J. Membrane Biol. 5, 20–50 (1971)

Junctional Membrane Permeability

Depression by Substitution of Li for Extracellular Na, and by Long-Term Lack of Ca and Mg; Restoration by Cell Repolarization

BIRGIT ROSE and WERNER R. LOEWENSTEIN

Cell Physics Laboratory, Department of Physiology,
Columbia University College of Physicians & Surgeons,
New York, New York 10032

Received 17 December 1970

Summary. Substitution of extracellular Na^+ by Li^+ causes depression of junctional membrane permeability in *Chironomus* salivary gland cells; within 3 hr, permeability falls to so low a level that neither fluorescein nor the smaller inorganic ions any longer traverse the junctional membrane in detectable amounts (uncoupling). The effect is Li-specific: if $choline^+$ is the Na^+ substitute, coupling is unchanged. The Li-produced uncoupling is not reversed by restitution of Na^+. Long-term exposure (>1 hr) of the cells to Ca, Mg-free medium leads also to uncoupling. This uncoupling is fully reversible by early restitution of Ca^{++} or Mg^{++}. Coupling is maintained in the presence of either Ca^{++} or Mg^{++}, so long as the total divalent concentration is about 12 mM. The uncoupling in Ca, Mg-free medium ensues regardless of whether the main monovalent cation is Na, Li or choline.

The uncouplings are accompanied by cell depolarization. Repolarization of the cells by inward current causes restoration of coupling; the junctional conductance rises again to its normal level. The effect was shown for Li-produced uncoupling, for uncoupling by prolonged absence of external Ca^{++} and Mg^{++}, and for uncoupling produced by dinitrophenol. In all cases, the recoupling has the same features: (1) it develops rapidly upon application of the polarizing current; (2) it is cumulative; (3) it is transient, but outlasts the current; and (4) it appears not to depend on the particular ions carrying the current from the electrodes to the cell. The recoupling is due to repolarization of nonjunctional cell membrane; recoupling can be produced at zero net current through the junctional membrane. Recoupling takes place also as a result of chemically produced repolarization; restoration of the K gradients in uncoupled cells causes partial recoupling during the repolarization phase.

An explanation of the results on coupling is proposed in terms of known mechanisms of regulation of Ca^{++} flux in cells. The uncouplings are explained by actions raising the Ca^{++} level in the cytoplasmic environment of the junctional membranes; the recoupling is explained by actions lowering this Ca^{++} level.

Previous work has shown that injection of Ca^{++} into cells, or excessive influx of Ca^{++} through mechanically or chemically produced leaks in cell surface membrane or in junctional seal causes depression of permeability

of junctional membrane (Loewenstein, Nakas & Socolar, 1967; Loewenstein, 1967*b*). These results have led to the hypothesis that the permeability of the junctional membrane depends on the amount of Ca bound to it and, hence, on the free Ca concentration in the cytoplasm in contact with this membrane. The permeability is high in the low Ca^{++} concentration normally prevailing in cytoplasm ($<10^{-6}$ M); it falls when the Ca^{++} concentration rises (Loewenstein, 1966, 1967*a*).

The present work was prompted by this hypothesis. We studied the junctional permeability of *Chironomus* salivary gland cells in two experimental situations in which cytoplasmic Ca^{++} may be expected to rise: when Li^{+} substitutes for Na^{+} in the external medium, and when the external medium lacks Ca^{++}. Ca^{++} influx is known to rise in the first situation and Ca^{++} efflux to fall in both situations in several types of cells (Niedergerke, 1963; Baker, Blaustein, Hodgkin & Steinhardt, 1967; Baker & Blaustein, 1968; van Breemen & van Breemen, 1968). It will be shown that junctional membrane permeability does, in fact, fall markedly in both situations.

In the course of this study, the fall of junctional membrane permeability, which was accompanied by cell depolarization, was found to be reversible by cell repolarization. An examination of this effect in several situations of uncoupling will show that the permeability of junctional membrane depends on the potential across nonjunctional membrane.

Brief accounts of some of the results have already appeared (Rose & Loewenstein, 1969; Rose, 1970).

Materials and Methods

Preparation and Media

Salivary glands of 10 to 12-day-old *Chironomus thummi* larvae were isolated as described in the preceding paper (Rose, 1971), and set up in a perfusion chamber. The perfusion system allowed vibration-free exchange of medium; about 96% of the chamber's medium was exchanged within 3 min, as shown by tests with colorant solutions.

The composition of the media is given in Table 1. The control medium approximates hemolymph with respect to content of cations and chloride, pH and osmolarity; it contains some organic components of hemolymph (Politoff, Socolar & Loewenstein, 1969). The cells maintained transparency, high resting potentials and coupling for at least 4 hr in this medium. In the experiments with 2,4-dinitrophenol, this substance was dissolved in control medium in concentrations of 1 to 10×10^{-5} M.

Electrical Measurements

Three microelectrodes were inserted into two contiguous cells for measurement of coupling (Fig. 1, *top*). One electrode served to pass rectangular current pulses (1 to 4×10^{-8} amp; 100 to 200 msec duration; base-line leakage $<10^{-11}$ amp) and the other

Table 1. *Composition of media (Concentration in* mM*)*

Medium	Abbreviation	KCl	NaCl	Na_2-Fumarate	$CaCl_2$	Mg-Succinate	Glutamine	TES[a]	Na_2-Succinate	LiCl	Li_2-Succinate	Choline Cl	Choline-Succinate	KH-Fumarate	Succinic acid	Titrated to pH 7.4 with (mM)
Control	(Na^{+Ca}_{+Mg})	2	28	28	5	7	80	5								NaOH(4–5)
Ca, Mg-free	(Na^{-Ca}_{-Mg})	2	38	28			80	5	7							NaOH(4–5)
Li	(Li^{+Ca}_{+Mg})	2			5	7	80	5		28	28					LiOH(4–5)
Ca, Mg-free Li	(Li^{-Ca}_{-Mg})	2					80	5		38	38					LiOH(4–5)
Choline	$(Chol^{+Ca}_{+Mg})$	2			5	7	80	5				28	28			KOH(4–5)
Ca, Mg-free choline	$(Chol^{-Ca}_{-Mg})$	2					80	5				38	38			KOH(4–5)
Li-choline	$(Li\text{-}Chol^{+Ca}_{+Mg})$	2			5	7	80	5			28	28				LiOH(4–5)
Ca, Mg-free Li-choline	$(Li\text{-}Chol^{-Ca}_{-Mg})$	2					80	5			38	38				LiOH(4–5)
K	(K^{+Ca}_{+Mg})	30			5	7	80	5						28		KOH(32)
Ca, Mg-free K	(K^{-Ca}_{-Mg})	50					80	5						28	7	KOH(44)

[a] N-Tris-(hydroxmethyl) methyl-2-amino-ethanesulfonic acid.

two electrodes to measure the resulting steady-state displacement (V) in membrane potential (E). In some experiments on membrane polarization, a fourth microelectrode was used for applying d.c. steps (Fig. 12, *inset*) (*see* preceding paper for microelectrode characteristics). Current and potentials were displayed on a four-channel storage oscilloscope at slow sweep (7.2 min/cm) and, simultaneously for accurate measurement, on a four-channel oscilloscope at much faster sweep (50 msec/cm). Fig. 1 shows record samples. A strip chart recorder provided a third time axis for some experiments (e.g., Fig. 12A).

The accuracy of the measurements of the absolute E values was probably not much better than the range of the electrode tip potential (5 mV). In the case of the E changes at zero current, the accuracy was probably better. The accuracy of the (fast) V measurements (limited only by the sensitivity of the amplifier system) was 1 to 2 mV; the size of the V dots on the figures is roughly that of the error. The accuracy of the current measurements was 1×10^{-9} amp.

Chelator Injection

Ethylenediamine-tetraacetic acid (EDTA) was used as a saturated solution of the di-sodium salt or as a 3 M solution of the tri-potassium salt. Ethyleneglycol-bis-β(amino-ethylether)-N,N′-tetraacetic acid (EGTA) was used as a saturated solution of the acid. The pH of the solutions was adjusted to 7 with $KHCO_3$. Micropipettes of about 1-μ tip diameter were used for injection. The pipettes were first filled with distilled water, which was then displaced by the chelator solution. The chelators were injected into the cells with hydraulic or electric pressure. In the former case, 10 mM fluorescein was added to the chelator solution as a marker.

Electrical injection was done with pulses of 20 to 40 mV (2 to 4×10^{-8} amp), tip negative, 100 to 200 msec duration, at a rate of 2.3 to 6.3/sec; or with d.c. steps several minutes long. Injection with d.c. had the disadvantage that the pipette resistance sometimes increased so much during current passing that only 10^{-9} amp could be delivered by the generator system.

The chelators were injected on one or both sides of a junction, while coupling was monitored. In the case of hydraulic injection, an injection pipette was used in addition to the three measuring microelectrodes. In the case of electric injection, the chelator-filled pipette had also the function of a current-passing electrode. The arrangement of Fig. 1, *inset*, applies to unilateral injection; that of Fig. 14, *inset*, to bilaterial injection.

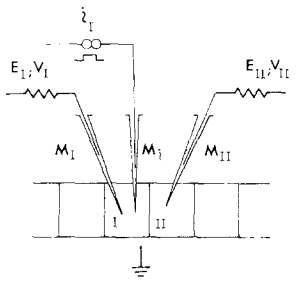
To bring the chelator anions as close as possible to the junctional membrane and to avoid causing changes in (nonjunctional) membrane potential, equal current of opposite direction was passed in some experiments of bilateral injection. Current direction was then changed frequently so as to inject chelator into both cells. This method was used in experiments in which we tried to prevent uncoupling; the junctional resistance was still low at the time of injection.

All experiments were performed on the gland's giant border cells at room temperature, generally 20 °C.

