	$\gtrsim 10^{-5}$ M	7.2	7.44	7.17	−0.27	0.85	0.15	32.1
C *a)*	Ca/EGTA[d]	$\gtrsim 10^{-5}$ M	7.2	7.30	7.17	−0.13	0.91	0.64	5.69
b)	Ca/EGTA	$\gtrsim 10^{-5}$ M	7.2	7.26	7.21	−0.05	0.80	0.61	2.56
c)	Ca/EGTA	$\gtrsim 10^{-5}$ M	7.2	7.35	7.25	−0.10	0.87	0.77	2.00

[a] Cell pairs A, B, C were in different glands; *a*, *b* ... *e*: repeated (separate) injections into the same cell.

[b] $Ca(OH)_2$ 0.06 M; K_3-citrate 0.1 M.

[c] $CaCl_2$, 2 mM; EGTA, 10 mM.

[d] $CaCl_2$, 9.5 mM; EGTA, 10 mM; in PIPES 0.2 M.

[e] Factor α, by which junctional conductance g_j is decreased, calculated according to

$$\alpha = \frac{((V_1 - V_2)/V_2)_{\text{control}}}{((V_1 - V_2)/V_2)_{\text{injection}}}.$$

conductance although pH_i had fallen by 0.82 units. This result was obtained in the same cell pair (*A*, Table 4) in which a fall in junctional conductance was observed with little ΔpH_i upon injection of the citrate solution of high free $[Ca^{2+}]$.

e) Cell Alkalinization

The aforegoing series of experiments indicate that decrease of pH_i is not necessary for the various uncouplings; uncoupling ensued with little or no decrement in pH_i. In the following experiments, we show that uncoupling can occur even when pH_i is markedly elevated. We increase pH_i by exposing the cells to NH_4HCO_3, TRIS, or by sudden withdrawal of propionate from a propionate-containing medium. $[Ca^{2+}]_i$, pH_i, and coupling are monitored.

i) NH_4HCO_3. Treatment with NH_4HCO_3 caused pH_i to rise rapidly to 8.3–8.5. This rise was accompanied by a short-lived $[Ca^{2+}]_i$ elevation

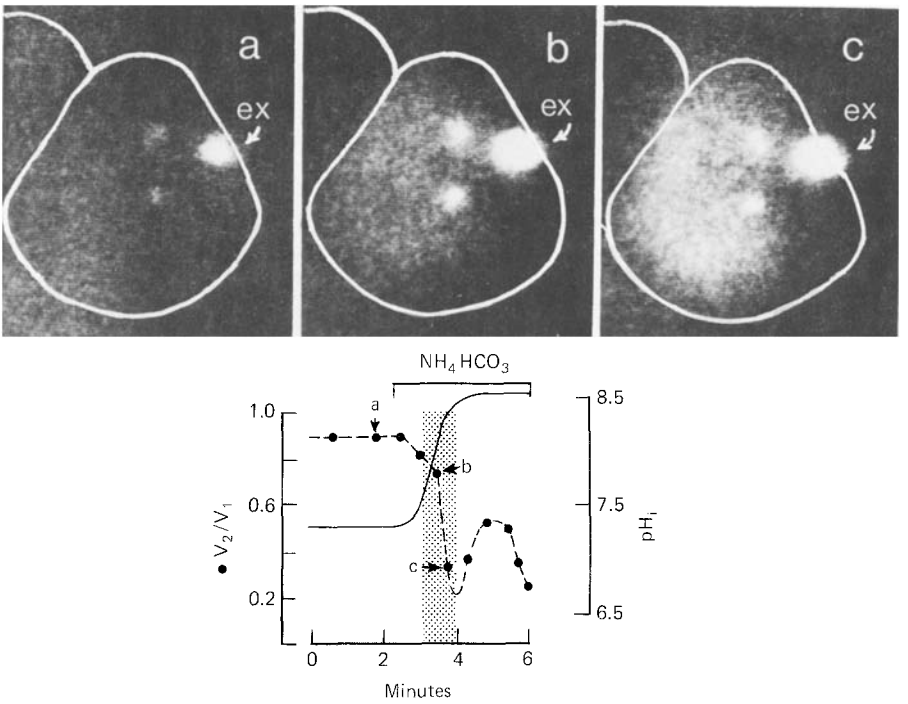


Fig. 6. $[Ca^{2+}]_i$ elevation and decrease in coupling coefficient during cell alkalinization: NH_4HCO_3 application. *Top:* videophotos of aequorin-injected cell (*a*) before application of NH_4HCO_3-saline; Ca, Mg-free; (*b*) 1 min, and (*c*) 1.5 min after NH_4HCO_3 application. (The bright spot of elevated $[Ca^{2+}]_i$ was identified in brightfield as an exocytosis bleb (*ex*) being extruded from the cell. The other two spots of $[Ca^{2+}]_i$ elevation appearing in *b* and *c*, are at the electrode insertion sites – often the areas where the $[Ca^{2+}]_i$ elevation appears first, including when the cells are in Ca-free medium; *see also* Rose & Loewenstein, 1976). Below, plot of V_2/V_1 and pH_i record. In this experiment, the fall in V_2/V_1 was not accompanied by a rise in V_1. Nonjunctional conductance here increased so quickly that a decrease in junctional conductance could not be evidenced by a rise in V_1. This figure serves not so much to illustrate the change in junctional conductance, as the associated elevation of $[Ca^{2+}]_i$ during alkalinization

and by uncoupling (Fig. 6). These cell alkalinizations were in one case achieved by direct application of NH_4HCO_3 medium, and in two other cases by removal of CO_2 from NH_4HCO_3 medium saturated with 100% CO_2. In all cases, when pH_i rose to >7.8, V_2/V_1 decreased with associated rise in $[Ca^{2+}]_i$. In these experiments, nonjunctional conductance increased so rapidly upon NH_4HCO_3 application that a decrease in junctional conductance could not be evidenced by a rise in V_1. Upon washout of NH_4HCO_3, pH_i swung to 6.49 (*see* Boron & DeWeer, 1976,

Table 5. $[Ca^{2+}]_i$ and coupling during treatments producing cell alkalinization

Cell pair	Pretreatment			Treatment						
	Medium	pH_i	V_2/V_1	Medium	Steady-state pH_i	Transient V_2/V_1[a]	Steady-state V_2/V_1	pH_i at onset of uncoupling	Onset of uncoupling (min)[b]	Period of aequorin glow (min)[b]
A	Saline	7.27	0.85	NH_4HCO_3-saline	8.49		0.15	7.7	0.5	0.5–1
A	100% CO_2 in NH_4HCO_3-saline	6.64	0.3	NH_4HCO_3-saline	8.3	0.8	0.01	7.8	0.9	1–2
B	100% CO_2 in NH_4HCO_3-saline	6.66	0.05	NH_4HCO_3-saline	8.29	0.35	0.03	7.8	1.2	ND
C	Saline	7.50	0.88	TRIS-saline	8.16		0.77	7.8	5	ND
C	100% CO_2 in TRIS-saline	6.48	0.06	TRIS-saline	7.98	0.76	0.69	7.7	8	ND
D	Propionate-saline Ca, Mg-free (78 min)	7.39	0.32	Washout with saline, Ca, Mg-free	8.74	0.55	0.02	7.65	2.2	2.2–4
E	Propionate-saline, Ca, Mg-free (10 min)	7.33	0.92	Washout with CHEER	8.33		0.08	7.7	6	ND
F	Propionate-saline, Ca, Mg-free (12 min)	ND	0.95	Washout with saline	ND		0.60	ND	1.1	1.2–2.3

[a] In several experiments, the first effect of the *treatment* was a transient recoupling with subsequent renewed uncoupling. In this column we list V_2/V_1 at peak of recoupling of these cases.
[b] Measured from arrival of *treatment* medium in bath.
ND: not determined.

