Permeability of a Cell Membrane Junction

*Dependence on energy metabolism*

A.L. POLITOFF, S.J. SOCOLAR, and W.R. LOEWENSTEIN

From the Cell Physics Laboratory, Department of Physiology, Columbia University College of Physicians and Surgeons, New York 10032

ABSTRACT The ion permeability of the membrane junctions between *Chironomus* salivary gland cells is strongly depressed by treatments that are generally known to inhibit energy metabolism. These treatments include prolonged cooling at 6°-8°C, and exposure to dinitrophenol, cyanide, oligomycin, and *N*-ethylmaleimide. Intracellular injection of ATP appears to prevent depression of junctional permeability by dinitrophenol or to reverse it. Ouabain, azide, *p*-chloromercuriphenylsulfonic acid, reserpine, and acetazolamide fail to depress junctional permeability. Thus the ion permeability of the junctional membranes appears to depend on energy provided by oxidative phosphorylation. Possible energy-linked processes for maintaining junctional permeability are discussed, including processes involving transport of permeability-modifying species such as Ca++. 

INTRODUCTION

The hypothesis has been proposed that the ionic permeability of junctional membranes in various cell systems depends on the free Ca++ concentration of the cytoplasm in contact with them (Loewenstein, 1966, 1967). The permeability is high in the Ca++ concentrations normally prevailing in cytoplasm; apparently when cell [Ca++] is raised, junctional permeability falls, as has been evidenced most directly in the salivary gland of *Chironomus* (Loewenstein et al., 1967). The normal Ca++ concentration in cytoplasm of a variety of cells is, by direct or indirect evidence, several orders of magnitude lower than in the normal extracellular media (Hodgkin and Keynes, 1957; Portzehl et al., 1964; Hagiwara and Nakajima, 1966). This situation is maintained far from electrochemical equilibrium throughout the life of a cell despite some Ca++ leakage into the cytoplasm, a fact that already in 1957 led to the recognition that Ca++ must be actively extruded from the cell (Gilbert and Fenn; Hodgkin and Keynes). If the normal cytoplasmic Ca++ level is similarly limited in the connected gland cells, the normal junctional membrane permeability must then ultimately depend on the supply of metabolic energy. One might anticipate,
therefore, that interruption of the energy supply would cause depression of junctional membrane permeability. These considerations led to the present experiments in which we inhibited cell metabolism by cooling and by chemical agents, and studied the effects on junctional membrane permeability in Chironomus salivary gland cells. Preliminary reports have already appeared (Politoff et al., 1967, 1968).

METHODS

Preparation

Isolated salivary glands of third and fourth instar larvae of Chironomus thummi were used. For the present experiments it was important that the cell surface membranes be intact in all cells, since injury to nonjunctional surface membranes is known to lead rapidly to changes in permeability of the junctional membranes (Loewenstein et al., 1967). Contact between cells and dissecting tools was therefore avoided during gland isolation and mounting. The procedure was to cut the larva into three pieces; one cut was made between the second and third body segments, and the other at midbody. The salivary glands and the esophagus or the intestine were then protruding from the center piece. The esophagus and intestine were pulled out, whereupon the green elements that join the glands to the body could be seen and severed. The gland was then floating free in the medium of the superfusion chamber. It was then immobilized on the bottom by the weight of a tiny stainless steel pin (Minutien insect pin, 0.012 x 1 cm), bent to a V and laid over the gland so that the (tapered) tip of the pin rested just over the edge of the gland, without much pressure, and the rest of the pin lay on the bottom of the chamber.

Electrical Arrangement

Three microelectrodes were inserted into two adjacent cells (Fig. 1, inset). One electrode passed rectangular current pulses (2 x 10^-8 amp, 100-150 msec duration, base line leakage < 10^-11 amp) at the rate of 1/min, and the other two recorded the membrane voltages. An electrode in the bath served as current sink and ground reference. The voltages were fed into DC amplifiers through field-effect transistor input stages (leakage current < 10^-13 amp). Current pulses and membrane potentials of the two cells were displayed (normally automatically 1/min) on an oscilloscope with 200 msec sweep duration and photographed. Simultaneously the three variables (only two in early experiments; current was omitted) were recorded continuously on a storage oscilloscope, with a much slower sweep (7 min/cm); thus continuous records of some 40-50 min duration could be photographed. Occasionally the potentials were recorded also on a potentiometric pen recorder, at intermediate speeds. The illustrations in the Results show storage oscilloscope records, except for Fig. 8, which gives a record

1 It was important to keep leakage current low; in some long experiments in which leakage exceeded 10^-10 amp of depolarizing current, this appeared sufficient to depolarize and eventually to uncouple the cells. This phenomenon has been explored further, and a preliminary report is in preparation.
from the pen recorder. The fast records were used for more accurate measurement of currents and for monitoring pulse forms.