Results

Uncoupling in Li Medium

Fig. 2 illustrates an experiment in which the Na in the medium is replaced by Li. The coupling coefficient, normally near 1, began to fall after 100-min exposure to the Li medium and reached near zero within 3 hr. By that time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$E_I; V_I$
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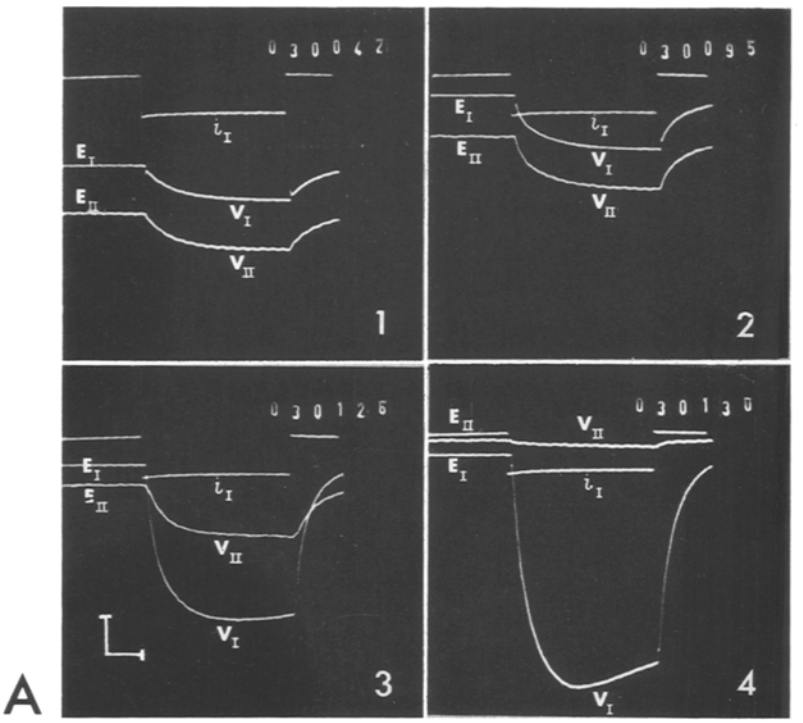
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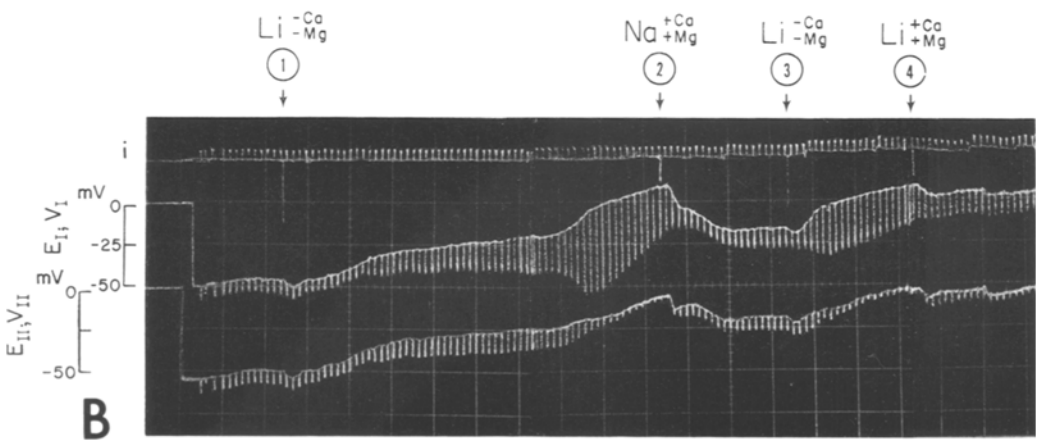
Li -Ca -Mg
Na +Ca +Mg
Li -Ca -Mg
Li +Ca +Mg
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2
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i
mV
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$E_i; V_I$
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Fig. 1

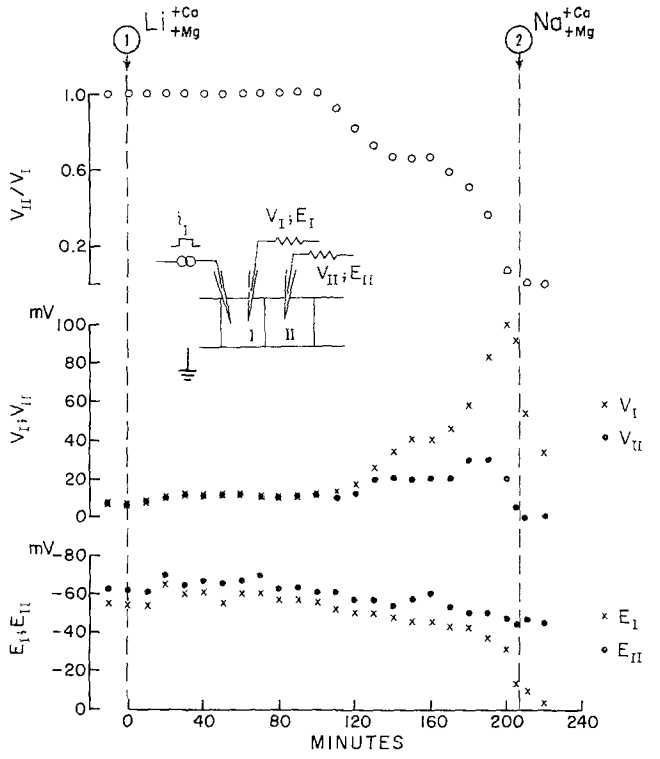


Fig. 2. Uncoupling in Li medium. Time course of E_I, E_{II}, V_I, and V_{II}, and coupling coefficient V_{II}/V_I. Arrow *1*, exchange of control medium by a medium in which Li substitutes for Na. *2*, restitution of control medium. The points before time zero are the corresponding stable values in control medium at the start of the experiment. Current, 2×10^{-8} amp; 100 msec

Fig. 1. *Top*, diagram of arrangement for electrical measurement of coupling. M_i microelectrode for passing pulses of inward current between interior of cell I and medium (grounded); M_I, M_{II} microelectrodes for measuring potential between interiors of cell I and II and the medium; E potential at zero current (membrane potential); V (steady state) change in E produced by current. Unless stated otherwise, this measuring arrangement applies to all subsequent figures. A, samples of oscilloscope records from an experiment of uncoupling in dinitrophenol medium. *1*, cell system in normal medium; *2*, *3*, in the course of uncoupling; *4*, fully uncoupled. $i_i = 4 \times 10^{-8}$ amp. Calibrations: 10 mV; 50 msec. B, sample of storage oscilloscope records (the complete time course of a 2-hr experiment of uncoupling by Ca, Mg-free Li medium). The two lower oscilloscope records show E_I and E_{II} as base lines, and V_I and V_{II} as downstrokes. The upper beam records the current pulses (2×10^{-8} amp, inward, 200 msec duration, 1/min). The arrows signal application of the media

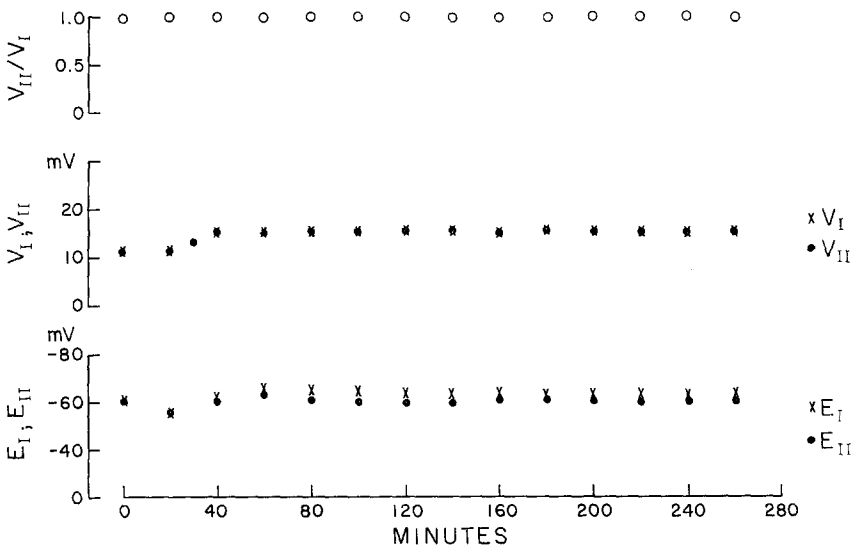


Fig. 3. Control run. Coupling in control medium. Current, 2×10^{-8} amp; 200 msec

the junctional conductance had fallen very markedly: V_I rose above its initial level and V_{II} became undetectably small (*see* Loewenstein *et al.*, 1967 for a quantitative analysis of an uncoupled cell chain).

The uncoupling is typically accompanied by cell depolarization. Thus, the uncoupling of a set of cells is often discernible by differences in their resting potentials (E). The resting potentials of all cells in a given gland are equal in the coupled state, within the accuracy of the measurements. In the uncoupled state, the resting potentials of the various cells often deviate (e.g., Fig. 2); each cell is now an isolated unit with respect to the ions determining the potential.

Coupling and resting potential remain constant for at least 4 hr in control medium under otherwise identical experimental conditions (Fig. 3).

In another series of experiments, electrode impalement was begun after the glands had already been in the test medium for several hours. Under these conditions, too, the cells uncoupled in all cases, but uncoupling was slower than in the experiments in which the cells were continuously impaled (Table 2). Apparently, the uncoupling process is sped up by continuous impalement. In control medium, cells remained coupled and had high resting potentials for up to 5.5 hr (Table 2).

The uncoupling is due to Na^+ substitution by Li^+, and not simply to lack of Na^+; coupling stays unaltered if Na^+ is substituted by choline$^+$ (2 to 3 hr observations) (Fig. 4).

Table 2. *Coupling and membrane potential in control and Li medium*

Medium	Length of treatment[a] (hr)	V_{II}/V_I	Membrane potential (mV)	Cell pairs
Control medium	2.0	≈1	30–40	2
	4.5	≈1	60–70	2
	5.0	≈1	60	1
	5.5	≈1	50	4
Li medium	2.5	0.86	5	1
	3.5	0.15	20–40	2
	3.5	0.6	50	1
	4.5	0.16	30–40	1
	5.0	≈0	5–15	4
	5.5	≈0	5–15	2

[a] Time from gland isolation in the corresponding medium to measurement. Unlike the experiments in Table 3 and all Figures, the cells were impaled for electrical measurement only at the end of the treatment time. The glands were directly isolated in the corresponding media.

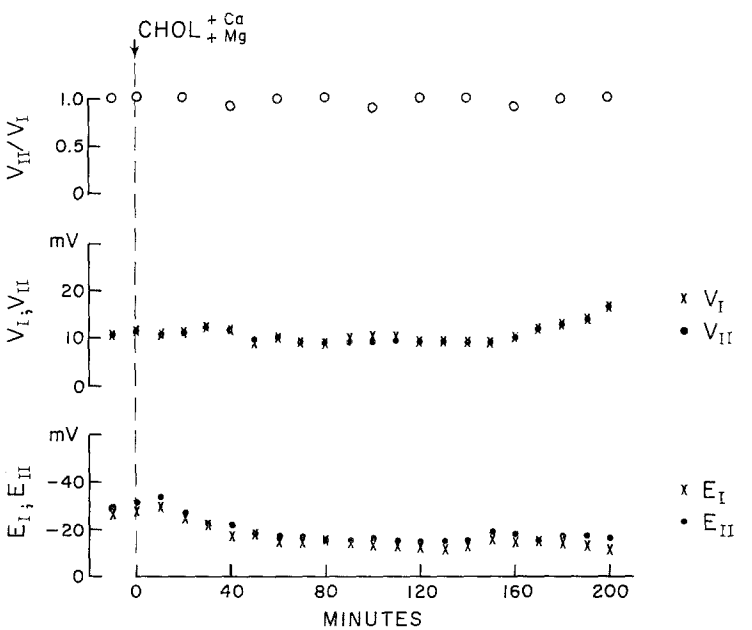


Fig. 4. Coupling in choline medium. At arrow, control medium is exchanged by medium in which choline substitutes for Na. Current, 2×10^{-8} amp; 200 msec

Uncoupling in Ca, Mg-free Medium

The preceding experiments were guided by the idea that depression of junctional conductance produced by substitution of Na^+ for Li^+ was caused