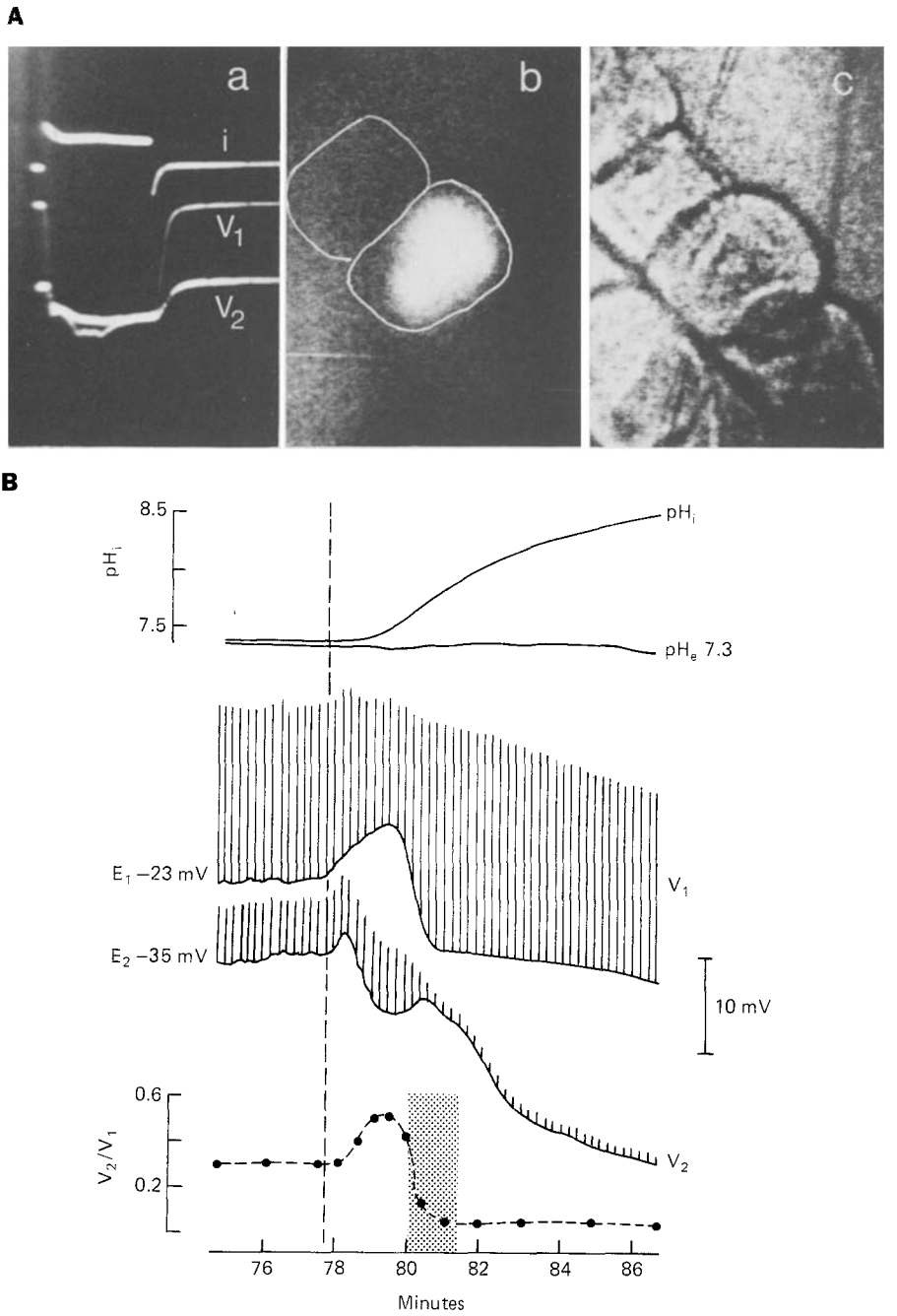


Fig. 7. $[Ca^{2+}]_i$ elevation and uncoupling during cell alkalinization: propionate washout. Cells were pretreated with propionate-saline, Ca, Mg-free, which then was washed out with saline, Ca, Mg-free. (*B*): From top to bottom: pH_i and pH_e; voltage records of cell *1* and cell *2*; plot of V_2/V_1. Cells were pretreated for 78 min, during which time V_2/V_1 fell to 0.3 (same experiment as in Fig. 17*A*, and Table 5*D*). Washout of propionate

for mechanism of this acidification), $[Ca^{2+}]_i$ stayed below detection, and recoupling began, with pH_i at about 6.5. The recoupling here clearly signalled an increase in g_j because V_1 fell as V_2/V_1 rose.

ii) TRIS. In TRIS medium pH_i rose from 7.5 to 8.16, accompanied by a slight depression of coupling (Table 5; $[Ca^{2+}]_i$ was not monitored here).

iii) Propionate withdrawal. Cells treated with propionate saline (Cl^--free) and subsequently placed in normal (Cl^--containing) saline, became alkaline. In one experiment in which cells were pretreated for 10 min with propionate saline, pH_i rose to 8.33 upon propionate washout, with concomitant uncoupling (cells had not uncoupled during the brief pretreatment). In a second experiment of this kind (77 min propionate pretreatment), propionate washout raised pH_i to 8.74. Here cells had uncoupled during the pretreatment, and propionate washout led first to a phase of recoupling, followed by renewed uncoupling at $pH_i > 7.65$, which was associated by $[Ca^{2+}]_i$ elevation (Fig. 7).

These experiments bring out the interesting point that alkalinization causes a fall in junctional conductance, associated with $[Ca^{2+}]_i$ rise. Clearly, uncoupling can occur at these rather high pH_i values. Moreover, as described in the experiment of cell acidification upon NH_4HCO_3-washout, cells can recouple at low pH_i. The experiments of cell alkalinization are summarized in Table 5.

Baker and Honerjaeger (1978) have shown that Ca^{2+} influx increases during alkalinization in squid axon. This may be the underlying mechanism of $[Ca^{2+}]_i$ elevation in the salivary gland, too. The brevity of the aequorin glows ($\approx$0.5–1.0 min) in the alkalinization experiments conceivably reflects the decrease in aequorin sensitivity to Ca^{2+} at pH > 8 (Shimomura & Johnson, 1973; Baker & Honerjaeger, 1978).

$[Ca^{2+}]_i$ during Junctional Uncoupling Produced by Treatments Lowering pH_i

Crucial to the question whether a pH_i decrease *per se* is sufficient to cause uncoupling, is whether $[Ca^{2+}]_i$ is elevated during cell acidifica-

(starting at stippled line) produces pH_i rise and an initial recoupling-phase followed by renewed uncoupling, associated with $[Ca^{2+}]_i$ elevation (shaded period). (*A*): Videophotos from a similar experiment (pretreatment here was for 12 min) 2 min after propionate washout: *a*, record of electrical coupling; *b*, simultaneous videophoto of aequorin glow (only the glowing cell contained aequorin); *c*, brightfield photo of cells

tion. We acidified cells by H^+ iontophoresis, exposure to H^+-transporting ionophore, or to CO_2, while monitoring $[Ca^{2+}]_i$, pH_i, and coupling.

a) $[Ca^{2+}]_i$ Measuring Sensitivity during Lowering of pH_i

First it was necessary to find out what effect changes in pH might have on the sensitivity of our Ca^{2+}-indicator aequorin. The results in the literature differ in this regard: Shimomura & Johnson (1973) report a stable aequorin response in the pH range 6–8; Moisescu, Ashley and Campbell (1975), using a different pH buffer, find a marked fall of sensitivity between pH 7.1 and 6.8; and Baker and Honerjaeger (1978), under yet another condition, describe a rise in sensitivity between pH 8 and 6.5. For the particular case of acidification by CO_2 exposure, Baker and Honerjaeger (1978) conclude that 5% CO_2 had no effect on the aequorin reaction; and Shimomura (*personal communication*) finds depression by $\approx 20\%$ with 100% CO_2 (constant pH).

The above information on aequorin sensitivity was obtained in *in vitro* conditions. *In vivo*, the situation could be more complex, since cell parameters other than pH_i may be affected, possibly changing the optical conditions in the cell. In fact, exposure to 100% CO_2 caused visible nuclear swelling and change in cell translucence. For these reasons, we tested the sensitivity of aequorin to Ca^{2+} inside the cell during CO_2 exposure. Standard Ca^{2+}-test pulses were iontophoresed into the cell and the resulting aequorin glows were videotaped together with the oscilloscope display of the Ca current.

We found a reduced response in a significant number of cases: in 5 out of 10 experiments, the aequorin glows in response to the standardized Ca^{2+}-current pulses decreased markedly, both in intensity and in spatial extent, during exposure to 100% CO_2 (Fig. 8); in 3 further experiments, the decrease was marginal (*see*, e.g., Fig. 14); and in 2 experiments there was no detectable decrease.

We also have evidence, although less direct, that aequorin sensitivity can diminish during H^+ injection. As described below, H^+ injection produces $[Ca^{2+}]_i$ elevation. The aequorin glow signalling that elevation typically brightened upon termination of the H^+-current (*c.f.* Figs. 9 *Bd*, 10 *Bd*). This brightening occurred over most of the cell, but it was at times delayed in the immediate vicinity of the H^+ pipette. We interpret this as recovery of the $[Ca^{2+}]_i$ measuring sensitivity due to pH_i recovery, and the presence of a H^+ gradient around the H^+ injection pipette. A similar