Most experiments were done with microelectrodes (tip diameter below 0.5 μ) filled with a mixture of 3 M KCl and 2 mM potassium citrate. These electrodes usually had much lower resistances (10–25 MΩ) and tip potentials (<3 mV) than equivalent electrodes filled with KCl alone. Moreover, the resistance of these electrodes did not increase during storage for several days, as was common with electrodes filled with KCl alone.

In experiments with iontophoretic injection of ATP,2 micropipettes filled with saturated potassium-ATP replaced the usual KCl current electrodes; and cathodal current pulses of the order of 10⁻⁸ amp and of 100 msec duration were passed at the rate of 30–100/min. (The transference number of ATP⁻ was not known.) We met with various technical difficulties. The ATP electrodes had high resistances (about 150 MΩ) and often failed to deliver current pulses of acceptable rectangularity. With some electrodes, the plateau height of the current diminished from pulse to pulse when the repetition rate was as high as 120/min; and there were often annoying fluctuations in electrode resistance. The latter problems could sometimes be circumvented by reducing the pulse repetition rate, but often such reduction was insufficient to stop fluctuations. These difficulties limited severely the number of useful experiments.

For filling with substances for iontophoretic injection, micropipettes were filled first with distilled water. The water in the pipette shank was then displaced with a saturated solution of the iontophoretic material, and the tip was immersed in a solution of the same kind and left for several hours to equilibrate. In the case of filling with ATP, the filled micropipettes were stored with tips in distilled water; sometimes cathodal current pulses were applied to hasten the movement of ATP⁻ into the tip after the latter was in saline.

Application of Inhibitors

The superfusion system allowed vibration-free continuous or intermittent flow. The time required for exchanging 99% of the chamber medium was of the order of 5 min or less. The marks on the records in the Results indicate the beginning of an exchange. Times given in the text and tables for exposure to inhibitor media are referred to starting time of the exchange. Except for the cooling experiments, all experiments were done at room temperatures ranging from 19° to 21°C. Generally, inhibition procedures were applied only after cell resting potentials, $V_I$, and $V_{II}$ (see below), had displayed stability in the control medium for at least 5 min, and usually for 10 min or more.

Cooling of the superfusion chamber was by means of a Peltier effect semiconductor device. Temperature of the bath was monitored by a thermistor placed about 4 mm from the gland.

2 The following abbreviations are used in this paper: ATP, adenosine-5'-triphosphate; DNP, 2,4-dinitrophenol; EGTA, ethylene glycol-bis-[β-(N,N-dicarboxymethylamino)-ethyl ether]; NEM, N-ethylmaleimide; PCMBS, p-chloromercuriphenylsulfonic acid; TES, N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid.
**Media**

The control medium had the following composition: KCl, 2 mM; disodium fumarate, 28 mM; CaCl₂, 5 mM; NaCl, 28 mM; magnesium succinate, 7 mM; glutamine, 80 mM; TES (as buffer), 5 mM. NaOH to bring pH to 7.40 (about 5 mM). This medium was formulated to approximate *Chironomus* larval hemolymph in respect to reported content of chloride and cations (Boné and Koch, 1942; Duchâteau et al., 1953), osmolality (Harnisch, 1934, 1943), and pH (Boche and Buck, 1942). The choice of anions besides chloride was guided by the reported prominence of organic acids of the tricarboxylic acid cycle in the hemolymphs of other endopterygote larvae (Wyatt, 1961; Florkin and Jeuniaux, 1964), and glutamine was used to fill the osmotic deficit because of its abundance in these hemolymphs (Florkin and Jeuniaux, 1964). Among the various media tested in trial runs, this gave the best morphological preservation and the highest membrane potential (56 ± 16 mV ± SD; 30 cases), and showed excellent maintenance of membrane potential and of cell-to-cell coupling for at least 3–4 hr.