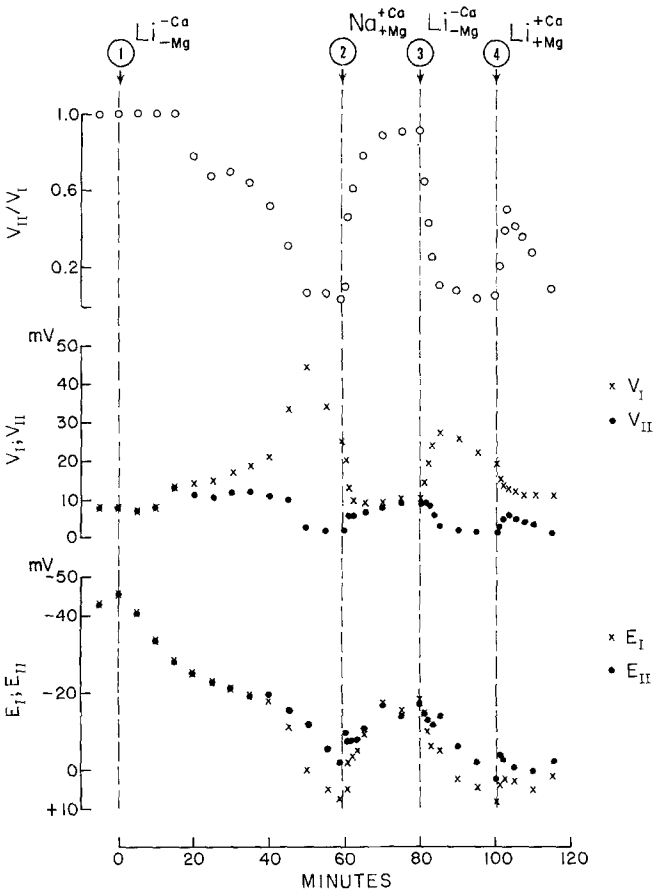


Fig. 5. Uncoupling in Ca, Mg-free Li medium. *1*, application of Ca, Mg-free Li medium. *2*, restitution of control medium. *3*, re-application of Ca, Mg-free Li medium. *4*, Ca, Mg-containing Li medium. The *same experiment* as in Fig. 1. Current, 2×10^{-8} amp; 200 msec

by a rise in cytoplasmic Ca^{++} (Mg^{++}) concentration. Thus, the question came up if extracellular Ca^{++} is required for the uncoupling.

We first examined the effects of a Li medium lacking all divalent cations. The cells uncoupled in this medium (Fig. 5; Table 3). However, this uncoupling, in contrast to that in Ca, Mg-containing Li medium, is not Li-specific. Exposure of the cells to Ca, Mg-free Na medium leads to uncoupling, too (Fig. 6; Table 3).

Coupling also declines if the main cation in Ca, Mg-free medium is $choline^{+}$ instead of Na^{+} or Li^{+}. However, coupling then stays relatively high for a long time. (The coupling coefficient was 0.5 to 0.44 for 1 to 2 hr

Table 3. *Latency of uncoupling and depolarization in various media (mean value ± SE)*

Group [a]	Medium [b]	Uncoupling delay [c] (min)	Membrane potential at		No. of expts.
			start of experiment (mV)	start of uncoupling (mV)	
A	Li^{+Ca}_{+Mg}	70 ± 6	−40 ± 8	+ 1 ± 4	3
	Li^{-Ca}_{-Mg} [d]	18 ± 8	−41 ± 3	−31 ± 2	12
	Na^{-Ca}_{-Mg}	83 ± 14	−44 ± 4	−25 ± 4	8
	$Chol^{-Ca}_{-Mg}$ [e]	92 ± 16	−49 ± 3	−17 ± 3	4
	$(Li\text{-}Chol)^{-Ca}_{-Mg}$	31 ± 9	−42 ± 3	−23 ± 5	6
B	Li^{+Ca}_{+Mg}	105 ± 3	−53 ± 0	−42 ± 1	2
	Li^{-Ca}_{-Mg}	82 ± 10	−47 ± 3	−37 ± 1	22
	Na^{-Ca}_{-Mg}	120	−25	−15	1

[a] A and B are from two experimental groups several months apart; *see* footnote 1.

[b] See Table 1 for definition of media.

[c] Time from start of exposure to the corresponding medium to beginning of reduction of normal coupling coefficient (beginning of uncoupling).

[d] Excluded are two experiments in which the coupling coefficient was unchanged (≈ 1) for periods of 80 and 115 min of observation. The cells depolarized in both cases to zero (from 45 and 48 mV).

[e] Excluded is an experiment in which the coupling coefficient stayed nearly unchanged (≈ 1) for 210 min of observation; E_I and E_{II} fell from 50 mV to 6 and 9 mV during this time.

in three cases, and ≈ 1 for 3-1/2 hr in one case; *see* Table 3.) With Li and choline (equal parts) as the main cations, uncoupling is complete upon withdrawal of Ca^{++} and Mg^{++} (Ca, Mg-free Li-choline medium; Fig. 7; Table 3 [1]).

Coupling is well maintained with either Ca^{++} or Mg^{++} as the only divalent cation in the medium, as long as the total concentration is not substantially below 12 mM (Figs. 8 & 9).

Uncoupling in Ca, Mg-free medium was generally reversed by early restitution of these ions. Fig. 7 shows an example for Ca, Mg-free Li-choline

1 The data listed under A and B in Table 3 belong to two groups of experiments several months apart, in which the conditions of the gland probably differed. The results are thus only comparable within each given group. We do not put weight on the large (and statistically significant; $p < 0.001$) difference in latency between Ca, Mg-containing and Ca, Mg-free media in group A, in view of the small (and statistically not significant; $p > 0.01$) difference in the corresponding latencies in group B.

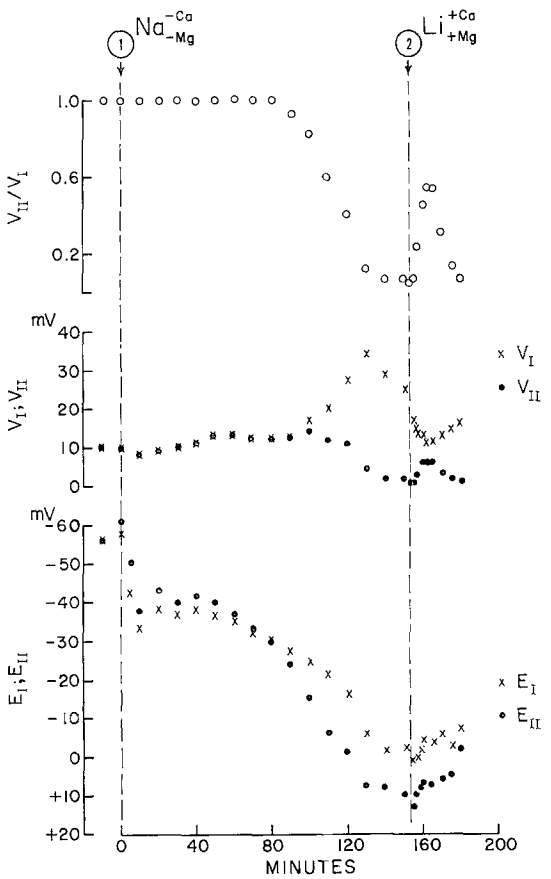


Fig. 6. Uncoupling in Ca, Mg-free Na medium. *1*, change from exchange of control medium to Ca, Mg-free Na medium. *2*, Ca, Mg-containing Li medium. Note the transient recoupling. Current, 2×10^{-8} amp; 200 msec

medium in which recoupling shows itself clearly in the V and E values. After an exchange of Ca, Mg-free medium for Ca, Mg-containing medium, V_{II} rises progressively while V_I falls; the two become nearly equal within 5 min, as they were at the start of the experiment before uncoupling. At the same time, the E values also equalize. The E values of the two cells, widely disparate here in the uncoupled state, change first in opposite (converging) directions as the coupling coefficient rises, before taking on their parallel course (Fig. 7, *2*). This is what one would expect if during reestablishment of coupling the ion concentrations equilibrated in the two cells and, as a result, the lower resting potential increased at the expense of the higher one.

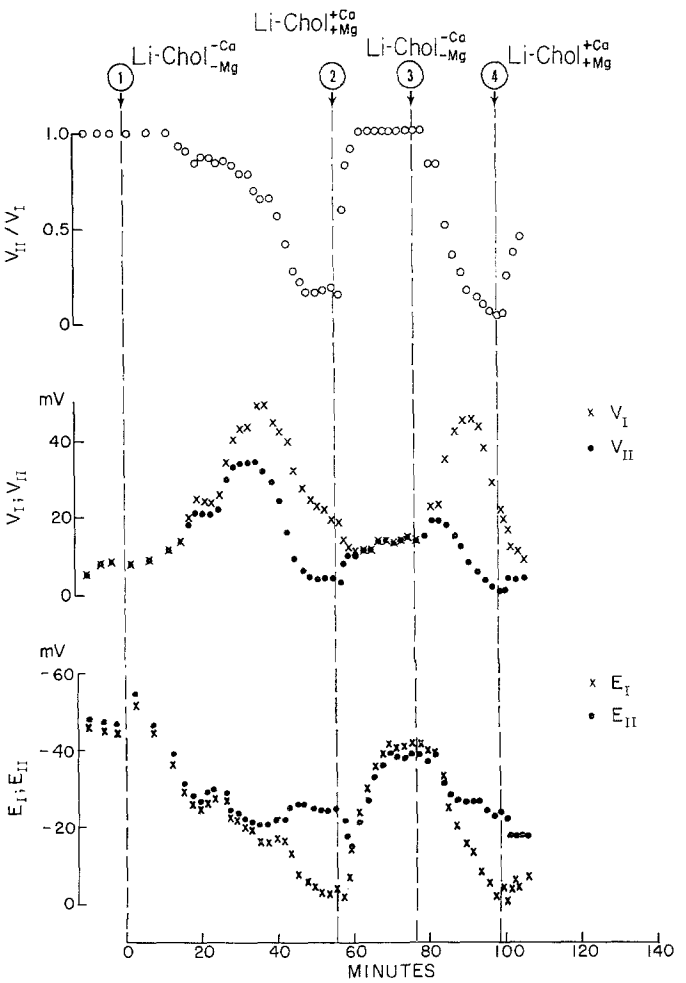


Fig. 7. Uncoupling in Ca, Mg-free Li-choline medium. Recoupling by Ca^{++}, Mg^{++}-restitution. *1*, application of Ca, Mg-free Li-choline medium. (Na^+ is replaced by equal parts of Li^+ and choline.) *2*, Ca, Mg-containing Li-choline medium. *3* and *4*, repetition of *1* and *2*. Current, 2×10^{-8} amp; 100 msec

The completeness and the speed of recoupling by restitution of Ca and Mg were typical for all restitution trials in Ca, Mg-free Na (two cases), Ca, Mg-free choline (one case) and Ca, Mg-free Li-choline medium (four cases). As in maintenance of coupling (Fig. 8), Ca^{++} and Mg^{++} substitute for each other in restoration of coupling (Fig. 9).