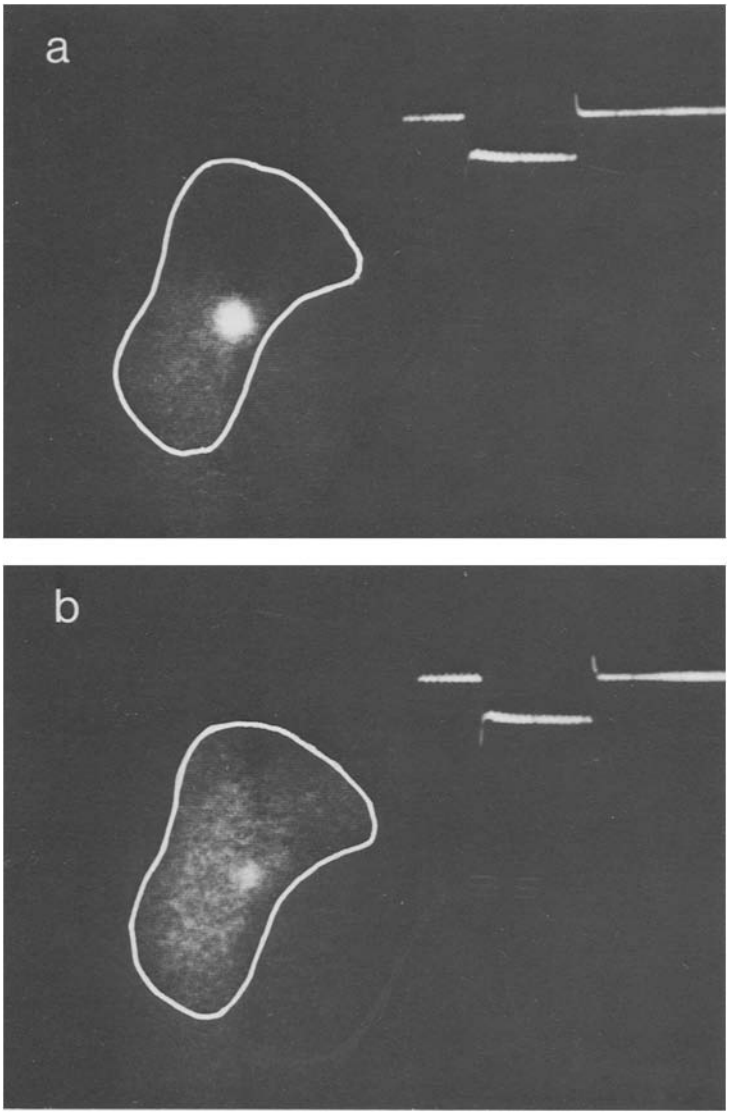
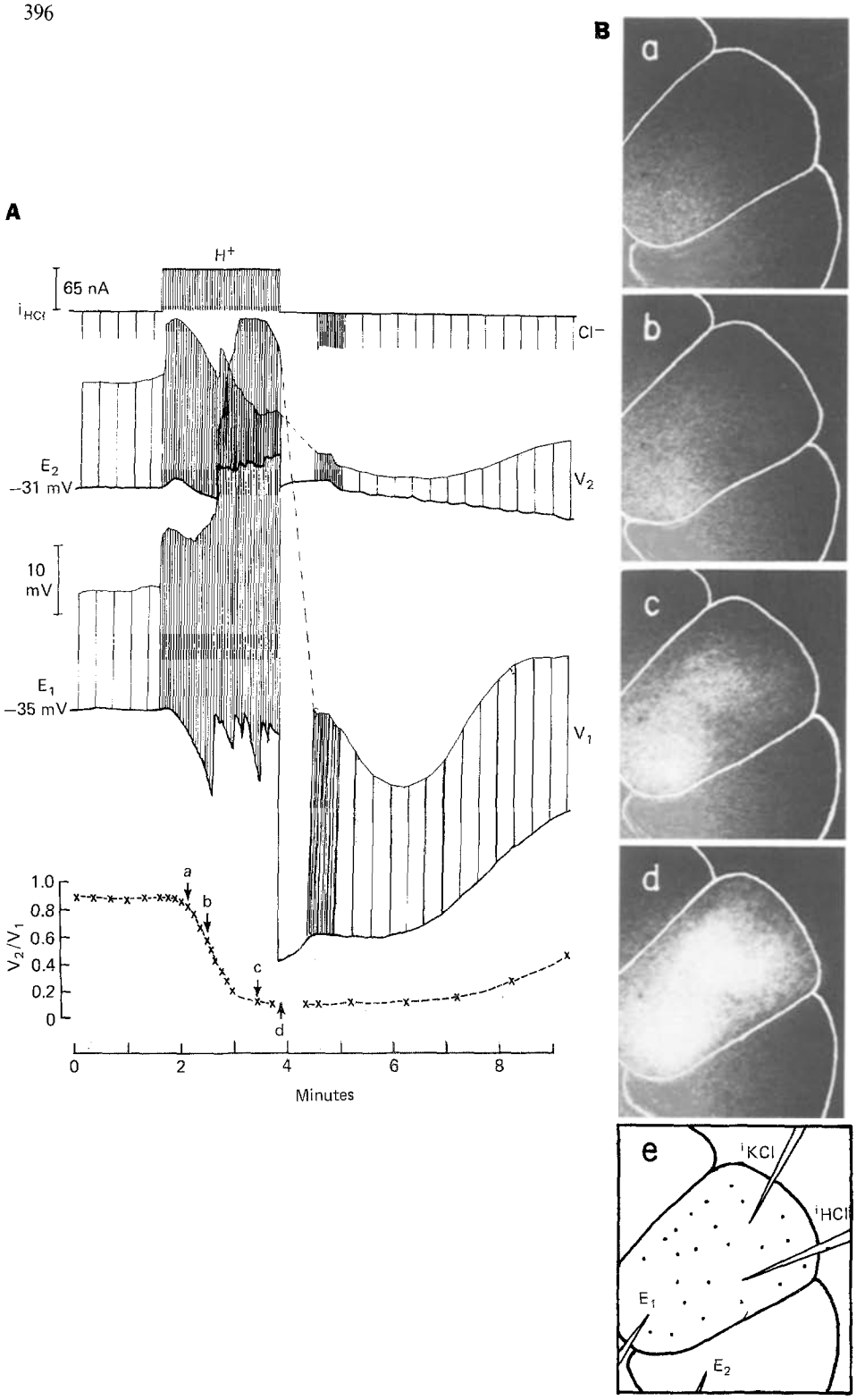


Fig. 8. Depression of $[Ca^{2+}]_i$ measuring sensitivity during cell exposure to 100% CO_2. Videophotos of aequorin-injected cells in microscope darkfield, and of the storage oscilloscope display of the standard Ca^{2+}-current test pulses delivered into one of the cells. The photos are taken at the time of maximal spatial spread of the aequorin glow. (*a*): Cells in CHEER, Ca, Mg-free. Aequorin glow response to the standard current test pulse (250 msec, 50 nA, 0.05 sec^{-1}). These shortlived responses were constant in intensity and spatial extent for test periods of at least 15 min. (*b*): Cells in 100% CO_2-saturated CHEER, Ca, Mg-free, 3 min after application. Note that brightness and spatial extent of the aequorin glow response to the standard Ca^{2+}-pulse are reduced. In addition, a weak glow is now visible throughout the cell. This glow is continuously present, independent of the Ca^{2+} test pulses; it is a CO_2-induced $[Ca^{2+}]_i$ elevation


A
B
H+
65 nA
iHCl
Cl−
E2
−31 mV
V2
10
mV
E1
−35 mV
V1
V2/V1
1.0
0.8
0.6
0.4
0.2
0
a
b
c
d
0
2
4
6
8
Minutes
a
b
c
d
e
iKCl
iHCl
E1
E2

brightening of the aequorin glow was observed many times during the 100% CO_2-washout period (*see* Table 6).

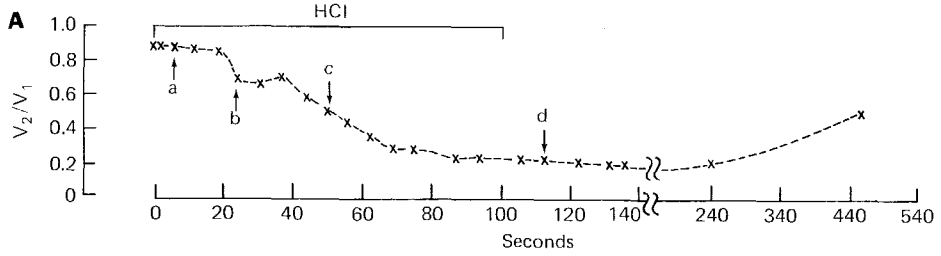
In summary, these tests reveal that our threshold for $[Ca^{2+}]_i$ detection can increase during cell acidification. The normal $[Ca^{2+}]_i$ level is below our $[Ca^{2+}]_i$ detection threshold of $1–2 \times 10^{-6}$ M at normal pH_i. There is thus a $[Ca^{2+}]_i$ range over which $[Ca^{2+}]_i$ elevations may remain undetected. This "dark" range—as shown by the tests—is widened at low pH_i. Hence a positive finding of $[Ca^{2+}]_i$ elevation in this condition is highly meaningful; a negative finding is little informative.

When comparing onset times of aequorin glow with onset times of uncoupling, the reader should bear in mind this normal "dark" range—and in the cases of cell acidification, a possibly widened "dark" range—which undoubtedly delays our detection of $[Ca^{2+}]_i$ elevation.

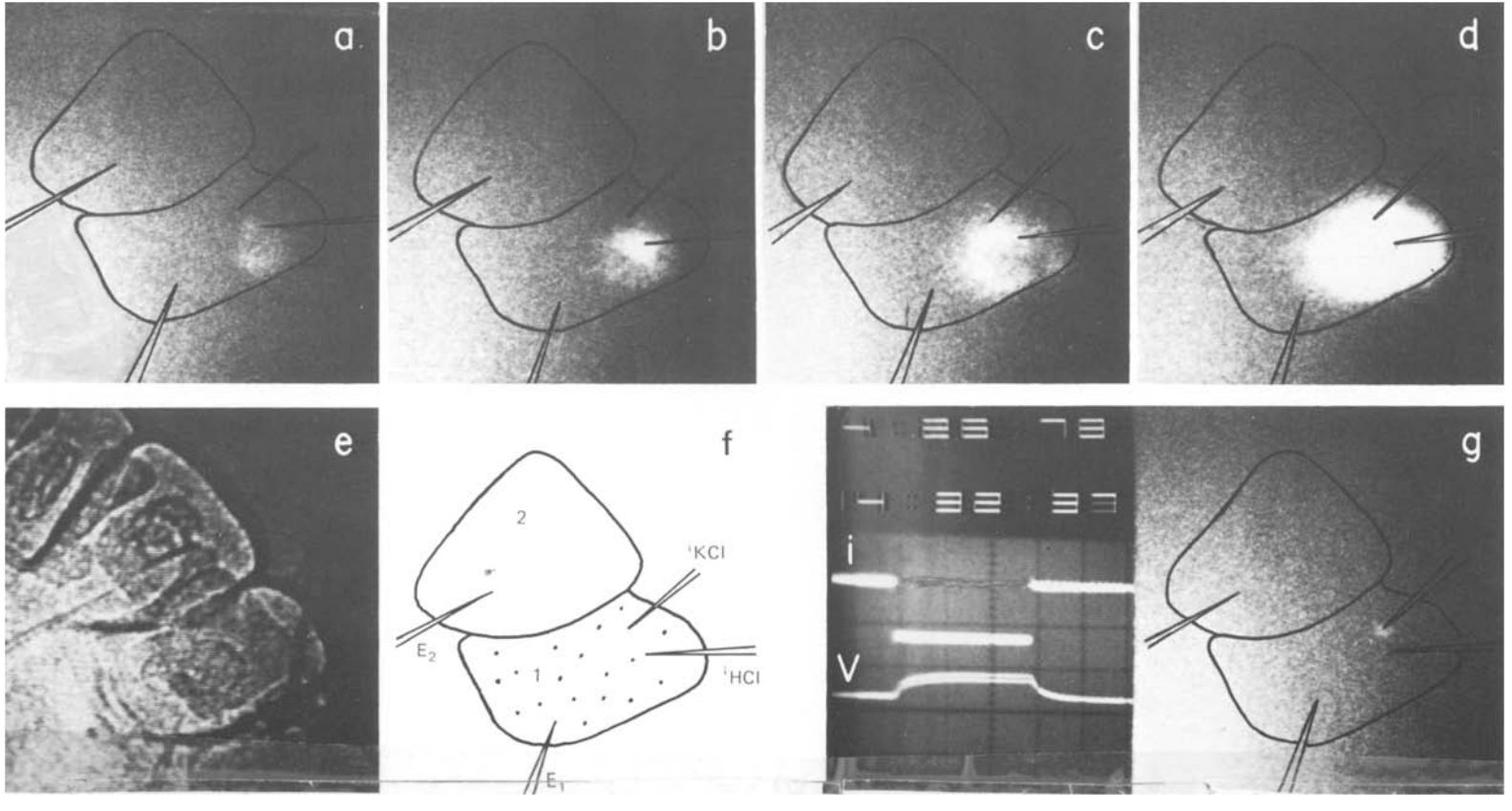
b) H^+ Injection

Proton injection is the most direct means for lowering pH_i. In one set of experiments we injected H^+ iontophoretically and monitored $[Ca^{2+}]_i$ and coupling. Since H^+ injection by outward current imposes membrane depolarization (which might affect the permeability characteristics of the junction (Socolar & Politoff, 1971) and of the cell membrane), we passed, in some experiments, a simultaneous inward current (Cl^-, from another electrode or from the second barrel of a double-barrelled electrode) into the same cell. In this way the membrane potential could be adjusted and held near the resting level (*see*, e.g., Fig. 9*A*).