When chemical inhibitors were used, they were dissolved in the control medium unless otherwise stated. In the case of oligomycin, this material was rendered soluble by mixing first with ethanol; the final ethanol concentration (in control medium) was 0.2% v/v. Control medium containing 1% ethanol showed no effect on junctional coupling or on resting potential.

The Ca-, Mg-free medium had, apart from salts of Ca and of Mg, the same solute composition as the control solution.

With NEM, solutions were prepared on the day of use to insure activity.

**RESULTS**

**Junctional Uncoupling by Cooling**

The general procedure here for probing cellular communication is to pass current from the inside of a cell (I) to the outside, while recording the resulting changes in membrane voltage (V) in this cell and in a contiguous one (II) (Fig. 1, inset). The ratio of steady-state membrane voltages, \( V_{II}/V_I \), (coupling coefficient), provides a convenient index of communication. In the normal cell system, the coupling coefficient is around 0.9 (0.93 ± 0.04 SD; 19 cases). Diminution of communication, due to fall in junctional membrane permeability, is indicated by a fall in \( V_{II}/V_I \) simultaneous with a rise in \( V_I \) (Loewenstein et al., 1967), and is termed junctional uncoupling.

Fig. 1 illustrates an experiment in which junctional uncoupling ensues upon cooling of the cell system to 6–8°C. The first reactions to cooling are a fall in cell resting potential and a rise in input resistance. Junctional coupling is initially unaffected (Fig. 1 A-B; C-D). But as the temperature is held low, the cells uncouple (D-E). Upon warming, the cells repolarize and normal coupling is restored (E-F). Subsequent cooling causes uncoupling practically without delay (F-G).
The initial delay in uncoupling varies. In the five preparations in which uncoupling was produced by cooling below 10°C, the delays were 30, 35, 60, 180, and 210 min. (But even longer cooling may be needed sometimes; in one experiment, 4 hr of cooling at 5°C failed to uncouple.) That even the longest de-

![Diagram](image)

**Figure 1.** Junctional uncoupling by cooling. Inset shows electrode arrangement (in contiguous cells, I and II) for this and subsequent experiments: electrodes serve to pass rectangular current pulses (i) and to record steady-state heights (Vr, Vr') of the resulting voltage pulses, as well as cell resting potentials (Ei, EII); Ei and EII are given by base lines (lower envelopes) of the upper pair of records; Vr, Vr' are given by heights of up-strokes from the base lines (downstrokes in subsequent figures). Vr, Vr' magnitudes on same scale as Ei, EII. Outward current pulses (2 x 10^-4 amp, 150 msec duration) are passed 1/min. Brief cooling (A-B) gives only cell depolarization, which is reversible (B-C) on warming. Prolonged cooling (C-E) eventually depresses junctional membrane conductance, as shown by the changes of Vr and Vr'. Warming (E-F) restores junctional coupling and, partially, cell resting potentials. Upon further cooling and warming, these phenomena repeat, but without the long delay of C-D. Potentials registered by recording electrodes on withdrawal from cells into bathing medium are shown by last segments at right (0 mv).

Published April 1, 1969
layed uncouplings were specific results of cooling and not of cell damage is evident from their reversibility; in every case uncoupling and depolarization were at least partly reversed on warming to 20°C. (Uncoupling by cell damage, on the other hand, is not reversible [Loewenstein et al., 1967].)

The delay in onset of junctional uncoupling in the early cooling period (Fig. 1 C-D) suggested that uncoupling is the result of a critical change in a metabolically dependent concentration of a cellular constituent. This suggestion was reinforced by the prompt occurrence of uncoupling in a subsequent period of cooling in the two experiments in which this was tried (Fig. 1 F-G). One would expect therefore that suitable chemical inhibitors of cellular metabolism can also produce junctional uncoupling. This in fact turned out to be the case, as shown by the following experiments.

**Figure 2.** Junctional uncoupling by DNP. In this and subsequent figures current pulses are inward (here and in Figs. 4-7 their amplitudes are shown by top record); pulse duration 100 msec unless stated otherwise; preparation is initially bathed in control medium; subsequent changes of medium are indicated by vertical marker lines. Here, control medium is restored at b just as uncoupling has evidently begun. Bottom of $V_I$ trace is offscreen beyond e.