The uncoupling in Ca, Mg-free Li medium was reversible only if restitution of Ca and Mg ensued early; and even then recoupling was at best only partial and transient (Fig. 5, *4*). Recoupling was complete and persistent

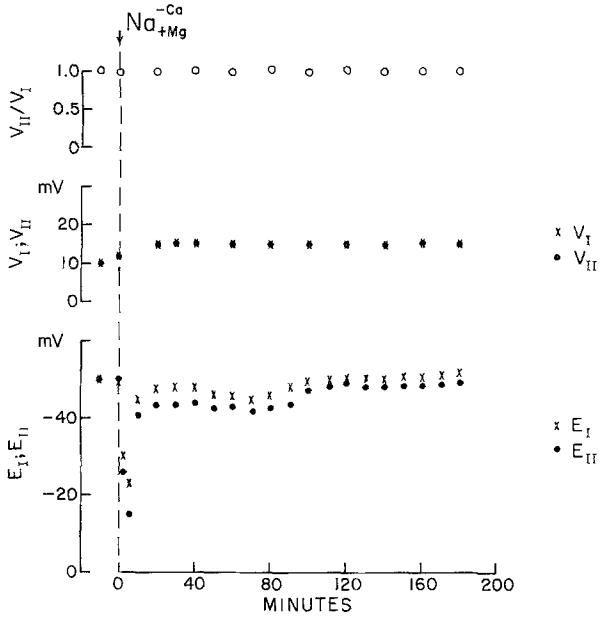


Fig. 8. Maintenance of coupling by Mg^{++}. At arrow, exchange of control medium by a medium whose Ca^{++} content is replaced by Mg^{++}. Current, 3×10^{-8} amp; 100 msec

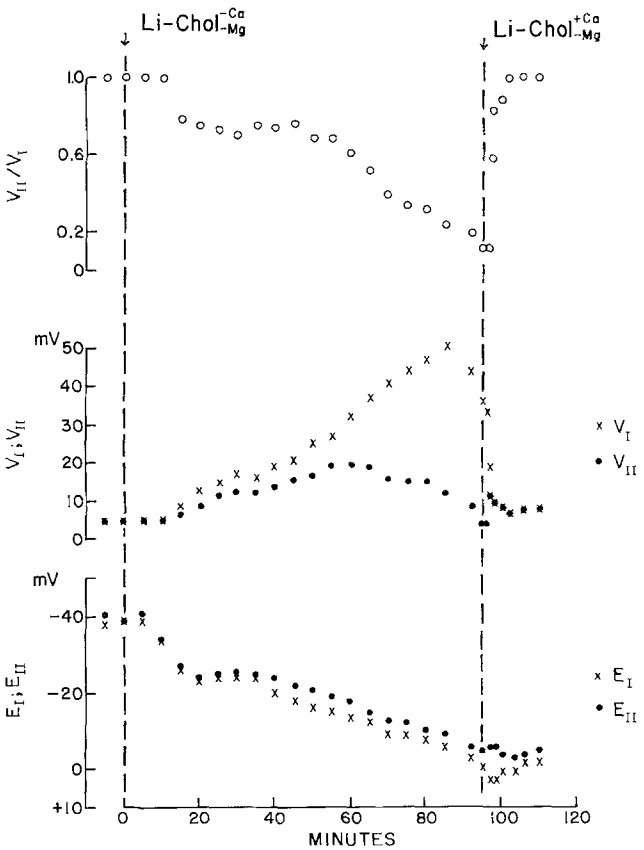


Fig. 9. Restoration of coupling by Ca^{++}. *1*, Ca, Mg-free Li-choline medium. *2*, Ca-containing (Mg-free) Li-choline medium (Ca, 12 mM). Current, 1×10^{-8} amp; 100 msec

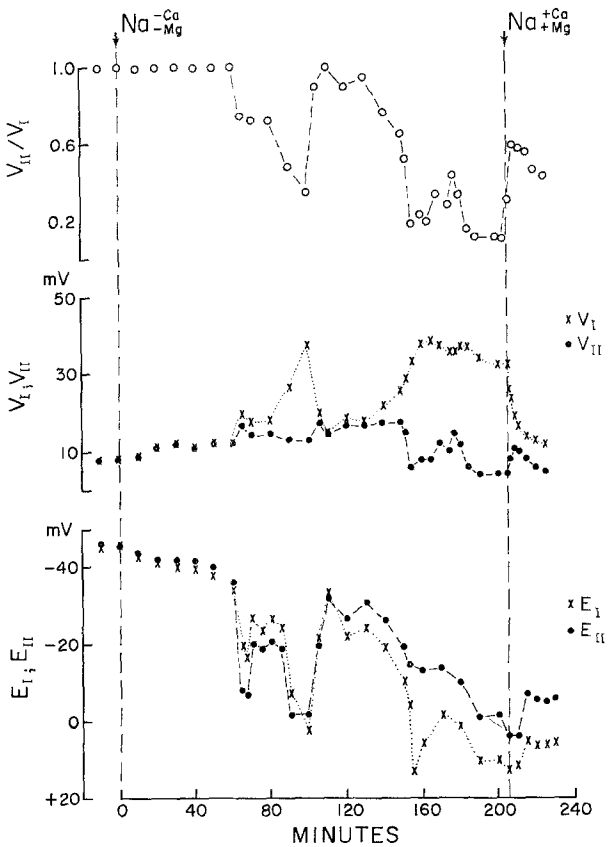


Fig. 10. Fluctuations in coupling. Current, 2×10^{-8} amp; 100 msec

when Na^+, in addition to divalent cations, was restored to the medium (Fig. 5, *2*). Uncoupling in Ca, Mg-containing Li medium was not reversible by such restitution (Fig. 2).

Typically, the coupling coefficient (and resting potential) declined rather continuously in the various 'uncoupling' media. In a few cases (Ca, Mg-free medium), the coupling fluctuated; the coupling coefficient recovered periodically in the course of uncoupling, and this was paralleled by periodic recovery in resting potential (Fig. 10).

Block of Junctional Passage of Fluorescein

The uncoupling can also be demonstrated by using fluorescein as a tracer. Fig. 11 gives an example for uncoupling in Ca, Mg-free Li medium. A cell, shown by electrical measurement to be uncoupled, was injected with fluorescein (mol. wt. 330). Fluorescein diffuses in the injected cell, but, in contrast to the situation in coupled cells (*see* Fig. 10 of Rose, 1971), the

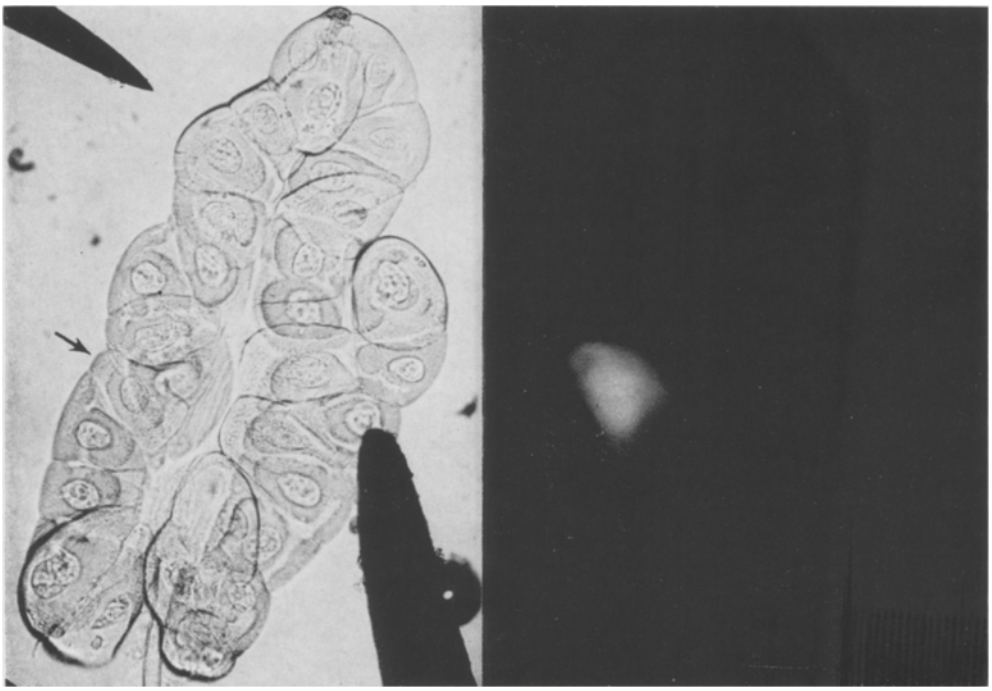


Fig. 11. Block of junctional passage of fluorescein. The cells were exposed to Ca, Mg-free Li medium. After development of uncoupling, as determined by electrical measurement, fluorescein was injected into a cell. *Left*, bright-field photomicrograph of the gland; the arrow marks the cell injected. *Right*, dark-field photomicrograph showing the fluorescence 15 min after injection. Compare with Fig. 10 of Rose, 1971

diffusion is confined to this cell alone. Evidently the junctional permeability to fluorescein is reduced, just as it is to the small inorganic ions that carry the electrical current. The good correlation between electrically measured uncoupling and block of fluorescein passage underlines once again the likelihood that the larger molecules follow the same junctional path as the smaller ones (Kanno & Loewenstein, 1966; Payton, Bennett & Pappas, 1969).

Chelator Injection

In another set of experiments, we injected the chelators EDTA and EGTA into uncoupling cells. These experiments were designed to sequester Ca from the junctional membrane and, thereby, to counteract uncoupling. EDTA binds Ca rather specifically (log-affinity constant of Ca-EGTA, 11.0, and of Mg-EGTA, 5.2, at pH 7.1); EDTA binds Ca and Mg (log-affinity constant of Ca-EDTA, 10.59, and of Mg-EDTA, 8.69, at pH 7.1; Schwar-

zenbach, 1955; Chaberek & Martell, 1959). The chelators were injected either hydraulically (mixed with fluorescein as marker) or electrically (alone as anions) (*see* Methods). The injected quantity of chelator was not known, but the fluorescent marking provided the certainty that the chelator was in the cells.

The effects of chelator were examined on preparations uncoupled in Ca, Mg-free Li medium (EDTA, 15 cases; EGTA, 9 cases), or by dinitrophenol, 5 to 10×10^{-5} M in the control medium[2] (EDTA, 3 cases; EGTA, 8 cases). The time of injection was varied: before uncoupling while the cells were still in control medium; at the first signs of uncoupling; and when uncoupling had already developed. In no instance was uncoupling significantly delayed or reduced. There was also no detectable reduction in nonjunctional membrane resistance.

Recoupling by Repolarization

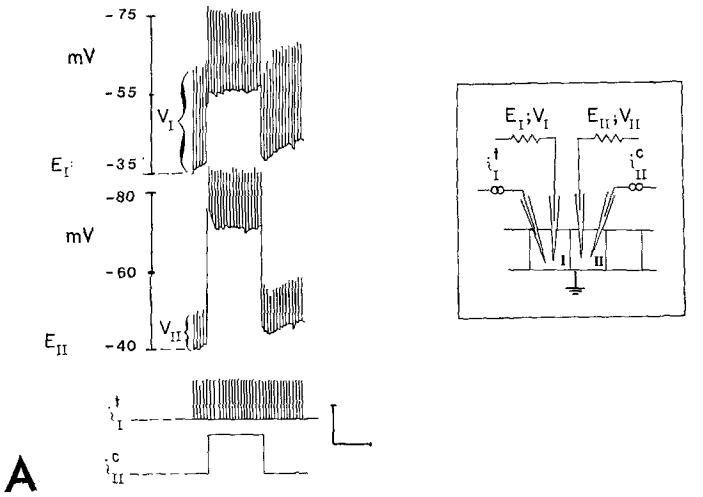
Nonetheless, there was also a positive side to the experiments with chelators. They led to the finding that the coupling process depends on (nonjunctional) membrane polarization. In the experiments in which EGTA and EDTA anions were injected with the aid of inward electrical current, we obtained, in fact, recoupling. But it soon turned out that this was an effect of the inward current, not an effect of chelation: when Cl^-, instead of EDTA or EGTA ions, was the current carrier, recoupling occurred just the same.