Fig. 9. Uncoupling and $[Ca^{2+}]_i$ elevation during HCl-injection. (*A*) (top to bottom): Chart recorder tracings of the current (i_{HCl}) of the HCl-filled electrode; voltage record of cell *2*; voltage record of cell *1*; and plot of coupling coefficient V_2/V_1. (*B*, *a–d*): Darkfield videophotographs of the aequorin-injected cell (*1*), taken at the times indicated on the V_2/V_1 curve. (*e*): Diagram of cells and electrode arrangement. HCl is injected iontophoretically: H^+ by outward current (65 nA; upward deflection) from an HCl-filled electrode (i_{HCl}); Cl^-, by a simultaneous inward current from a KCl-filled electrode (i_{KCl}) (current trace not shown) passed into the same cell. This current is adjusted several times to keep the cell's membrane potential near resting level for the period of H^+ injection. Upon termination of the currents, the cell resumes its own potential (sudden large shift of E_1). Coupling is tested by inward (Cl^-) pulses (300 ms; 50 nA, increased to 65 nA during H^+ injection) from the HCl electrode. The envelopes of V_1 and V_2 are drawn in to facilitate trace identification. Shortly after onset of HCl injection, cells begin to uncouple. This uncoupling is accompanied by a diffuse $[Ca^{2+}]_i$ elevation, increasing in intensity and area as uncoupling proceeds (*a–c*). (*d*): Aequorin glow brightens upon termination of HCl injection (*see* text). Cells in CHEER, Ca, Mg-free


A
HCl
1.0
0.8
0.6
0.4
0.2
0
V_2/V_1
a
b
c
d
0
20
40
60
80
100
120
140
240
340
440
540
Seconds

B


a
b
c
d
e
f
2
KCl
E_2
1
HCl
E_1
g
i
V

Cell pair[a]	Medium	Number of cells containing aequorin	CO_2 application			CO_2 washout	
			$[Ca^{2+}]_i$ elevation (number of cells)	Onset aequorin glow (min)[b]	Onset uncoupl.[b] (min)	$[Ca^{2+}]_i$ elevation[c] (number of cells)	Onset aequorin glow (min)[d]
A *a*	CHEER; Ca, Mg-free	1	0	–	1	1	2.0
b	CHEER; Ca, Mg-free	1	0	–	1.2	1	0.5
c	CHEER	1	1	2	1.3	1	3.5
B *a*	$NaHCO_3$-CHEER	3	2	9; 10	2.4	1	3.5
b	$NaHCO_3$-CHEER	3	1	12	1.7	0	–
C	CHEER; Ca, Mg-free	2	0	–	1	1	1.5
D *a*	Saline; Ca, Mg-free	2	1	3	ND[e]	2	1.0
b	Saline; Ca, Mg-free	1	1	1	ND	ND	–
E *a*	$NaHCO_3$-CHEER; Ca, Mg-free	2	2	2; 2	ND	1	5
b	$NaHCO_3$-CHEER; Ca, Mg-free	2	2	3; 3	ND	ND	–
F *a*	Saline; Ca, Mg-free	3	0	–	2	2	1.8
b	Saline; Ca, Mg-free	3	0	–	2.2	–	–
G	CHEER	1	0	–	1.5	–	–

[a] Cell pairs A, B ... G were in different glands. *a*, *b*, *c*: repeated CO_2 exposure of same cells.

[b] Measured from arrival of CO_2 solution in bath as signalled by change in pH_e.

[c] $[Ca^{2+}]_i$ elevation here refers to either the intensification of the CO_2-elicited aequorin glow, or its first appearance upon CO_2 washout.

[d] Time from onset CO_2 washout (signalled by pH_e) at which glow intensified or appeared for the first time.

[e] ND: not determined.

Fig. 10. Uncoupling during HCl injection correlates with $[Ca^{2+}]_i$ elevation at junction. HCl is injected iontophoretically as in Fig. 9 ($i_{H+} = 40$ nA). Here the $[Ca^{2+}]_i$ elevation is, for some time, localized at a region away from junction. (*A*): Plot of coupling coefficient V_2/V_1. Bar denotes duration of HCl injection. (*B*, *a–d*): Darkfield videophotos of aequorin-injected cell, taken at the times indicated on the V_2/V_1 curve; (*e*): brightfield videophoto of cells; (*f*): diagram of cells and electrode arrangement; (*g*): videophoto *(right)* of same cell in darkfield during iontophoretic injection of KCl (3 min duration, 40 nA) inverting the polarity of the i_{KCl} and i_{HCl} current electrodes, and *(left)* of simultaneous storage oscilloscope display of a current pulse (*i*) for testing coupling, and the resulting nearly equal voltage deflections V_1, V_2 (superimposed here, and marked *V*). Shortly after beginning of HCl injection, $[Ca^{2+}]_i$ elevation is first seen only in the vicinity of the H^+ source and does not extend to the junctional region (*a*). As the elevation increases in magnitude and spatial extent (*b*, *c*), uncoupling becomes marked. From time 50 sec to 100 sec the aequorin glow remains sensibly the same in intensity and extent. (The actual *spatial* extent in *c*, as seen on the video monitor, was equal to that in *d*, but the peripheral glow then was too faint to register on the photograph.) (*d*): Upon HCl current termination, the aequorin glow brightens, presumably reflecting the lifting of the depression of $[Ca^{2+}]_i$ measuring sensitivity. $[Ca^{2+}]_i$ elevation outlives the HCl injection, becoming localized again at the HCl electrode, associated with recoupling, and eventually subsides

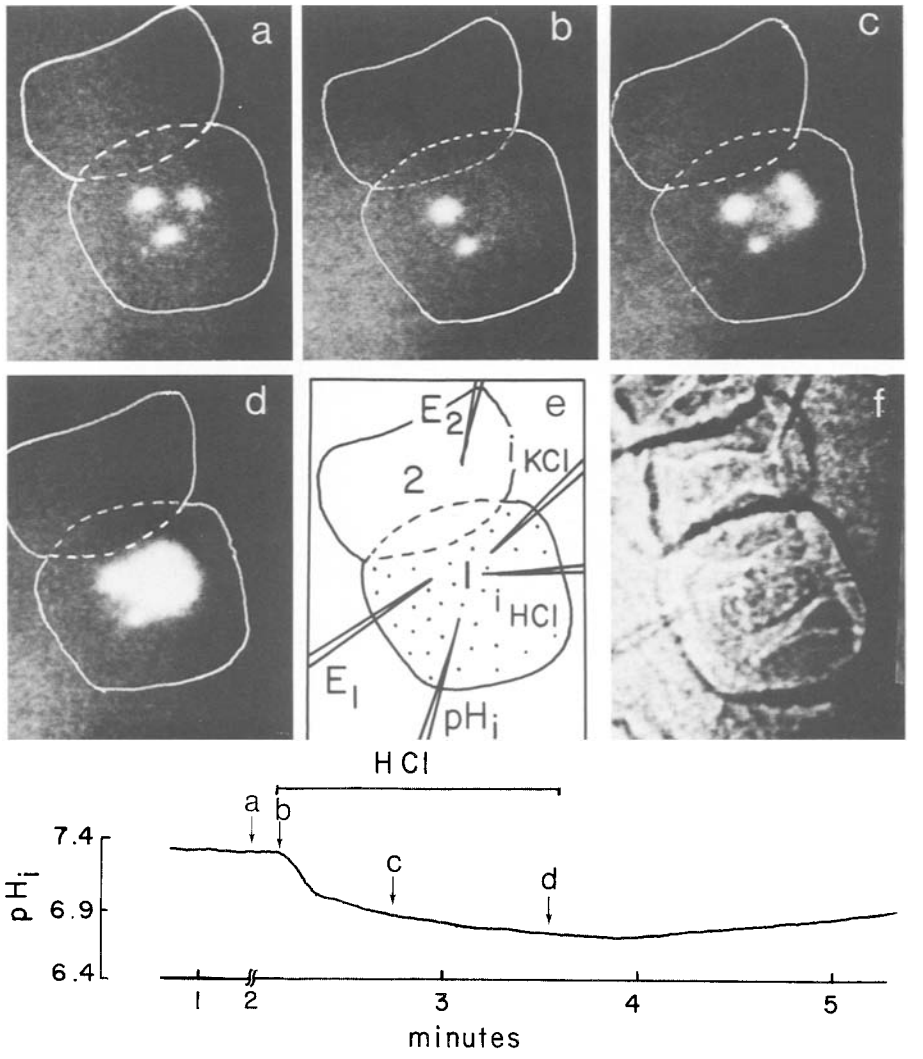


Fig. 11. pH_i and $[Ca^{2+}]_i$ during HCl injection. HCl is injected iontophoretically (35 nA) as in the experiment of Fig. 9. pH_i is continuously monitored (bottom record). *Top*, video photos: (*a*) before the injection, 3 bright spots of local $[Ca^{2+}]_i$ elevations are seen; these are due to Ca^{2+} leaking into the cell from the medium at the electrode insertion sites (they disappear in Ca^{2+}-free medium). (*b*) Upon injection, the spot at the i_{HCl} electrode disappears, presumably reflecting depression of $[Ca^{2+}]_i$ measuring sensitivity. (*c*) After 20 sec of continued injection, a marked $[Ca^{2+}]_i$ elevation is visible, most intensely at the electrode insertion sites, increasing with time in intensity and spatial extent (*d*). Cells in CHEER