### Junctional Uncoupling by Dinitrophenol

DNP in concentrations of $5 \times 10^{-5}$ M or higher produces junctional uncoupling (11 experiments). At concentrations of $1-2 \times 10^{-4}$ M, junctional uncoupling occurred in 5-40 min of exposure (nine experiments); at $5 \times 10^{-3}$ M, in 70-80 min (two experiments); and at $1 \times 10^{-5}$ M, there was no sign of uncoupling after a 90 min exposure (one experiment).

An example of junctional uncoupling by DNP is illustrated in Fig. 2. Bathing of the cell system with $5 \times 10^{-5}$ M DNP is seen to cause depolarization and increase in input resistance $(a-b)$, and eventually the start of junctional uncoupling of the cells (around $b$). At $b$, when the change in $V_I$ was

---

3 Not included in this tally are 22 other cases in which DNP produced junctional uncoupling in preparations that had first been treated with other agents which themselves failed to uncouple, for example, Ca-, Mg-free media; acetazolamide; iontophoretically injected citrate, orthophosphate, EGTA, or ATP.
reflecting an uncoupling trend, the cell system was superfused with control solution. This was followed by complete restoration of coupling; \( V_I \) and \( V_{II} \) returned to normal (c-d). Return to DNP caused junctional uncoupling again, more completely and with much shorter delay than before (beyond d). Washout of the DNP by control medium again (e) led, after 20 min, to a decline of \( V_I \) without rise of \( V_{II} \).

Apart from the experiment of Fig. 2, one other attempt was made to reverse uncoupling by washing away the DNP. In the latter case, with \( 10^{-4} \text{ M DNP} \), the coupling coefficient recovered from 0.1 to 0.4 (initial value 0.9), while \( V_{II} \) regained about 0.65 of its initial value.

fig3.jpg

Figure 3. Junctional uncoupling by DNP in Ca-, Mg-free medium. Only the (upper) voltage base lines and ends of downstrokes are recorded. DNP in Ca-, Mg-free medium (a) causes junctional uncoupling, indicated here by large rise of \( V_I \); early return of control medium (b) restores junctional coupling, apparently preventing the (usually delayed) decline of \( V_{II} \). Current, \( 2 \times 10^{-8} \text{ amp} \), 130 msec pulse duration.

Earlier results suggested that excessive Ca++ influx, leading to an elevated intracellular concentration of free Ca++, may be a general forerunner of experimentally induced junctional uncoupling (Loewenstein et al., 1967). Hence it was of interest to investigate the action of DNP in an extracellular medium free of Ca++ (and of Mg++). In three out of four trials, with DNP at a concentration of \( 1-2 \times 10^{-4} \text{ M} \), junctional uncoupling occurred in 4-40 min (Fig. 3). Coupling was restored on replacement of the DNP medium by the control solution (normal Ca++ and Mg++). This reversibility was confirmed in each of two trials. In one experiment, \( 2 \times 10^{-4} \text{ M} \) DNP in the Ca-, Mg-free medium failed to produce junctional uncoupling in 70 min.

As a control on the foregoing DNP experiments in the absence of external
Ca and Mg, six preparations were observed in the Ca-, Mg-free medium for periods of 40–65 min. In four cases (40–50 min), no uncoupling was seen; but in a fifth, pronounced uncoupling had developed in 40 min, after 10–15 min during which the coupling coefficient fluctuated slowly following the first clear

<table>
<thead>
<tr>
<th>Table 1</th>
<th>EFFECTS OF INHIBITORS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agent</td>
<td>Concentration</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>DNP</td>
<td>$5 \times 10^{-5}$, $2 \times 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^{-4}$ in Ca-, Mg-free</td>
</tr>
<tr>
<td>Oligomycin</td>
<td></td>
</tr>
<tr>
<td>NaCN</td>
<td>$2 \times 10^{-2}$</td>
</tr>
<tr>
<td>NEM</td>
<td>$10^{-5}$–$10^{-3}$</td>
</tr>
<tr>
<td>PCMBs</td>
<td>$10^{-4}$</td>
</tr>
<tr>
<td>NaN₃</td>
<td>$3 \times 10^{-3}$</td>
</tr>
<tr>
<td>Ouabain</td>
<td>$10^{-4}$</td>
</tr>
<tr>
<td>Reserpine</td>
<td>$8 \times 10^{-8}$-saturated</td>
</tr>
<tr>
<td>Acetazolamide</td>
<td>$4.5 \times 10^{-8}$</td>
</tr>
<tr>
<td></td>
<td>$4.5 \times 10^{-4}$§§</td>
</tr>
</tbody>
</table>