Fig. 12 illustrates the effect for a set of cells uncoupled in Ca, Mg-free Li medium. One of the cells (II) was repolarized with a step of current (carried by Cl^- out of the micropipette), and the coupling was measured with short test pulses. The coupling coefficient, which had fallen from near 1 to 0.33 in the Ca, Mg-free medium, rose within 10 sec to 0.55 and within 40 sec to 0.7. As in the cases described in the preceding section, this rise is determined by a rise in V_{II} and a simultaneous fall in V_I and, consequently, reflects an increase in junctional conductance.

The example (Fig. 12) shows the features of this kind of restoration of coupling: (1) recoupling develops rapidly after onset of the polarizing current; (2) it is transient; and (3) it outlasts the current. Moreover, as already mentioned, (4) the recoupling does not depend on the particular ion species carrying the current from the microelectrodes into the cells.[3]

2 Dinitrophenol and several other metabolic inhibitors cause uncoupling (Politoff *et al.*, 1969).

3 This would seem to apply also to the cations that may carry the current to the cell from the extracellular electrode. Recoupling occurred equally well in media containing Li or Na and lacking Ca and Mg.



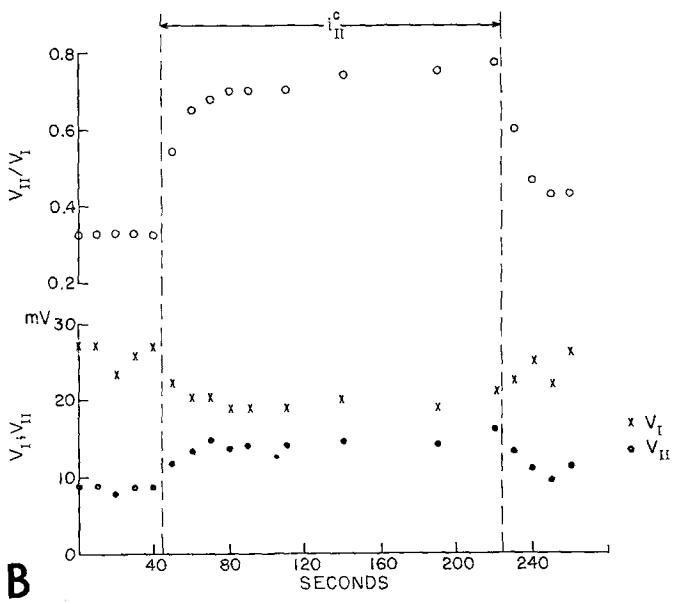


Fig. 12. Restoration of coupling by repolarization. The cells were uncoupled in Ca, Mg-free Li medium. (Before the treatment, the coupling coefficient was near 1.) *Inset*, electrode arrangement. For testing of coupling, inward current pulses (i^t_I, 2×10^{-8} amp, 100 msec duration, 6/min) are passed into cell I. For polarization, a step of inward current (i^c_{II}, 2×10^{-8} amp) is applied for 3 min with a fourth electrode in cell II. A, strip chart records of E and V; i^t_I and i^c_{II} are represented on the same time axis. Calibration: 2×10^{-8} amp; 2 min. B, plot of time courses of the V values and of the coupling coefficient V_{II}/V_I

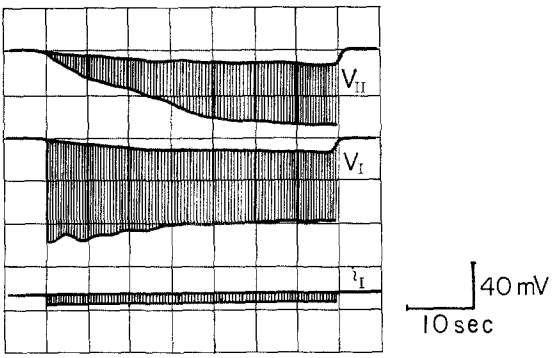


Fig. 13. Cumulative restoration of coupling by brief repolarizing current pulses. The cells were fully uncoupled ($V_{II}/V_I \approx 0$) and depolarized (E_I, $E_{II} = 0$) by treatment with Ca, Mg-free Li medium and remain in this medium throughout the experiment. Inward current pulses i_I (2×10^{-8} amp, 200-msec duration) are applied in cell I with a frequency of 2.3/sec, and the resulting V values are measured in this cell and an adjacent one, II (storage-oscilloscope tracing)

Restoration of coupling by repolarization was studied in four uncoupling situations: when the cells were uncoupled in Li medium (3 cases), in Ca, Mg-free Li medium (27 cases), in Ca, Mg-free Na medium (2 cases), and when they were uncoupled by dinitrophenol (10^{-4} M, 2 cases). In all instances, the cells had depolarized during uncoupling (at a coupling coefficient of 0.3, the depolarization ranged from 10 mV to total loss of membrane potential) and, in all instances, the cells recoupled upon repolarization.

The third feature of the recoupling – its lingering – is shown particularly clearly by experiments in which the restoration of coupling is brought about cumulatively by brief pulses of inward current. Fig. 13 illustrates this for a preparation which had been uncoupled in Ca, Mg-free Li medium. The coupling, initially nearly zero, improved with each of a series of current pulses, reaching a maximum coefficient of 0.85. [Current maintained continuously in the same cell produced complete recoupling (Fig. 15).]

The inward current flowed through both the nonjunctional and junctional membrane in the preceding experiments. Which part of the current determines the recoupling? In a series of experiments directed to this question, we approximated a symmetric arrangement of the electrical field on both sides of a junction by use of two intracellular current sources (Fig. 14, *inset*). Here, the nonjunctional membrane was repolarized with not more than about 1 mV potential across the junctional membrane at steady state. The cells recouple nonetheless, and they do so even faster and more completely than with the asymmetric arrangement. Thus, clearly, the recoupling is a result of current flowing through the nonjunctional membrane.

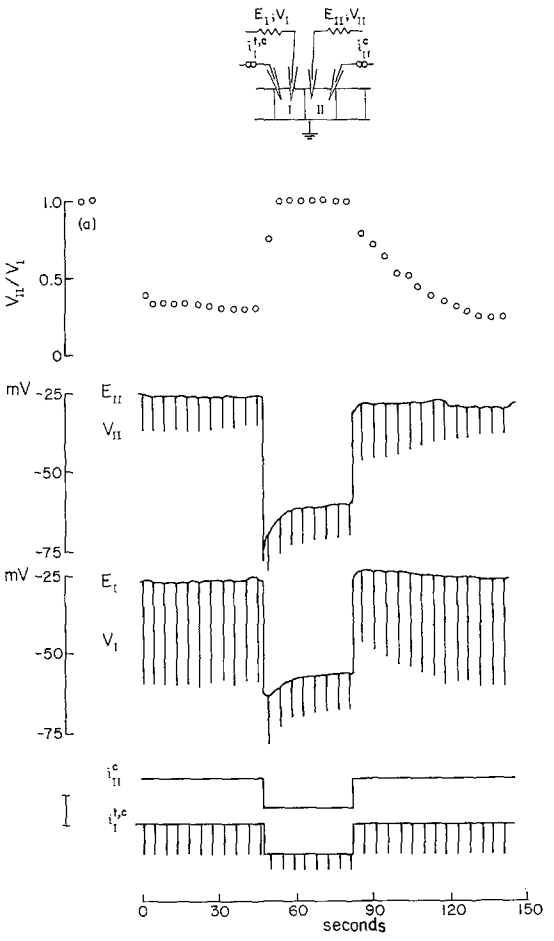


Fig. 14. Restoration of coupling at zero current through junctional membrane. Cells in Ca, Mg-free Li medium. *Inset*, for polarization, a 36-sec step of d.c. current (4×10^{-8} amp, inward) is applied (with two current electrodes) in each of two adjacent cells (i_I^c, i_{II}^c). For determination of coupling, brief current pulses (i_I^t, 12/min, 100-msec duration, 4×10^{-8} amp; and 2×10^{-8} amp, when superimposed on i_I^c) are passed in cell I with one of these electrodes. *Top*, time course of coupling coefficient V_{II}/V_I. *Middle*, strip chart records of E_I, E_{II}, V_I and V_{II}. $i_I^{t,c}$ and i_{II}^c are represented below on the same time axis. (i_I^t was reduced from 4×10^{-8} to 2×10^{-8} amp during superposition on i_I^c, because the current generator system was near saturation)

The recoupling effect can extend beyond the junctions of the cell containing the repolarizing current source. In the experiment of Fig. 15, coupling was tested simultaneously in three adjacent cells. The cells recouple in succession: the cell nearest to the current source within 1 min, and the other within 4 min of continuous current application.

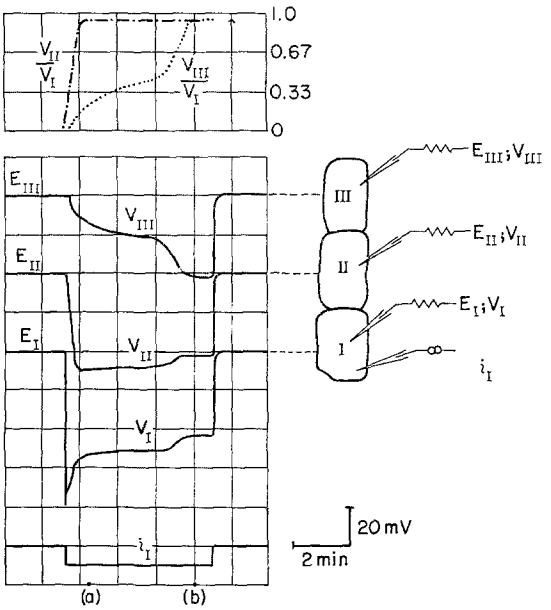


Fig. 15. Recoupling of a junction series. The same gland as in Fig. 13, uncoupled ($V_{II}/V_I \approx 0$) and depolarized (E_I, $E_{II} = 0$ mV) in Ca, Mg-free Li medium. For polarization, a d.c. step (i_I, 2×10^{-8} amp, inward) is applied for 5 min in cell I. V values are measured in this cell and two neighboring ones, II and III. *Bottom*, storage oscilloscope record of V_I, V_{II}, V_{III} and i_I, with diagram of electrode arrangement. *Top*, time course of the values of V_{II}/V_I and V_{III}/V_I. The cells recouple completely at *a* and *b*, respectively

Discussion

A striking result of the present experiments is that the depression of junctional membrane permeability produced by three entirely different means – dinitrophenol poisoning, substitution of extracellular Na^+ (Na_e^+) by Li^+, or long-term exposure to divalent cation-free media – is reversed by a single procedure, i.e., repolarization of the nonjunctional membrane. This suggests, as did earlier results from this laboratory (Loewenstein, 1966; Loewenstein *et al.*, 1967; Politoff *et al.*, 1969) that the depressions are mediated by a common factor. A possible mediator is Ca^{++}. The hypothesis that guided the present work is that the permeability of the junctional membrane is inversely related to the amount of Ca^{++} bound to it and, hence, is inversely related to the cytoplasmic Ca^{++} concentration (Loewenstein, 1967*a*). The hypothesis is based on the results of experiments wherein depression of junctional permeability ensued under conditions in which the normally low Ca^{++} level in cytoplasm had risen (intracellular injection of Ca^{++}, or influx of Ca_e^{++} through leaks in nonjunctional membrane or

in junctional seal; Loewenstein *et al.*, 1967) or could be expected to have risen (inhibition of metabolism on which Ca efflux depends; Politoff *et al.*, 1969). We shall try to interpret the present results on depression and restoration of junctional permeability in the light of this hypothesis.