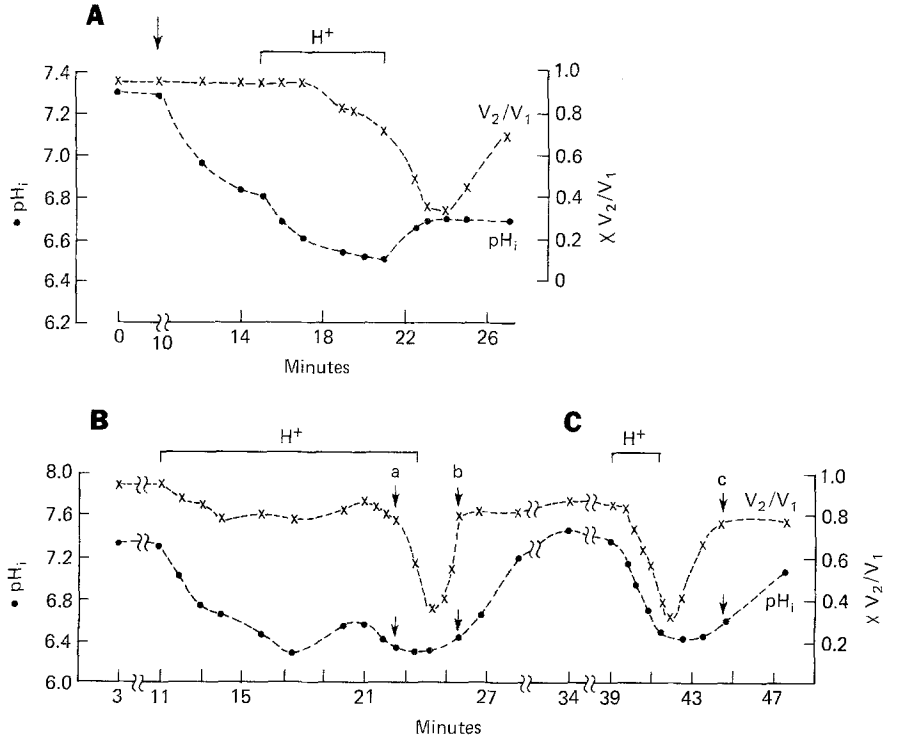


Fig. 12. Coupling changes occurring out of phase with pH_i changes during H^+ injection. H^+ injected by outward current from an HCl-filled microelectrode. (*A*): The HCl electrode is somewhat leaky; its insertion alone (without current) causes pH_i to decrease to 6.8. This decrease is not associated with uncoupling. H^+ current injection (25 nA) decreases pH_i to 6.5, associated with uncoupling. After current termination, pH_i recovers to 6.7, but uncoupling continues. Eventually cells recouple, with pH_i stable at 6.7. Cells in CHEER. (*B*): In this experiment, the HCl electrode is inserted at time 0, without affecting pH_i. Injection (25 nA) decreases pH_i to a level fluctuating between 6.3 and 6.6. The major uncoupling (*a*) occurs only after pH_i had been reduced many minutes; and upon H^+-current termination, recoupling is nearly complete at pH_i 6.5 (*b*). (*C*): Uncoupling follows pH_i change closely (i_{H^+} 35 nA), but recoupling is nearly complete at a time pH_i only begins to recover (*c*). Cells in CHEER, Ca, Mg-free

Invariably, such H^+ injections resulted in $[Ca^{2+}]_i$ elevation (4 experiments), including when the cells were in Ca-free medium. Fig. 9 illustrates an experiment in which an aequorin glow appears diffusely throughout the cell upon H^+ injection, as the cells uncouple. The glow brightens during continued H^+ injection, and coupling falls to near 0. The $[Ca^{2+}]_i$ elevation outlives the H^+ injection by about 2.5 min; thereafter the elevation subsides, and the cells recouple. (The last video picture illustrated in Fig. 9*Bd* is taken 5 sec after H^+-current termination. The stronger brightness probably reflects the lifting of aequorin sensitivity depression; *see* preceding paragraph.)

In two cases of H^+ injection the aequorin glow first appeared locally at the H^+-source (away from junction), indicating a substantial, local $[Ca^{2+}]_i$ rise. Eventually $[Ca^{2+}]_i$ elevation spread to the junction—associated with marked uncoupling (Fig. 10). Here, too, the $[Ca^{2+}]_i$ elevation outlived the H^+ current; and as the glow became localized again at the H^+ source, the cells recoupled.

In one experiment of H^+-injection, we monitored pH_i as well as $[Ca^{2+}]_i$: pH_i fell from 7.36 to 6.73; $[Ca^{2+}]_i$ again rose locally at the H^+-source (Fig. 11); V_2/V_1 fell slightly from 0.85 to 0.64. $[Ca^{2+}]_i$ elevation and uncoupling outlived the H^+ injection, while pH_i recovered to 7.0.

The $[Ca^{2+}]_i$ elevations here were not due to an unspecific effect of ionic current: injecting K^+ and Cl^- instead of H^+ and Cl^-, by reversing electrode polarity (one electrode contained HCl, the other KCl; *see Methods*) failed to produce a detectable, diffuse $[Ca^{2+}]_i$ elevation. Only a faint glow appeared occasionally in the immediate vicinity of the K^+ electrode, stemming possibly from an impurity of the KCl electrode solution (Fig. 10*Bg*).

In another set of experiments we monitored pH_i (but not $[Ca^{2+}]_i$) during H^+ injection. H^+ injections of magnitudes comparable to those of the above experiments lowered pH_i to 6.43 ± 0.24 SD (9 experiments). In respect to coupling, the results were of two kinds. In one kind (2 experiments), there was correlation between pH_i and uncoupling, both in the falling and rising phases. In the other kind, there was no correlation; the pH_i change preceded uncoupling (Fig. 12*A* and *C*) and/or recovery of pH_i and coupling were out of phase (Fig. 12*A–C*).

c) H^+ Transporting Ionophore

The ionophore Nigericin catalyzes rather specifically electroneutral K^+ movement through membrane in exchange for protons (Pressman, 1969). We used this ionophore as a means for lowering pH_i, by loading the cells with H^+ from the medium. Application of 2 μM Nigericin—in one case at pH_e 6.8, in the other at pH_e 6.5—resulted in a pH_i of 6.7 and 6.8, respectively. In the first case, coupling remained rather stable. In the case of the lower pH_e, uncoupling ensued, accompanied by a diffuse $[Ca^{2+}]_i$ elevation (experiment of Fig. 13).

In the absence of Nigericin, as in other tissues (Thomas, 1974; Boron & DeWeer, 1976; Turin & Warner, 1977), pH_i was quite independent

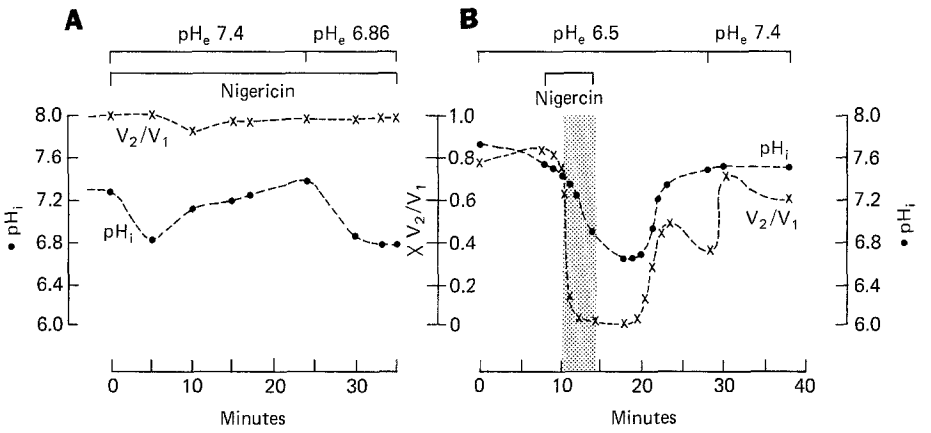


Fig. 13. Uncoupling by H^+-transporting ionophore Nigericin correlates with $[Ca^{2+}]_i$ elevation, not with pH_i. (*A*): The cells are treated with 2 μM Nigericin first at bath pH (pH_e) 7.4, and then at pH_e 6.86. In both cases, pH_i falls to 6.8 (transiently in the first case) without affecting coupling significantly. Cells in CHEER. (*B*): 2 μM Nigericin at pH_e 6.5 lowers pH_i to 6.6. Cells uncouple (beginning at $pH_i \approx 7.4$), associated with $[Ca^{2+}]_i$ elevation (shaded period). Cells in CHEER, 1 mM Ca

of pH_e in the range of 6–8.4; and coupling was stable over this pH_e range (*see also* Loewenstein *et al.*, 1967).

d) CO_2 Exposure, 100%

Cell acidification by CO_2 is a long-known phenomenon. First shown in the pioneering work on plant and animal cells naturally endowed or stained with pH indicator dyes by Jacobs (1920, 1922), the phenomenon was confirmed by measurement with intracellular pH microelectrodes by Thomas (1974, 1976) and Boron and DeWeer (1976). Although it is an indirect approach with possible side effects due to CO_2, cell exposure to CO_2, because of its convenience, has been widely used as a tool for cell acidification. Turin and Warner (1977) used it to study its effect on cell coupling. We examine here $[Ca^{2+}]_i$, pH_i, and coupling in this condition.