* In all but two cases, a "-k-" entry connotes at least an 8-fold fall of $V_{II}/V_I$ (in some cases, greater than 15-fold) accompanied by a rise of $V_I$. In two of the experiments with DNP in Ca-, Mg-free medium, $V_{II}/V_I$ had declined only about fourfold when washout of the DNP led to recovery (Fig. 3).† Number preceding slash represents total of experiments giving the results indicated in "Uncoupling" column and, unless otherwise noted, in "Depolarization" column. Number following slash is total of all experiments.‡ All four trials gave depolarization.|| This material was described by the supplier as 15% oligomycin A and 85% oligomycin B.¶ In one of these two experiments, the preparation had, 3 h before CN⁻ treatment, been exposed to 10 mg% diphenylhydantoin, in 1% ethanol, for purposes unrelated to the CN⁻ experiment.‡‡ This is exclusive of four other cases, in which NEM led to junctional uncoupling after other agents had failed. Fig. 6 shows one of the latter experiments. Also, 5 mM NEM led to junctional uncoupling in two experiments in a medium with reduced Ca²⁺ and Mg²⁺.‡‡‡ Reserpine was used at the suggestion of W. Hasselbach. It inhibits Ca uptake by sarcoplasmic vesicles (Balzer et al., 1967).§§ Followed 1.5 hr of cooling at 7°C.

sign of uncoupling. In the sixth experiment, the coupling coefficient fell progressively from 0.9 to 0.7 over the course of an hour and was still falling at the end of that interval. When the Ca-, Mg-free solution was replaced by control medium, the coupling coefficient returned promptly to its original value. The
low incidence of junctional uncoupling here, as compared with the higher incidence in generally shorter times in the presence of DNP, supports the interpretation that DNP was instrumental in junctional uncoupling even in the absence of external Ca\(^{++}\) and Mg\(^{++}\).

**Junctional Uncoupling by Other Inhibitors**

Aside from DNP, eight other compounds were tested. Their effects are summarized in Table I, and examples of the time course of action of some of the agents appear in Figs. 4 to 6.

Of the eight compounds, oligomycin, cyanide, and NEM led to junctional uncoupling. Apart from the results appearing in Table I and the figures, a few additional comments seem worthwhile.

![Figure 4. Junctional uncoupling by oligomycin.](image)

1. In every case of junctional uncoupling, depolarization began before any sign of uncoupling appeared.
2. Ouabain caused depolarization without junctional uncoupling. In addition, in four cases out of five, it caused roughly a doubling of input resistance without significant change in \(V_{\text{r}}/V_{i}\); this presumably reflects increased nonjunctional membrane resistance.
3. The actions of the two sulfhydryl reagents, NEM and PCMBS, offer an interesting contrast. While NEM consistently gave junctional uncoupling, the apparently more reactive but far less permeant PCMBS (as found in other materials: Katz and Mommaerts, 1962; Jacob and Jandl, 1962; Van Steveninck et al., 1965; Rothstein and Weed, 1965) failed to uncouple. This suggests that the sulfhydryl groups relevant to maintenance of junctional coupling are intracellular, or at least below the membrane's outer surface.
4. In one of the two CN\(^{-}\) experiments, an attempt was made to restore
junctonal coupling by washout of the poison with control medium; no recoupling ensued. In the two NEM experiments in which this was tried (NEM concentrations $10^{-5}$ to $10^{-4}$ M and $2 \times 10^{-4}$ M), clear-cut restoration of coupling failed, although in the latter case, in which exposure to NEM had been briefer, there was a slight suggestion of recoupling. No tests were made of reversibility in the experiments with oligomycin.