Uncoupling and the Regulation of Cytoplasmic Ca^{++}

It appears possible to account for the permeability depression findings in terms of known mechanisms regulating Ca^{++} fluxes in other cells. In all cells heretofore examined, the level of cytoplasmic free Ca^{++} lies normally below 10^{-6} M (Hodgkin & Keynes, 1955; Portzehl, Caldwell & Ruegg, 1964; Luxoro & Yañez, 1968), several orders of magnitude below that of the extracellular liquids (10^{-3} to 10^{-2} M) and, hence, in cells with electrically negative interiors, far from electrochemical equilibrium. The permeability of the cell membrane to Ca^{++} is normally low (Hodgkin & Keynes, 1955; Rummel, Seifen & Baldauf, 1962; Baker *et al.*, 1967), yet high enough to allow considerable Ca^{++} influx in the life span of a cell (in squid axon, the Ca^{++} influx amounts to 10^{-13} moles cm^{-2} sec^{-1}; Baker *et al.*, 1967). The high concentration difference between cell inside and outside is maintained by metabolically driven Ca^{++} efflux (Schatzmann, 1966). Also, Ca^{++} efflux is strongly dependent on extracellular Ca, Mg and Na (van Breemen, Daniel & van Breemen, 1966; Baker *et al.*, 1967; van Breemen & van Breemen, 1968). In squid axon, for instance, the Ca^{++} efflux falls to about one-half if Na_e^+ is replaced by $choline^+$ or Li^+, and to about one-tenth if, in addition, Ca_e^{++} and Mg_e^{++} are withdrawn (Baker, 1970). The Ca^{++} influx, on the other hand, depends on Na_e^+ also. The influx increases steeply when Na_e^+ is reduced (skeletal muscle, Cosmos & Harris, 1961; heart muscle, Niedergerke, 1963, and Reuter & Seitz, 1968; liver, Judah & Ahmed, 1964; smooth muscle, Goodford, 1967; nerve, Baker *et al.*, 1967, and Baker & Blaustein, 1968). In the example of squid axon, the influx rises about twofold when $choline^+$ substitutes for Na_e^+, and fivefold when Li^+ is the substitute (Baker, 1970). Short-term buffering of the Ca^{++} level is provided by the cellular Ca depots, the mitochondria and the cell membrane. The well-known Ca-storing capacity of mitochondria depends on the K:Na ratio in the cytoplasm. Dransfeld, Greef, Schorn and Ting (1969) have shown that isolated mitochondria which have a high Ca storage capacity at high K:Na ratio, such as normally prevailing in cytoplasm, lose Ca^{++} at low K:Na ratio. (The situation for mitochondrial Mg is quite similar in this respect; Gear & Lehninger, 1968.) The Ca (and Mg) storing capacity of the cell membrane is also very high. For example, erythrocyte

membrane binds 1.6 mequiv Ca or Mg per g nitrogen (Carvalho, Sanui & Pace, 1963). The enormity of this quantity is perhaps best seen if it is expressed as atomic density on a two-dimensional membrane sheet: the distance between Ca atoms on such a membrane is 15 Å (K. S. Cole, *personal communication*). The Ca-holding capacity of the membrane seems to depend on the membrane potential. Work on barnacle muscle fiber (Hagiwara & Takahashi, 1967; Ashley & Ridgway, 1969) and on squid axon (Baker, Hodgkin & Ridgway, 1970) shows that free Ca^{++} is increased in the cell interior upon depolarization. There is evidence suggesting a similar situation for other axons (Frankenhäuser & Hodgkin, 1957; Narahashi, 1964), heart muscle (Niedergerke, 1963; Niedergerke & Orkand, 1966), and smooth muscle (Goodford, 1967; Bülbring & Tomita, 1970). For frog heart muscle, which lacks tubules and has little sarcoplasmic reticulum, Staley and Benson (1968) make a good *a priori* case for Ca^{++} release from cell membrane during depolarization, which is not immediately dependent on Ca_e^{++}: the amount of Ca^{++} required for maximal activation of actomyosin (73.4 μmoles/liter) is far in excess of the amount of maximal Ca_e^{++} influx (3 μmoles/liter) per contraction (*see* also Niedergerke, 1963).

With this background, it is possible to formulate a plausible explanation of the depression of junctional permeability produced in Li- and in Ca, Mg-free media. Assuming that the salivary gland cells here behave like the other cells, on the aforegoing grounds one may expect – in the case of the Li medium – an increased Ca^{++} flux into cytoplasm from the exterior, and from mitochondria and nonjunctional cell membrane, as Li^+ accumulates inside the cells, displaces K^+ (Carmeliet, 1964) and causes depolarization. At the same time, there is depression of the Na_e^+-dependent Ca^{++} efflux. Moreover, Li^+, which blocks respiration and glycolysis (Lindahl, 1940), may conceivably also in this way disturb the cellular Ca^{++} balance. Upon exhaustion of energy reserves and interruption of energy production, the mitochondria lose their Ca (Drahota, Carafoli, Rossi, Gamble & Lehninger, 1965; Carafoli, Patriarca & Rossi, 1969) and the ATP-dependent fraction of Ca^{++} efflux is blocked. All these factors concur in raising the cytoplasmic Ca^{++} level. Since Li^+ is not pumped out of the cells (Schou, 1957; Keynes & Swan, 1959; Obara & Grundfest, 1968), the perturbations of mitochondrial and membrane Ca stores are persistent even when the cells are exposed again to control medium. The result is an irreversible increase in cytoplasmic Ca^{++} and, hence, in the light of the Ca hypothesis, an irreversible decrease in junctional permeability.

The depressions of junctional permeability in Ca, Mg-free media fall into another category with respect to their reversibilities. Here, no major

Table 4. *Coupling and cellular Ca^{++} regulation*

Ca^{++} Flux	Origin of Ca^{++}	flux dependent on:	Medium[a]								
			Li^{+Ca}_{+Mg}	Li^{-Ca}_{-Mg}	$Chol^{+Ca}_{+Mg}$	$Chol^{-Ca}_{-Mg}$	Na^{-Ca}_{-Mg}	K^{+Ca}_{+Mg}	K^{-Ca}_{-Mg}	$(Li\text{-}Chol)^{-Ca}_{-Mg}$	DNP
A. Expected effects of media on Ca^{++} flux											
Efflux (out of cytoplasm)	Cytoplasm	$(Na)_e$	↓	↓	↓	↓		↓	↓	↓	
	Cytoplasm	$(Ca, Mg)_e$		↓		↓	↓		↓	↓	
	Cytoplasm	ATP	↓?	↓?						↓?	↓
Influx (into cytoplasm)	External med.	$(Na)_e$	↑	↓	↑	↓	↓	↑	↓	↓	
	Cell memb.	Potential	↑	↑	↑	↑	↑	↑	↑	↑	↑
	Mitochond.	K:Na; ATP	↑	↑	↑	↑	↑	↓?	↓?	↑	↑
B. Effects of media on coupling											
Uncoupling			yes	no	no	yes	yes	no[b]	yes	yes	yes
Recoupling by restitution of		Ca, Mg	—	transient	—	transient	yes	—	yes	yes	—
		Norm. medium	no	yes	—	yes	yes	yes	yes	yes	yes

(↓) Decrease of flux; (↑) increase of flux. It is assumed here that the salivary gland cells behave like certain other cells for which the corresponding effects were shown experimentally (*see* text).

[a] *See* Table 1 for composition.

[b] Applies to fresh gland only (*see* text).

disturbing factor of the Ca^{++} balance is persistent, at least not when Ca^{++} or Mg^{++} are restored early. In Ca, Mg-free medium, Na^{+} (and presumably also Li^{+}) accumulates in cells (Frankenhäuser & Hodgkin, 1957; Adelman & Moore, 1961; Bolingbroke & Maizels, 1959; Morril, Kaback & Robbins, 1964; Reuben Brandt, Girardier & Grundfest, 1967). The entry of Na^{+} or Li^{+}, into cytoplasm causes an increased Ca^{++} flux into cytoplasm from the mitochondria and membrane stores for the reasons already discussed for Li medium. Simultaneously, the $(Ca, Mg)_e$-dependent component of Ca^{++} efflux drops out. Upon restitution of Ca^{++} or Mg^{++} to a Na medium, the efflux becomes normal again (or overshoots; van Breemen & van Breemen, 1968), and all causes for the cytoplasmic Ca^{++} rise are removed as soon as Na^{+} is pumped out of the cells. In Ca, Mg-free Li medium, the Na_e-dependent component of Ca^{++} efflux falls away in addition to the alterations in Ca, Mg-free Na medium. Otherwise, the situations in the two media are probably initially similar, including with respect to reversibility when Ca^{++} and Mg^{++} [Figs. 5 (*4*) & 7 (*2*)] or Na^{+}, Ca^{++} and Mg^{++} [Figs. 5 (*2*) & 10] are restored. Upon prolonged exposure to Ca, Mg-free Li medium, sufficient Li^{+} accumulates in the cytoplasm and, as in the case of Ca, Mg-containing Li medium, the corresponding perturbations on mitochondrial and cell membrane Ca stores persist.

Table 4 summarizes the expected effects on cell Ca^{++} regulation in the various media.

Coupling and Cell Membrane Potential

The above interpretations assign the cell membrane potential an important role in the cellular Ca^{++} balance and, hence, in the coupling process. We met thus with the experimental question whether coupling can be altered by cell depolarization. In a series of experiments, freshly isolated glands were exposed to high K_e. The cells depolarized to near zero within 3 min of exposure to K medium (the polarity reversed transiently), but they did not uncouple over 18 min of observation (longer observations were not practicable because cell membrane resistance fell excessively) (Fig. 16, *1*). However, there was rapid and complete uncoupling upon application of K medium, when the cells had been pretreated with Ca, Mg-free Li medium (Fig. 16, *2* & *4*). Exposure to a Ca, Mg-free K medium causes uncoupling even without pretreatment, and this uncoupling develops faster than in Ca, Mg-free Na- or Li medium (Fig. 17; *compare* with Figs. 5, 6 & 10 and Table 3). From these observations, we got the impression that the depolarization by K medium facilitated uncoupling, although it was not sufficient to cause uncoupling by itself during the period of observation.