i) $[Ca^{2+}]_i$ elevation. Exposure to 100% CO_2 elicited diffuse $[Ca^{2+}]_i$ elevation in 10 out of 25 trials performed on 14 different cells (from 7 glands). This effect ensued in Ca-containing as well as in Ca-free medium (Figs. 8 and 14). $[Ca^{2+}]_i$ elevation was seen in most cases within 1–3 min of CO_2 application (Table 6). While this work was in progress,

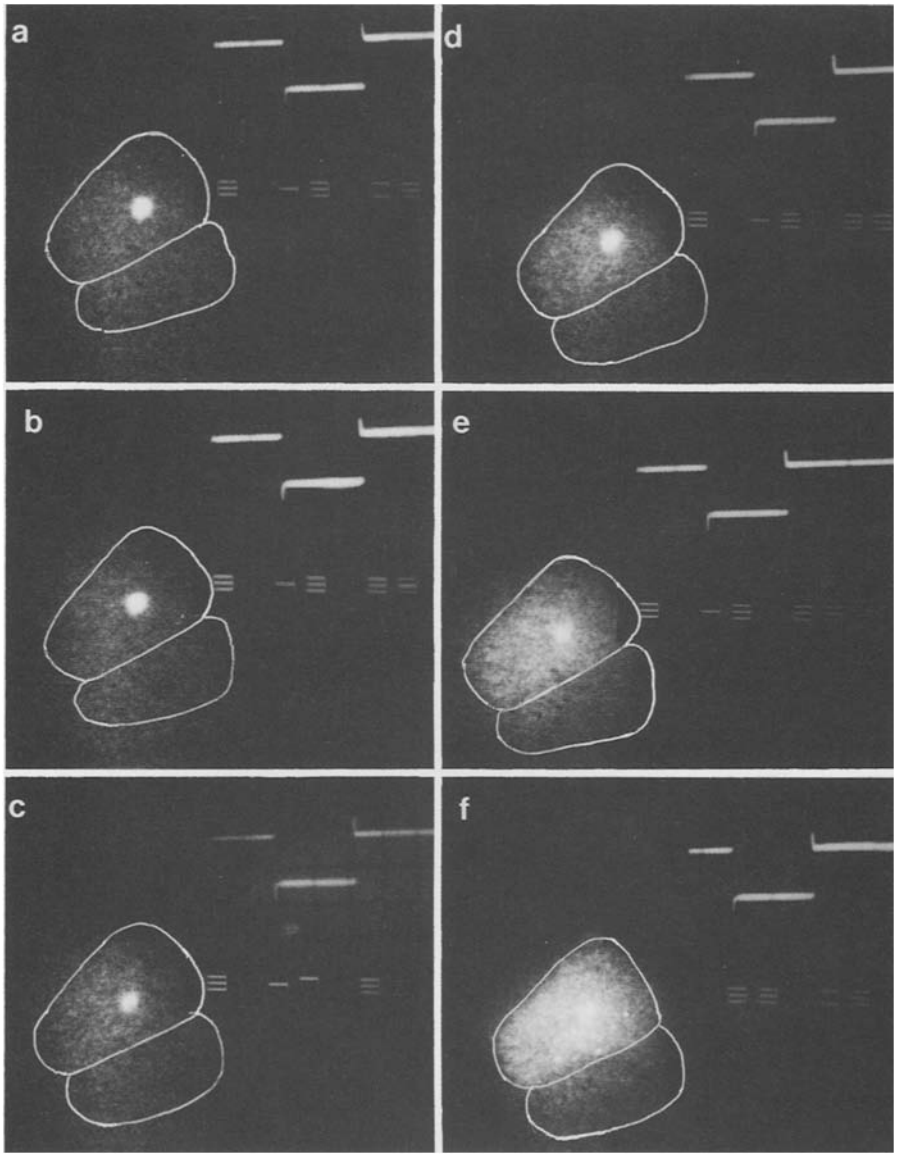


Fig. 14. $[Ca^{2+}]_i$ elevation during 100% CO_2 application. Videophotos of 2 aequorin-injected cells in darkfield. (As in the experiment of Fig. 8, standard Ca^{2+} test pulses (70 nA, 250 msec; 0.2 sec^{-1}) are iontophoresed into one cell, producing the bright, local glows.) (*a*) Cells in saline, Ca, Mg-free; (*b*) start application of 100% CO_2 in saline, Ca, Mg-free; (*c*) 2 min in 100% CO_2; (*d*) 4 min: a diffuse glow is now clearly visible throughout the cell. (The first diffuse glow was already detectable in the actual TV mode at 2 min, but on the videophoto it cannot be distinguished. Likewise, a faint diffuse glow in cell 2 after 6 min does not show up on photos.) (*d*) 4 min, and (*e*) 6.4 min in CO_2. (*f*) 1.5 min of CO_2 washout; the glow in the cell has brightened

Lea and Ashley (1978) reported a similar result of $[Ca^{2+}]_i$ elevation during 100% CO_2 exposure for barnacle muscle.

During the first 3 min or so of CO_2 washout, the aequorin glow at times became more intense than during the period of CO_2 exposure, and in two cases it only then made its first appearance. It is noteworthy that in these two cases the $[Ca^{2+}]_i$ measuring sensitivity was found depressed during CO_2 application, as shown by tests with standard Ca^{2+} pulses.

$[Ca^{2+}]_i$ elevation during CO_2 exposure was seen mostly in those cells containing an electrode or two, but in three cases it also occurred in cells not impaled on electrodes. Continuous electrode insertion clearly renders cells more vulnerable; it may affect their metabolic state as well as, e.g., the load of intracellular Ca^{2+}-stores (Brinley *et al.*, 1977). A certain variability in the $[Ca^{2+}]_i$ elevation is, of course, to be expected (even in the absence of depression of $[Ca^{2+}]_i$ detection). The elevation will depend, among other factors, on the Ca^{2+} load of the various intracellular Ca-stores [which in turn depends on the cell metabolic state (Baker, 1972) and on cell integrity (Brinley *et al.*, 1977)]. On top of this come variations in the degree of depression of $[Ca^{2+}]_i$ measuring sensitivity by CO_2. However, the important point here is that a $[Ca^{2+}]_i$ elevation was detectable. An elevation—in the face of a depression of $[Ca^{2+}]_i$ detection sensitivity— weighs more heavily than a negative result; a negative result is ambiguous.

ii) pH_i and coupling. The effect of 100% CO_2 was to rapidly decrease pH_i, and to drastically diminish the junctional conductance—results similar to those obtained in *Xenopus* embryo cells (Turin & Warner, 1977). The final pH_i levels ranged 6.00–6.60 (in CHEER) (Table 7).

The first effect on coupling usually was a slight improvement followed by a steep decline. Uncoupling began in most cases within 1–3 min of CO_2 application, a timing within the general range of the $[Ca^{2+}]_i$ elevations (*see* preceding paragraph and Table 6).

Two trials were made with 52 and 36% CO_2, respectively. The effect of 52% CO_2 was similar to that of 100% CO_2 treatment. 36% CO_2 reduced junctional conductance as well as pH_i to a much lesser degree (Table 7).

Upon washout of CO_2, both pH_i and coupling recovered (Fig. 15). Noteworthy here is the finding that recoupling set in at pH_i levels ranging as low as 6.32 to 6.88, with a mean pH_i of 6.65 ± 0.15 SD (10 experiments). Uncoupling, on the other hand, began at a mean pH_i of 6.80 ± 0.17 SD (12 experiments), ranging 6.51 to 7.06. Significant here is the fact that

Table 7. Effect of 100% CO_2 on pH_i and coupling

Cell pair[a]	Medium	% CO_2	pH_i				V_2/V_1		
			Control[b]	CO_2	At onset uncoupling[c]	At onset recoupling[c]	Control[b]	CO_2	Onset of uncoupling[d] (min)
A	CHEER	100	7.68	6.28	6.77	6.68	0.97	0.04	2.4
B	CHEER	100	7.52	6.05	6.51	n.d.	0.88	0.13	1.7
C	CHEER	100	7.27	6.00	6.79	6.56	0.81	0.04	1.6
D	CHEER; Ca, Mg-free	100	7.64	6.56	6.70	7.32	0.78	0.01	1.3
E	CHEER; 0.5 mM Ca	100	7.54	6.06	6.65	6.88	0.93	0.02	0.8
F	CHEER+1 mM DIAMOX	100	7.56	6.28	6.87	6.70	0.89	0.05	1.6
G	$NaHCO_3$-CHEER	100	7.17	6.20	6.52	6.54	0.91	0.02	1.5
H	TRIS-saline	100	8.16	6.48	7.06	6.68	0.78	0.05	2.0
F	$NaHCO_3$-saline	100	7.66	6.48	6.91	6.78	0.82	0.09	2.0
I	NH_4HCO_3-saline	100	7.24	6.66	6.92	ND	0.91	0.05	1.5
J	Saline; Ca, Mg-free +2 mM EGTA	100	7.55	6.07	6.78	6.32	0.92	0.02	1.0
H	CHEER	52	7.72	6.34	6.87	6.67	0.84	0.07	1.8
H	CHEER	36	7.76	6.60	6.96	6.68	0.91	0.74[e]	2.4

[a] Cell pairs A, B ... J were in different glands.
[b] Values in respective medium in absence of CO_2.
[c] Corrected for response time of respective pH_i electrode.
[d] As measured from time of arrival of CO_2-medium (signalled by pH_e—*see*, e.g., Fig. 15); current test pulse frequency (0.1 sec^{-1}) here limits accuracy to ± 0.2 min.
[e] During the first minutes of CO_2 exposure, V_2/V_1 transiently fell to 0.63.

many of the individual cells began to *re*couple at a pH_i lower than the one at which their *un*coupling had set in (*see* Table 7).

e) CO_2 Exposure, 5%. $[Ca^{2+}]_i$, pH_i, Coupling

Exposure to 5% CO_2 lowered pH_i on the average of 9 cases by 0.29 ± 0.09 SD (the starting pH_is ranged 6.69 to 7.98) and depressed coupling—with one exception—in only those cells of a low initial (before 5% CO_2 application) coupling coefficient ($V_2/V_1 < 0.82$). $[Ca^{2+}]_i$ was seen elevated during 5% CO_2 application in only one case (significantly, the above-mentioned exception), in which coupling was depressed in a cell pair of initially high V_2/V_1 (0.91) (*see* Table 8, cell pair *D*, and Fig. 16).