5. In one of the six azide experiments ($5 \times 10^{-4}$ M, 14 min) and in one of the four reserpine experiments (saturated, 6 min) junctional uncoupling occurred. For the present we are inclined to attribute these two exceptions to cell injury independent of any actions of the inhibitors (see Loewenstein et al., 1967).

6. The fact that cyanide caused junctional uncoupling might lead one to expect that azide also should, since both are often considered to be specific inhibitors of the same enzyme complex in the electron transport chain. There is, however, evidence that CN$^-$ inhibits other metalloenzymes as well (see, for example, Mahler and Cordes, 1966). The essential absence of effect with azide here recalls, for example, the findings that azide fails to inhibit either the resting uptake of oxygen or sodium extrusion of frog skeletal muscle, both of which are cyanide-sensitive (Stannard, 1939; Carey et al., 1959). Since the undissociated acid molecules may well enter the cells more rapidly than the ions, azide may be ineffective here simply because its pK is so low compared to the pH.

**Metabolic Inhibitors and Spontaneous Electrical Activity**

Some preparations (14 cases) presented electrical activity of unknown origin in the form of depolarizations of irregular frequency on the order of 1/min, variable ampli-
tude as large as 25 mv, and 20-60 sec duration. This activity was synchronous in records made from contiguous cells. DNP (six cases) and NEM (one case) abolished it with little delay (Figs. 2, 6, and 7).

**Restoration of Junctional Coupling by ATP Injection**

A common effect of cooling and of the various chemical agents which caused junctional uncoupling is inhibition of cellular synthesis of ATP. It was therefore natural to ask whether cellular injection of ATP would prevent junctional uncoupling or restore coupling in metabolically inhibited cells. In the experiments exploring this question, DNP was used as the inhibitor. We preferred a chemical inhibitor to cooling, because uncoupling by cooling often has very long delays. (DNP, $10^{-4} \text{M}$, had caused junctional uncoupling unfailingly, usually within 20 min and always within 40 min.) Moreover, there is a distinct possibility that junctional uncoupling on cooling may reflect a block of energy-utilizing systems (e.g., ATPases) rather than depletion of high energy intermediates such as ATP (see, for example, Willis, 1964; Ahmed and Judah, 1965; Gruener and Avi-Dor, 1966).

In five preparations, the procedure was to preinject cell I iontophoretically with ATP, then to expose it to $10^{-4} \text{M}$ DNP for 0.5-2 hr while continuing to inject with ATP at a rate of at least 30 pulses/min ($2 \times 10^{-8} \text{amp}, 100 \text{msec}$ duration). In two of these cases, ATP injection prevented or reversed junctional uncoupling. In the case shown in Fig. 7, it restored an uncoupled junction to a normal level of coupling: the coupling coefficient, which had fallen markedly (c-d) during DNP treatment, increased progressively (d-f) upon

---

**Figure 6.** Effect of reserpine. After failure of reserpine to affect junctional coupling, action of NEM demonstrates that the preparation can exhibit uncoupling. (At extreme right, $V_T$ downstrokes go offscreen.) Note the spontaneous synchronous depolarizations, which disappear after NEM treatment.
massive injection of ATP at 30 pulses/min, finally \( g \) reaching its original value (0.85) after about 50 min of ATP injection. (During the control period \( a-c \), ATP injection was at the rate of 1/min.) In the case shown in Fig. 8, ATP injection appeared to prevent junctional uncoupling: the coupling coefficient stayed essentially normal during 1.5 hr of treatment with \( 5 \times 10^{-5} \text{ M DNP} \) plus an additional 45 min in \( 10^{-4} \text{ M DNP} \). A sixth preparation, not preinjected, \[ \begin{align*}
\text{Figure 7. Restoration of junctional coupling by ATP injection. Current microelectrode filled with saturated potassium ATP. Cell I is preinjected with ATP (1 pulse/min, 2 \times 10^{-8} \text{ amp}) for 1.8 hr. (Recording of current pulses (upper trace) fails in interval b-d.) DNP (c) effects junctional uncoupling (c-d). Beginning 2–3 min after c, current is passed at 30 pulses/min, leading (e-g) to full recoupling. Note that the interval of reduced ATP delivery (f-g) is associated with transient decline of \( V_{II}/V_I \). Current pulse height, usually held constant in our procedures, was varied in this case in an effort to maintain acceptable pulse form; therefore a plot of \( V_{II}/V_I \) is presented as an aid in seeing changes of coupling. In vicinity of e, bottom of \( V_I \) trace is offscreen. Displacement of current trace above base line (a-b and just after d) shows brief outward overshoot at end of pulse; integral of overshoot is in all cases \( \leq 15\% \) of integral of inward current. (3/4 hr before the experiment, the gland was exposed for 5 min to \( 1.4 \times 10^{-4} \text{ M atropine}, \) for purposes unrelated to this experiment.)
\end{align*} \]
but pulsed with ATP at a rate of 95/min (3 \times 10^{-8} \text{ amp, 100 msec}) during DNP exposure, remained coupled throughout 1.5 hr of observation. Intracellular injections of HPO_{4}^{2-} (two experiments with preinjection) did not counteract junctional uncoupling by DNP even though the molar quantity in-