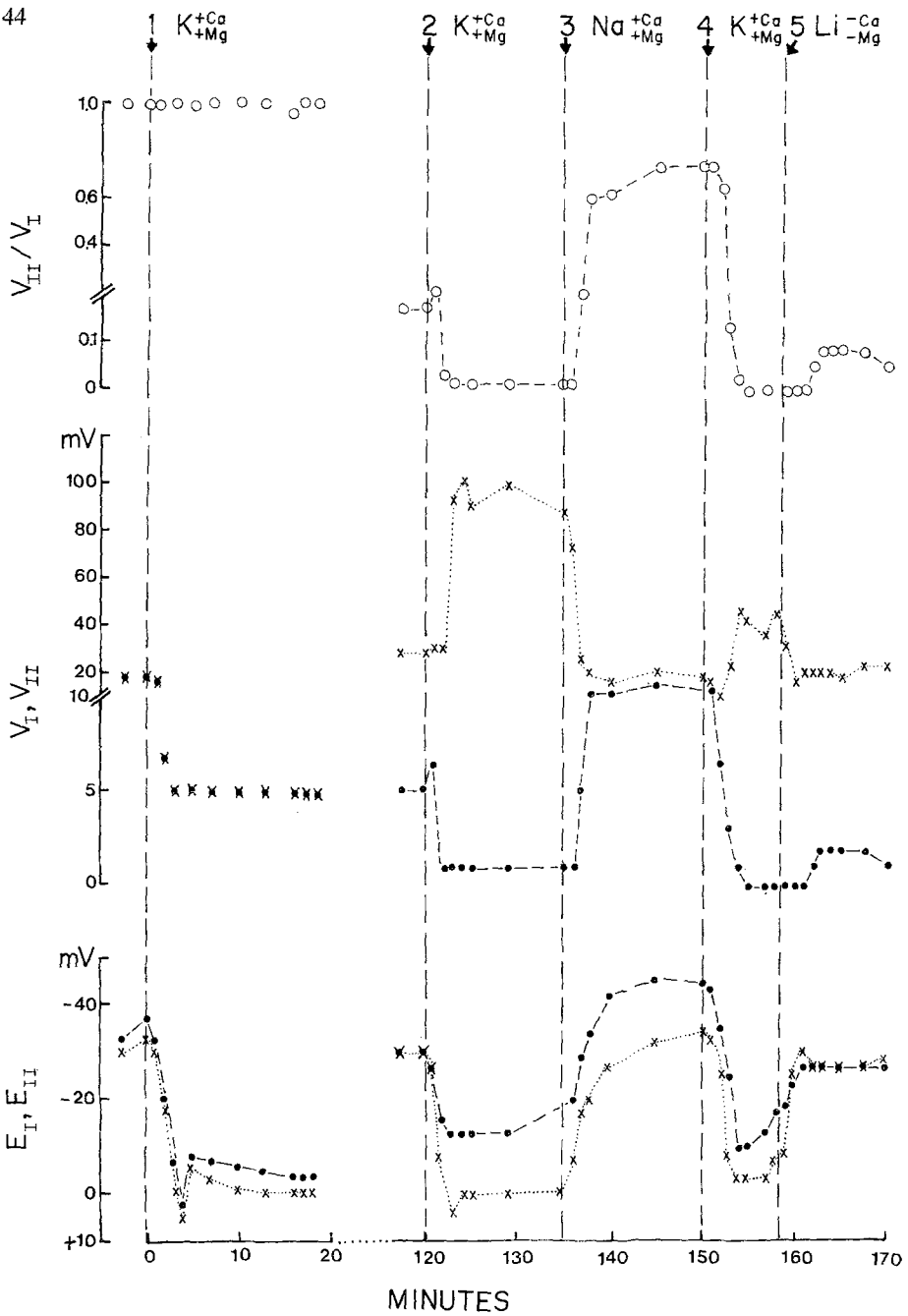


Fig. 16. Depolarization by K_e; effects on coupling. *1*, control medium exchanged by a medium in which K^+ substitutes for Na^+. The coupling stays unaltered during the 20-min exposure to this medium. Subsequently, the gland is exposed to Ca, Mg-free Li medium in which the coupling coefficient fell progressively (not plotted) to 0.15 within 100 min. At arrow *2*, the Ca, Mg-free Li medium is replaced by K medium; at *3*, by control medium; at *4*, by K medium; and at *5*, by Ca, Mg-free Li medium. Note the rapid and complete uncoupling at *2* and *4* and the partial recoupling at *5*. Scale expanded on lower part of the two upper ordinates. Current, 4×10^{-8} amp; 200 msec

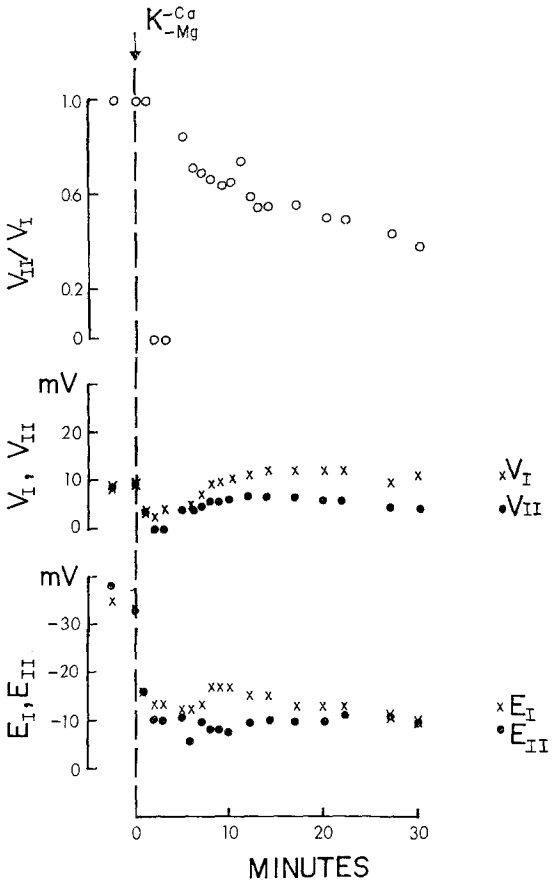


Fig. 17. Uncoupling in Ca, Mg-free K medium. At arrow, the control medium is replaced by a medium in which Na^+, Ca^{++} and Mg^{++} are replaced by K^+. Current, 4×10^{-8} amp; 200 msec

This impression was reinforced by the result shown in Fig. 16, *5*, where coupling improved transiently upon repolarization by Ca, Mg-free Li medium. The improvement was small, but nonetheless clear; V_{II} increased from an undetectably small value to 1.7 mV, while V_I fell by 20 mV. This recoupling effect is informative: Ca, Mg-free Li medium in the long run is actually an 'uncoupling' medium (*see* Figs. 1 B & 5), yet coupling improves during the repolarization phase produced by the transient restoration of the K gradients across the cell membrane in this medium. A similar betterment of coupling was observed in a cell pair uncoupled in Ca, Mg-free choline medium when the membrane potential was transiently raised by exposure to a Ca, Mg-free Li medium.

These results might be explained as follows: in K medium lacking Na^+, the Na_e-dependent Ca^{++} influx rises and the Na_e-dependent Ca^{++} efflux declines. Moreover, Ca^{++} is released from the depolarized cell membrane (*see* also van Breemen & Daniel, 1966). On the other hand, the Ca^{++}-buffering capacity of mitochondria may be improved owing to increase in cytoplasmic K^+:Na^+ ratio. If, in addition, Ca_e^{++} and Mg_e^{++} are lacking, Ca^{++} efflux diminishes further as the $(Ca, Mg)_e$-dependent efflux component drops out; and this tips the Ca^{++} balance toward uncoupling. Upon exchange of Ca, Mg-free K medium by Ca, Mg-free Li medium, the cytoplasmic Ca^{++} excess is absorbed transiently by the repolarizing cell membrane.

The strongest indication of the importance of the cell membrane potential in the coupling process is provided by experiments on uncoupling and recoupling by electric current. These experiments reveal, on one hand, that cell repolarization by unspecific inward current can restore the high junctional conductance in uncoupled cells (*see* Figs. 12–15); and, on the other hand, as shown by Socolar and Politoff (1971 *a*, *b*), that cell depolarization by outward current can depress junctional conductance. The recoupling effect by repolarization was demonstrated in four situations of uncoupling: DNP poisoning, Li medium, Ca, Mg-free Li- and Ca, Mg-free Na medium. The first situation would affect the coupling process in many ways. Upon exhaustion of the ATP and other energy reserves, there is (1) Ca^{++} release from the energy-dependent mitochondrial Ca store (Drahota *et al.*, 1965), (2) block of active Ca^{++} transport through cell membrane (Schatzmann, 1966), and (3) as a consequence of block of Na^+, K^+ transport (Hodgkin & Keynes, 1955), Ca^{++} release from cell membrane and further Ca depletion of mitochondria owing to intracellular accumulation of Na (and cell depolarization). In spite of these drastic alterations, coupling is improved or even normalized by cell membrane repolarization.

The reverse effect presents itself upon depolarization with outward current. Here, normally connected cells uncouple (Socolar & Politoff, 1971 *a*). These uncoupling and recoupling effects may be explainable by the dependency of the Ca-holding capacity of cell membrane on potential, already discussed. The justification for attributing the uncoupling process to alteration in Ca-holding capacity of cell membrane (and thus for considering the process even at this level as the reverse of the recoupling process) comes from a series of experiments in which the cells were electrically depolarized in Ca, Mg-free medium (*see* also Socolar & Politoff, 1971 *b*). Under these conditions, where Ca_e^{++} influx is excluded, uncoupling ensued as in Ca, Mg-containing medium (Fig. 18). The same mechanisms may be

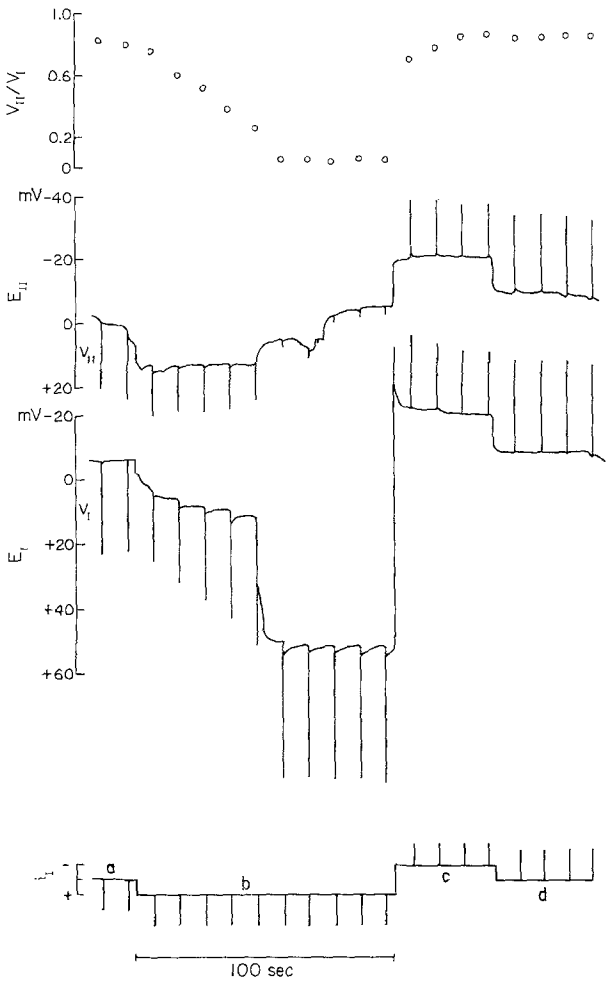


Fig. 18. Uncoupling by outward current in absence of external Ca and Mg. For depolarization (or polarization reversal), a step of outward d.c. is passed in cell I; for repolarization, current is reversed. For measurement of coupling, current pulses (4×10^{-8} amp, 100 msec, 6/min) are passed in this cell superposed on the d.c. step. Electrode arrangement as in Fig. 1 inset. d.c. current, *a*, zero; *b*, 4×10^{-8} amp outward; *c*, 4×10^{-8} amp: inward; *d*, zero. From top to bottom: coupling coefficient V_{II}/V_I; strip chart records of E_I, E_{II}, V_I and V_{II}; representation of the currents (i_I: +, outward). Note the marked uncoupling in *b* and its prompt reversal in *c*. (Test pulses in *c* smaller than in *a*, *b*, *d*.) The experiment was made shortly after exposure of the cells to Ca, Mg-free Li medium at a time when coupling was only slightly affected by this medium

at play here as those effecting Ca^{++} release from membranes of cells that normally carry electrical signals such as cardiac and smooth muscle, and nerve.