Upon CO_2 washout, pH_i recovered, generally overshooting the initial control pH_i. In 6 cases, $[Ca^{2+}]_i$ elevation was detected during the washout period, and this elevation was (with one exception) associated

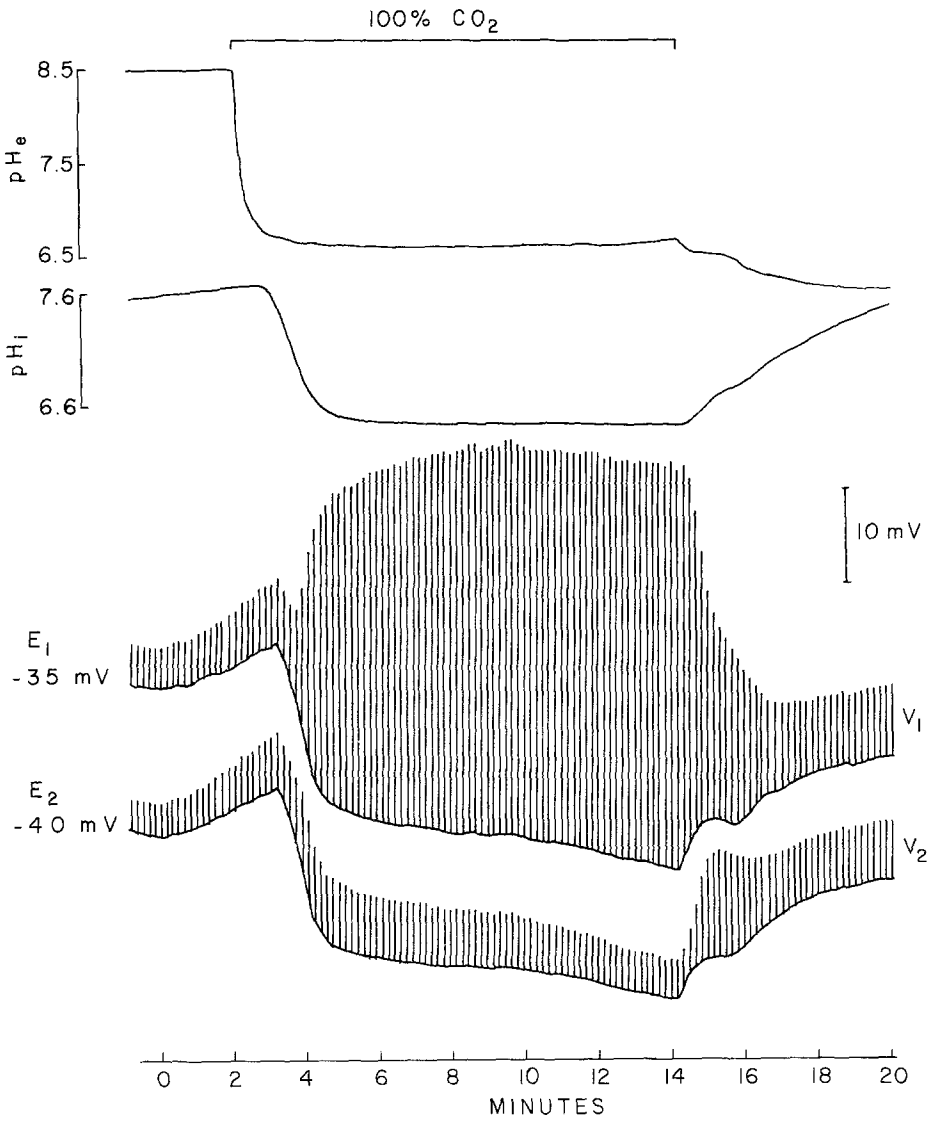


Fig. 15. Uncoupling by cell exposure to 100% CO_2. Prior to 100% CO_2 application, medium has been changed from saline to $NaHCO_3$-saline (pH_e 8.5), causing a slight pH_i increase. Application of 100% CO_2 (in $NaHCO_3$-saline) decreases pH_i and uncouples cells. CO_2 washout (with saline, at pH_e 6.3) produces fast recovery of coupling and pH_i

with a decrease in junctional conductance or a decrease in rate of recovery of junctional conductance. The simultaneously recorded pH_i then showed no decrease or change in recovery rate. Table 8 summarizes the results.

In one experiment, exposure to 10% CO_2 caused pH_i to fall from 7.64 to 6.87. Cell coupling here was not affected; in fact, junctional conductance appeared to improve (Fig. 4*C*).

Table 8. Effect of 5% CO_2 on pH_i, coupling, and $[Ca^{2+}]_i$

Cell pair[a]	Medium	Effect of 5% CO_2 exposure on pH_i, V_2/V_1, and $[Ca^{2+}]_i$						$[Ca^{2+}]_i$ elevation and associated changes in V_2/V_1 during CO_2 washout	
		pH_i			V_2/V_1		Period of aequorin glow (min)	Period of aequorin glow[b] (min)	Coupling changes during period of aequorin glow[c]
		Control	CO_2	ΔpH_i	Control	CO_2			
A	CHEER, 0.5 mM Ca	7.52	7.18	−0.34	0.43	0.17	None	3–7	Transient decrease: V_2/V_1 from 0.7 to 0.56
A	CHEER, 1 mM Ca	7.56	7.30	−0.26	0.54	0.31	None	1–4	No effect
B	CHEER, 1 mM Ca	7.22	6.93	−0.29	0.82	0.80[e]	None	6–7	Transient decrease: V_2/V_1 from 0.88 to 0.74
C	CHEER, 1 mM Ca	7.4	7.08	−0.32	0.99	0.99	None	2–4	Transient decrease: V_2/V_1 from 0.99 to 0.92
D	CHEER, 1 mM Ca	6.88	6.79	−0.09	0.91	0.74	Last min[f]	0–4	Retardation of recoupling onset
E	CHEER, 1 mM Ca	7.91	7.65	−0.34	0.99	0.99	None	None	
B	CHEER, 2 mM Ca[d]	7.0	6.87	−0.23	0.54	0.36	None	5–6	Decreased rate of recoupling
F	CHEER[d]	6.69	6.35	−0.34	0.68	0.12	ND	ND	
G	CHEER[d]	7.98	7.53	−0.45	0.87	0.89	ND	ND	

[a] Each cell pair A ... G was in a different gland.
[b] Time of first detection of aequorin glow after start CO_2 washout until time of disappearance.
[c] At all other times during CO_2-washout the cells continuously recoupled.
[d] Before CO_2 treatment, cell had been injected with HCl.
[e] V_2/V_1 transiently decreased to 0.59 and then recovered to 0.80 while in CO_2.
[f] *See* Fig. 16.

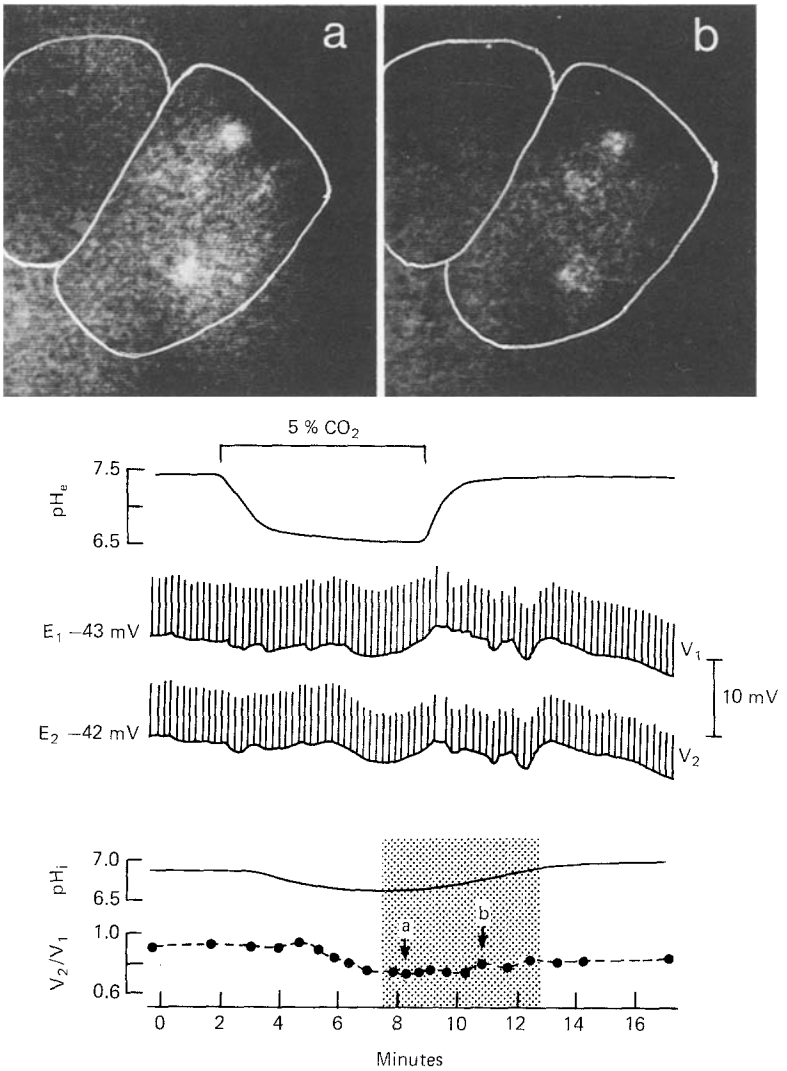


Fig. 16. A coupling depression during 5% CO_2 exposure associated with $[Ca^{2+}]_i$ elevation. Cell exposure to 5% CO_2 lowers pH_i slightly and decreases coupling, associated with a $[Ca^{2+}]_i$ elevation (*a* and *b*). Only cell 1 was aequorin-injected

Discussion

The present results show a good correlation between junctional uncoupling and elevation of $[Ca^{2+}]_i$, as did earlier experiments (*cf.* Loewenstein & Rose, 1978), but not, in general, between uncoupling and changes in pH_i. We will center the discussion of the results on the questions

of (i) whether H^+ ions are necessary for uncoupling induced by $[Ca^{2+}]_i$ elevation, and (ii) whether they are sufficient for uncoupling.