**BEFORE DNP**

-58 mv

**V_{II}**

**AFTER DNP**

-41 mv

**V_{II}**

**V_{I}**

10 mv

0.5 min

**FIGURE 8.** ATP injection prevents junctional uncoupling by DNP. Gland preloaded with ATP is exposed for 2.5 hr to DNP (concentration 5 \times 10^{-5} \text{ M for 1.5 hr, then increased to 10^{-4} \text{ M and maintained for 0.5 hr}}) while ATP injection continues. Contrary to the results of parallel experiments without ATP injection, normal junctional coupling persists. Figure shows samples of \( V_{I} \), \( V_{II} \) during a period before DNP exposure ("Before") and during a period (still in DNP) at the end of the 2.5 hr exposure ("after"). During the periods shown, the ATP microelectrode passes 90 pulses/min; during preloading (1 hr) approximately 200 pulses are delivered, and during DNP exposure approximately 1000 (rate varying). Current pulses 100 msec duration, approximately 5 \times 10^{-8} \text{ amp. Input resistance rises and current amplitude varies, each by a factor of } \sim 2, \text{ during the experiment; approximate equality of } V_{II} \text{ before and after, as well as that of } V_{I}, \text{ is thus fortuitous. Time reference points for the two channels are slightly offset.}
jected was undoubtedly far greater than in the case of ATP, because of greater ion mobility.

**DISCUSSION**

The present results show that a variety of agents known to inhibit energy metabolism, such as prolonged cooling, DNP, cyanide, oligomycin, and NEM, cause junctional uncoupling. Such uncoupling, at least in the case of DNP, can be prevented or reversed by cellular injection of ATP. (The present results, however, still leave open the possibility that the effect of ATP injection in supporting junctional coupling may be mediated by the complexing of metal ions, for example Ca++, or by a cytoplasmic pH change.) On the other hand, no junctional uncoupling is produced by ouabain (10^{-4} M), a specific inhibitor of (Na+ ÷ K+)-activated ATPase, or by PCMBS, a relatively active but probably poorly permeant sulfhydryl reagent. Although these limited findings allow a variety of possible interpretations, it may, nonetheless, prove helpful to consider what appears to be the simplest interpretation, namely, that the ionic permeability of the salivary gland junctional membranes depends on metabolic energy provided by oxidative phosphorylation.

As to how this energy might be used, no positive evidence is available. There is no need to consider metabolic energy as driving the ion movements in junctional conductance; such fluxes result from imposed electric fields. More likely, metabolic energy is a factor in the passive permeability of the junctional membrane.

Two general possibilities are apparent: (a) an endergonic membrane process is required for maintaining structurally labile permeable components of the junctional membrane; (b) metabolic energy is required for regulating concentrations (at the junction) of substances that modify permeability.

The hypothesis that led to the present series of experiments (see Introduction) implies a mechanism of type (b), with Ca++ the permeability-modifying substance. In this hypothesis, junctional uncoupling follows from elevation of cytoplasmic [Ca++] (Loewenstein, 1966, 1967). Here the most obvious sources of Ca++ are the extracellular medium and the mitochondria. In all likelihood, both are large Ca reservoirs from which the cytoplasm is shielded by energy-
linked transport. Thus, as evidence from other cells suggests, metabolic inhibition might be expected to promote a net Ca++ flux into the cytoplasm, through nonjunctional cell surface membrane (Luxoro and Yañez, 1968) and from mitochondria (Carafoli, 1967; for results on isolated mitochondria, see also Vasington and Murphy, 1962; Greenawalt et al., 1964; Drahota et al., 1965; also, compare Blaustein and Hodgkin, 1969).