The proposed explanations in terms of presumed effects on Ca^{++} fluxes and stores provide, of course, only a useful framework for a more direct study.

Experiments with Chelators

The negative results of the chelator injections on coupling provide little information; the ineffectiveness of EDTA and EGTA may have many causes, of course. Nevertheless, the chelator experiments had a result which is of much interest in itself. They showed that the injected chelators produced no change in conductance of the (nonjunctional) cell membrane. This stands in striking contrast to the marked conductance increase when the chelators are applied to the outside of the cells (Loewenstein *et al.*, 1967). A similar asymmetric behavior of the cell membrane was found by L.J. Mullins and J. Brinley *(personal communication)* on perfused squid axon where the intracellular chelator concentration could be controlled. There were no changes in membrane conductance in this cell even with internal concentrations of EGTA or EDTA as high as 10^{-2} M; the outer membrane side is here, too, very chelator-sensitive. Thus, the membrane-bound Ca is either not accessible to chelators from the inside, or the Ca^{++} affinity of the inner membrane side is much higher than that of the chelators. This property of the cell membrane, which may well apply also to its junctional portions (*see* Oliveira-Castro & Loewenstein, 1971), provides a simple explanation for the ineffectiveness of the chelator injections on coupling.

We thank Dr. S. J. Socolar for much valuable discussion, Mr. I. Baird for construction of electronic apparatus and technical assistance, and Dr. R. Baltzley (Burroughs Wellcome Co.) for preparing the choline succinate.

This paper is based on portions of a dissertation presented by B. R. to the University of Munich in partial fulfillment of the requirements for the degree of Doctor of Natural Sciences.

The work was supported by research grants from the U.S. National Science Foundation and the National Institutes of Health.

References

Adelman, W. J., Moore, J. W. 1961. Action of external divalent ion reduction on sodium movement in the squid giant axon. *J. Gen. Physiol.* **45**:93.

Ashley, C. C., Ridgway, E. B. 1969. Aspects of the relationship between membrane potential, calcium transient and tension in single barnacle muscle fibres. *J. Physiol.* **200**:74P.

Baker, P. F. 1970. Sodium-calcium exchange across the nerve cell membrane. *In*: Calcium and Cellular Function. A. W. Cuthbert, editor. p. 96. St. Martin's Press, New York.

– Blaustein, M. P. 1968. Sodium dependent uptake of calcium by crab nerve. *Biochim. Biophys. Acta* **150**:167.

– – Hodgkin, A. L., Steinhardt, R. A. 1967. The effect of sodium concentration on calcium movements in giant axons of *Loligo forbesi*. *J. Physiol.* **192**:43P.

– Hodgkin, A. L., Ridgway, E. B. 1970. Two phases of calcium entry during the action potential in giant axons of *Loligo*. *J. Physiol.* **208**:80P.

Bolingbroke, V., Maizels, M. 1959. Calcium ions and the permeability of erythrocytes. *J. Physiol.* **149**:563.

van Breemen, C., van Breemen, D. 1968. Stimulation of calcium efflux from smooth muscle by extracellular calcium. *Biochim. Biophys. Acta* **163**:114.

– Daniel, E. E. 1966. The influence of high potassium depolarization and acetylcholine on calcium exchange in the rat uterus. *J. Gen. Physiol.* **49**:1299.

– – van Breemen, D. 1966. Calcium distribution and exchange in the rat uterus. *J. Gen. Physiol.* **49**:1265.

Bulbring, E., Tomita, T. 1970. Calcium and the action potential in smooth muscle. *In*: Calcium and Cellular Function. A. W. Cuthbert, editor. St. Martin's Press, New York.

Carafoli, E., Patriarca, P., Rossi, C. S. 1969. A comparative study of the role of mitochondria and the sarcoplasmic reticulum in the uptake and release of Ca by the rat diaphragm. *J. Cell. Physiol.* **74**:17.

Carmeliet, E. E. 1964. Influence of lithium ions on the trans-membrane potential and cation content of cardiac cells. *J. Gen. Physiol.* **47**:503.

Carvalho, A., Sanui, H., Pace, N. 1963. Calcium and magnesium binding properties of cell membrane materials. *J. Cell. Comp. Physiol.* **62**:311.

Chaberek, S., Martell, A. E. 1959. Organic Sequestering Agents. John Wiley & Sons, New York.

Cosmos, E., Harris, E. J. 1961. In vitro studies of the gain and exchange of calcium in frog skeletal muscle. *J. Gen. Physiol.* **44**:1121.

Drahota, Z., Carafoli, E., Rossi, C. S., Gamble, R. C., Lehninger, A. L. 1965. The steady state maintenance of accumulated calcium in rat liver mitochondria. *J. Biol. Chem.* **240**:2712.

Dransfeld, H., Greef, K., Schorn, A., Ting, B. T. 1969. Calcium uptake in mitochondria and vesicles of heart and skeletal muscle. *Biochem. Pharmacol.* **18**:1335.

Frankenhäuser, B., Hodgkin, A. L. 1957. The action of calcium on the electrical properties of squid axons. *J. Physiol.* **137**:218.

Gear, A. R., Lehninger, A. L. 1968. Rapid, respiration-dependent binding of alkali metal cations by rat liver mitochondria. *J. Biol. Chem.* **243**:3953.

Goodford, P. J. 1967. The calcium content of smooth muscle of the guinea pig taenia coli. *J. Physiol.* **192**:145.

Hagiwara, S., Takahashi, K. 1967. Surface density of calcium ions and calcium spikes in the barnacle muscle fiber membrane. *J. Gen. Physiol.* **50**:583.

Hodgkin, A. L., Keynes, R. D. 1955. Active transport of cations in the giant axons from sepia and loligo. *J. Physiol.* **128**:28.

Judah, J. D., Ahmed, K. D. 1964. The biochemistry of sodium transport. *Biol. Rev.* **39**:160.

Kanno, Y., Loewenstein, W. R. 1966. Cell-to-cell passage of large molecules. *Nature* **212**:629.

Keynes, R. D., Swan, R. C. 1959. The permeability of frog muscle fibre to lithium ions. *J. Physiol.* **147**:628.

Lindahl, P. E. 1940. Neuere Beiträge zur physiologischen Grundlage der Vegetativisierung des Seeigelkeimes durch Lithiumionen. *Arch. Entw. Mech. Org.* **140**:168.

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

– 1967*a*. Cell surface membranes in close contact. Role of calcium and magnesium ions. *J. Colloid Interface Sci.* **25**:34.

– 1967*b*. On the genesis of cellular communication. *Develop. Biol.* **15**:503.

– Nakas, M., Socolar, S. J. 1967. Junctional membrane uncoupling: Permeability transformations at a cell membrane junction. *J. Gen. Physiol.* **50**:1865.

Luxuro, M., Yañez, E. 1968. Permeability of the giant axon of *Dosidicus gigas* to calcium ions. *J. Gen. Physiol.* **51**:1155.

Morrill, G. A., Kaback, H. R., Robbins, E. 1964. Effects of calcium in intracellular sodium and potassium concentrations in plant and animal cells. *Nature* **204**:641.

Narahashi, T. 1964. Restoration of action potential by anodal polarization in lobster giant axons. *J. Cell. Comp. Physiol.* **64**:73.

Niedergerke, R. 1963. Calcium movements in frog heart ventricles at rest and during contractions. *J. Physiol.* **167**:515.

– Orkand, R. W. 1966. The dual effect of calcium on the action potential of the frog's heart. *J. Physiol.* **184**:291.

Obara, S., Grundfest, H. 1968. Effects of lithium on different membrane components of crayfish stretch receptor neurons. *J. Gen. Physiol.* **51**:635.

Oliveira-Castro, G. M., Loewenstein, W. R. 1971. Junctional membrane permeability: Effects of divalent cations. *J. Membrane Biol.* **5**:51.

Payton, B. W., Bennett, M. V. L., Pappas, G. D. 1969. Permeability and structure of junctional membranes at an electrotonic synapse. *Science* **166**:1641.

Politoff, A. L., Socolar, S. J., Loewenstein, W. R. 1969. Permeability of a cell membrane junction. Dependence on energy metabolism. *J. Gen. Physiol.* **53**:498.

Portzehl, H., Caldwell, P. C., Ruegg, J. C. 1964. The dependence of contraction and relaxation of muscle fibres from the crab *Maia squinado* on the internal concentration of free calcium ions. *Biochim. Biophys. Acta* **79**:581.

Reuben, J. P., Brandt, P. W., Girardier, L., Grundfest, H. 1967. Crayfish muscle: Permeability to sodium induced by calcium depletion. *Science* **155**:1263.

Reuter, H., Seitz, N. 1968. The dependence of Ca efflux from cardiac muscle on temperature and external ion composition. *J. Physiol.* **195**:451.

Rose, B. 1970. Junctional membrane permeability: Restoration by repolarizing current. *Science* **169**:607.

– 1971. Intercellular communication and some structural aspects of membrane junctions in a simple cell system. *J. Membrane Biol.* **5**:1.

– Loewenstein, W. R. 1969. Depression of junctional membrane permeability by substitution of lithium for extracellular sodium. *Biochim. Biophys. Acta* **173**:146.

Rummel, W., Seifen, E., Baldauf, J. 1962. Aufnahme und Abgabe von Calcium an Erythrocyten von Menschen. *Arch. Exp. Pathol. Pharmakol.* **244**:172.

Schatzmann, H. J. 1966. ATP-dependent Ca-extrusion from human red cells. *Experientia* **22**:364.

Schou, M. 1957. Biology and pharmacology of the lithium ion. *Pharmacol. Rev.* **9**:17.

Schwarzenbach, G. 1955. Die komplexometrische Titration. Ferd. Enke Verlag, Stutgart.

Socolar, S. J., Politoff, A. L. 1971*a*. Uncoupling a cell junction in a glandular epithelium by depolarizing current. *Science* **172**: 492.

– – 1971*b*. Depression of conductance of a cell membrane junction by depolarization. *XXV Intern'l Congress of Physiol. Sci.* Munich, July 1971.

Staley, N. Benson, E. S. 1968. The ultrastructure of frog ventricular cardiac muscle and its relationship to mechanisms of excitation-contraction coupling. *J. Cell Biol.* **38**:99.