In regard to the first question, the answer given by the results is clearly negative. Experimental elevation of $[Ca^{2+}]_i$—as during injection of pH-buffered Ca^{2+}-solution, cell exposure to CN, Ca^{2+} ionophore, or CO_2 (5%) washout—caused uncoupling with little or no change in pH_i. And in those experiments in which a significant decrease in pH_i did occur—as during application of DNP where uncoupling is known to correlate with $[Ca^{2+}]_i$ elevation (Rose & Loewenstein, 1976)—the uncoupling and recoupling were out of phase with the fall and recovery of pH_i. Moreover, uncoupling ensued even when the elevation of $[Ca^{2+}]_i$ occurred in association with a substantial *increase* in pH_i—as in the experiments of cell alkalinization with NH_4HCO_3 and of propionate washout. There is thus no reason to think that the action of Ca^{2+} on junctional permeability is mediated by H^+.

The question of whether H^+ is by itself sufficient for uncoupling is more difficult to decide. The results of the experiments in which a substantial lowering of pH_i failed to produce uncoupling (except when concomitant with $[Ca^{2+}]_i$ elevation)—as in treatment with Nigericin, injection of a pH 6.4 solution of $< 10^{-6}$ M free Ca^{2+}, exposure to 10% CO_2 (Fig. 4*C*)—or where the cells began to recouple in the presence of low pH_i—as during the acidification phase ($pH_i \approx 6.5$) following removal of NH_4HCO_3 or upon washout of 100% CO_2 (pH_i 6.35, Table 7)—suggest that H^+ is not sufficient for uncoupling, at least not at these pH_i levels ($\gtrsim 6.5$). In the experiments of 100% CO_2 exposure and in some experiments of HCl injection, coupling and uncoupling appears to closely correlate with pH_i. However, the interpretation of these results is obscured by the insensitivity of $[Ca^{2+}]_i$ detection in these conditions; a possible $[Ca^{2+}]_i$ elevation (when not observed) may have been masked by the depressant effect of CO_2 on the $[Ca^{2+}]_i$ measuring sensitivity. Most significant here is that $[Ca^{2+}]_i$ was found elevated in a good number of the trials in spite of the depressed $[Ca^{2+}]_i$ measuring sensitivity, leading us to suspect that $[Ca^{2+}]_i$ may have been generally elevated during these cell acidifications. The results obtained with 5% CO_2, where a change in junctional conductance occurred only in cells with an initially low coupling coefficient, in the absence of detectable $[Ca^{2+}]_i$ elevation, may be interpreted in the same way, if we assume that the $[Ca^{2+}]_i$ elevation required to further depress the already lowered junctional conductance lay within our "dark" range of $[Ca^{2+}]_i$. We consider as support of this notion the finding that, in the one case of uncoupling by 5% CO_2 of an initially well-coupled cell pair, $[Ca^{2+}]_i$ elevation was detectable

during CO_2 exposure and that the $[Ca^{2+}]_i$ elevations observed during CO_2-washout were associated in all but one case with decrease of coupling (or recoupling rate)—again from comparatively high degrees of coupling (Table 8).

As to the Ca^{2+} sources for the $[Ca^{2+}]_i$ elevation during cell acidification, these are probably intracellular. One thinks first here of mitochondria which, *in vitro*, are known to release Ca^{2+} when the pH of their bathing medium is lowered from 8 or 7.4 to 6.8 (Åkerman, 1978). But other intracellular Ca^{2+} stores (probably endoplasmic reticulum, cell surface membranes, etc.; *c.f.* Moore *et al.*, 1974; Brinley *et al.*, 1977; Blaustein *et al.*, 1978; *see also* Hales *et al.*, 1974) may conceivably be sources, too. A contribution by extracellular Ca^{2+} stores appears less likely: in squid axon, Ca^{2+} influx is actually reduced at lowered pH_i (Baker & Honerjaeger, 1978).

In summary, the combined results of our pH_i and $[Ca^{2+}]_i$ observations provide no evidence that would support the idea of H^+ playing a role (independent of Ca^{2+}) in the regulation of *Chironomus* cell-cell channel permeability, at least not at $pH_i \gtrsim 6.5$. Channel closure occurs in the absence of a pH_i decrease and even in the presence of pH_i increase, as long as $[Ca^{2+}]_i$ is elevated; and pH_i decrements equivalent to those observed in certain kinds of experimental $[Ca^{2+}]_i$ elevation can fail by themselves (in the absence of $[Ca^{2+}]_i$ elevation) to produce channel closure.

A priori there are, of course, no reasons to believe that Ca^{2+} is the sole or universal agent for uncoupling. Loewenstein's hypothesis postulates that Ca^{2+} is one such agent and implies that this ion plays a physiological regulatory role of cell-cell communication (Loewenstein, 1967; Rose & Loewenstein, 1976; Loewenstein, Kanno & Socolar, 1978*b*). Experimental tests of the hypothesis have shown that elevation of $[Ca^{2+}]_i$ indeed causes channel closure, namely, graded closure (Rose, Simpson & Loewenstein, 1977; Loewenstein, Kanno & Socolar, 1978*a*), and that $[Ca^{2+}]_i$ is elevated in a variety of conditions depressing cell-to-cell channel permeability (Loewenstein & Rose, 1978). But this, of course, does not exclude the possibility that channel permeability is also affected by other agents. In fact, one may suspect this in the case of uncoupling produced by cell exposure to Li (Li substituting for Na_e, Rose & Loewenstein, 1971) or to propionate (propionate substituting for Cl_e^-, Dreyfuss, Girardier & Forssmann, 1966; Pappas, Asada & Bennett, 1971), in which there is no detectable $[Ca^{2+}]_i$ elevation (Rose & Loewenstein, 1976). However, whatever the uncoupling agent here may be, it does not seem to be H^+: we measured pH_i during uncoupling in Li-medium (1 experi-

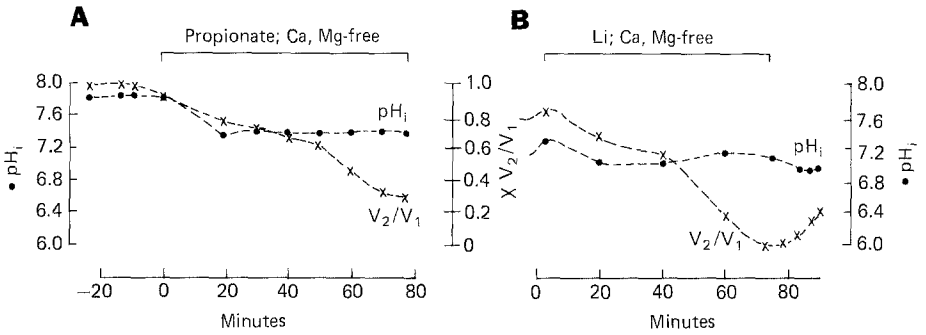


Fig. 17. Uncouplings associated neither with pH_i change nor with $[Ca^{2+}]_i$ elevation. (*A*): Cells are exposed to propionate-saline, Ca, Mg-free, pH_e 7.35; coupling, pH_i and $[Ca^{2+}]_i$ are monitored. pH_i falls to only 7.35 during propionate exposure; cells uncouple gradually to $V_2/V_1=0.3$. No $[Ca^{2+}]_i$ elevation is detectable during the exposure. (However, upon washout of propionate, $[Ca^{2+}]_i$ elevation is seen during the alkalinization phase, associated with uncoupling; *see* Fig. 7). (*B*): During cell exposure to Li-CHEER, Ca, Mg-free, pH_i does not change significantly; cells uncouple completely. As in the experiment of *A*, no $[Ca^{2+}]_i$ elevation is detectable (different gland)

ment) and in propionate medium (2 experiments) and found no significant change in pH_i (Fig. 17).

To end the discussion we will comment briefly on the usefulness and necessity of the image-intensifier system in conjunction with the aequorin technique. This method allowed us to determine the spatial Ca^{2+} distribution in the cytosol, and to correlate junctional conductance with the local Ca^{2+} concentration inside the cell. This information is a necessity for the present purposes, because Ca^{2+} spreads very poorly through the cytosol of normal cells (Hodgkin & Keynes, 1957; Podolsky & Costantin, 1964; Rose & Loewenstein, 1977), and a fully coupled junction can coexist with fairly high $[Ca^{2+}]_i$ elevation occurring some 10 μm away from the junctional region (Rose & Loewenstein, 1976). The use of the simpler technique of collecting the total aequorin light output inside the cell by means of a photomultiplier would be inadequate here. In fact, in many critical instances, the information thus provided would be misleading. For example, in the experiment of Fig. 10*Ba*, during the initial phase of $[Ca^{2+}]_i$ elevation at the iontophoresis source some 20 μm away from junction, such a technique would have signalled a marked $[Ca^{2+}]_i$ elevation concomitant with full coupling—a completely meaningless observation, since what matters here is the $[Ca^{2+}]_i$ at the junction. It would have been nice to have such a method for determining the spatial distribution of H^+, too. However, there are no reasons to

think that diffusibility of H^+ in the cytosol is anywhere nearly as restricted as that of Ca^{2+}. In any event, in the experiments of long-term gland exposure to uncoupling agents where an effect throughout the cell would be expected in steady state, such information would not seem necessary.

We are grateful to Dr. R. Thomas for his technical advice for the manufacture of pH-microelectrodes; to Dr. O. Shimomura for his gift of aequorin, and to Jim Gray for his unfailing assistance in design and maintenance of electronic equipment. We especially thank Drs. W.R. Loewenstein and S.J. Socolar for many helpful discussions and constructive criticism of the manuscript.

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