The idea that junctional uncoupling results from Ca++ action at the junctional membrane flowed from earlier experiments, in which junctional membrane permeability was depressed when the cytoplasmic [Ca++] was raised (Loewenstein et al., 1967). But there is no evidence as to whether the Ca++ in those experiments acted directly on the junctional membrane or indirectly, by affecting some other site. The present finding, that metabolic inhibition also depresses junctional permeability, points up alternative possibilities, in which Ca++ has no unique status as an effector of junctional uncoupling. Ca++ is known to inhibit a variety of enzymes, including ATPases (see, for example, Dixon and Webb, 1964; for Ca effects on ATPases, see also Rothstein, 1968). Thus it is possible that Ca++ achieves its depressing effect on junctional permeability by interfering with the utilization of metabolic energy. Complex dependence on Ca++ can set a critical [Ca++] requirement for optimum ATPase activity. An instructive example is the demonstration that a single cell can contain one ATPase that is Ca++-inhibited and another that is Ca++-activated at one site and Ca++-inhibited at another site (Dunham and Glynn, 1961; Wins and Schoffeniels, 1966).

Another conceivable indirect mode of Ca++ action would be by interference with mitochondrial function. Mammalian mitochondria exposed to [Ca+++] in the range 10^-4-10^-3 M show inhibition of ATP formation, inhibition of respiration, and even irreversible damage (see, for example, Wojtczak and Lehninger, 1961; Vasington and Murphy, 1962; Judah et al., 1965). This, however, seems an unlikely alternative: these effects, whose rates depend on [Ca+++] appear to be far too slow to account for the rapid uncoupling found at [Ca++] in the range 10^-5-10^-4 M (Loewenstein et al., 1967).

What may at first sight appear challenging to a simple scheme of Ca++-mediated junctional uncoupling is the result indicating that uncoupling occurs upon mere exposure to a Ca-, Mg-free medium. This can be accommodated, however, if we may suppose, for example, that, as in other cells, in the absence of divalent cations in the extracellular medium, cytoplasmic [Na+] rises (Maizels, 1956; Bolingbroke and Maizels, 1959; Frankenhaeuser and Hodgkin, 1957; Adelman and Moore, 1961; Morrill et al., 1964; Reuben et al., 1967; Baker et al., 1967, 1969), and that as

---

8 For reviews dealing with membrane transport of Ca++, see Harris et al., 1966; Weber, 1966; Pullman and Schatz, 1967; Lehninger et al., 1967; Caldwell, 1968; Rothstein, 1968.

9 Since this medium is just the control medium with Mg and Ca salts omitted, it represents a substantial reduction in ionic strength as well.
has been shown for isolated mitochondria, the Na⁺ causes mitochondrial release of Ca²⁺.¹⁰ This result could also be explained by the less specific hypothesis of junctional uncoupling consequent to interrupted energy supply, if prolonged exposure to the Mg²⁺-free medium depletes the cells of Mg²⁺ ions, which are known to be needed for enzyme activator functions, as for example in energy metabolism (see, for example, Dixon and Webb, 1964).

Dr. Politoff is a visiting scientist from the University of Chile, Santiago.
Mr. Ian Baird proposed and assembled the system for parallel storage display and for automatic recording. We are grateful to him also for continuing technical advice and assistance. We thank Mrs. Irene Young for maintaining the stock of Chironomus. Acetazolamide ("Diamox") was kindly given to us by Dr. John T. Litchfield, Jr., of Lederle Laboratories.
This work was supported by research grants from the National Institutes of Health and the Natural Science Foundation.

Received for publication 21 August 1968.

REFERENCES


¹⁰ B. Reynafarge and A. L. Lehninger (personal communication) have recently shown that, on addition of Na⁺, mitochondria bathed in [K⁺] approximating intracellular levels, unload much of their Ca.
steady state maintenance of accumulated Ca$^{++}$ in rat liver mitochondria. *J. Biol. Chem.* 240:2712.


POLITOFF, SOCOLAR, AND LOEWENSTEIN  Metabolism and Junctional Permeability  515


