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CALCIUM-INDUCED UNCOUPLING IN COMMUNICATING HUMAN LYMPHOCYTES

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SUMMARY

Microelectrode studies of the junctional conductance in human lymphocytes after phytohemagglutinin stimulation are described. A state of high intercellular permeability was detected after 12 to 48 h of incubation.

When cytoplasmic calcium concentration increases, after iontophoretic injection, these highly permeable junctions seal off. The coupling coefficient falls to zero within 2 to 4 min of Ca^{2+} application.

Current flow through the junctional membranes of the lymphocyte clump does not alter the transjunctional permeability.

Intercellular communication in this system derived from the aggregation of free-floating cells is compared with that prevailing in communities of epithelial cells. Possible mechanisms of permeability control and their relation with the structure of the contact regions are discussed.

INTRODUCTION

The high ion permeability of junctional membranes in epithelial cells is labile, falling several orders of magnitude when cytoplasmic Ca^{2+} concentration increases by iontophoretic injection [1] or by penetration from the bathing medium [2]. A decrease in the transjunctional conductance has also been demonstrated in association with metabolic poisoning, where the calcium-sensitive protein aequorin revealed a simultaneous increase in the cytoplasm-free calcium [3]. Human lymphocyte clumps, after stimulation with plant-derived mitogens, exhibit extensive intercellular communication [4]. The application of the mitogen phytohemagglutinin to adjacent lymphocytes induces the establishment of highly permeable junctions between them within 15 min [5]. The goal of this study is to compare the control mechanism of interlymphocyte junctional permeability with that of the better known epithelial contact regions. Although these systems have different reactions to current flow through their junctional elements, their response to the intracellular calcium injection is very similar.

METHODS

Peripheral blood lymphocytes from healthy human donors were cultivated in the presence of 0.1 ml phytohemagglutinin (Burroughs-Wellcome, Tuckahoe, N.Y.) for each 10^7 cells after gradient purification, in a standard medium (Roswell Park Memorial Medium 1640 from Grand Island Biological Co, Grand Island, N.Y., according to ref. 6) containing 20 % of fetal calf serum. After 12–48 h of incubation, cell clumps were added to 2 % agar in culture medium at 40 °C and a thin smear was prepared with a cover-glass on a plastic Petri dish previously covered with transparent dielectric resin Sylgard 184 (Dow Chemical, Midland, Mo.). The preparation was maintained at 35–37 °C and fresh medium was added every 30 min.

Glass micropipette electrodes filled with 3 M KCl (30 to 100 MΩ resistance) were inserted into different cells allowing continuous monitoring of membrane potentials and junctional conductance. In a typical experiment three pipettes were positioned in adjacent cells as follows: Pipette 1 was connected to a bridge-like circuit (WPI-Model M 4 A pre-amplifier) and was used to record voltage and simultaneously pass current pulses between the cytoplasm of Cell 1 and the extracellular fluid. Pipette 2 was positioned in a neighbouring cell (Cell 2) and was used either for current injection (connected to the stimulus isolator WPI-Isopulser Model PC-3) or for intracellular calcium application. Iontophoretic Ca^{2+} injection was performed through electrodes filled with 95 mM $Ca(OH)_2$, 100 mM EGTA and 100 mM Tris solution. Ca^{2+} was driven by rectangular pulses of 10^{-8} A and 50 ms duration at a rate of 5 to 10 pulses/s. Measurements of the transference number with radioactive calcium using this technique gave a value of 0.2 [7]. Pipette 3 was placed in a third cell and used to record membrane potential and its drop during current injection in Cells 1 or 2. The junctional conductances were indicated by coupling coefficients (ratio of voltage drops produced in both sides of junctions by a current pulse: V_2/V_1 or V_3/V_1).

The intensity of the current pulse (i), the intracellular potentials (E) and voltage drops (V_1, V_2 or V_1 V_3) were simultaneously displayed on a double-beam oscilloscope and on a slave storage scope. The time courses of the experiments were followed on a strip chart recorder with a slow time base.

A stable membrane potential and a state of high junctional permeability were considered indications of reliable impalements (see ref. 4 for details).

RESULTS AND DISCUSSION

Coupling coefficients of 0.18 to 0.60 were measured in adjacent cells using both depolarizing and hyperpolarizing current pulses of $2 \cdot 10^{-9}$ to $2 \cdot 10^{-8}$ amperes.

To test junctional permeability after a period of current injection a series of experiments with two electrodes in neighbouring cells was performed.

Fig. 1 illustrates one of these experiments, in which the cell clumps were immobilized by gentle suction (inset) using a larger pipette (around 5 μm tip). Voltage drops (V_1, V_2) were stable throughout the experiment, with a coupling ratio of 0.56. Small differences in transmembrane potentials in both cells and the simultaneous fluctuations seen in E_1 and E_2 can be regarded as another indication of intercellular

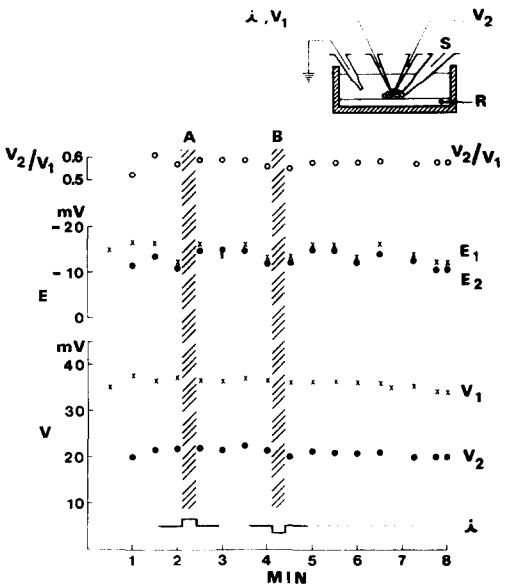

Fig. 1. Effect of current flow on junctional conductance. Transmembrane potentials (E_1, E_2) and voltage drops (V_1, V_2) resulting from current injection through KCl-filled microelectrode in Cell 1. The upper plot represents the coupling coefficient (V_2/V_1). Current pulses (i) applied at A (inward current) and B (outward current) did not affect intercellular conductance (pulses of 50 ms, 10^{-8} A at a rate of 5 pulses/s during 10 s). Inset: Scheme of the experimental set-up showing a lymphocyte clump resting on a neutral resin (R) and immobilized by the suction pipette (S). Microelectrodes (V_1 and V_2) and reference pipette (connected to ground) are also shown.

communication. Immediately after the inward or outward current injection no changes of junctional conductance were detected.

In another group of experiments designed to test junctional permeability during current flow, both electrodes were connected to the bridge-like arrangement. With this set up while current pulses of 80 ms were injected into Cell 1, synchronous pulses of 20 ms were superimposed through the electrode impaling Cell 2.

Fig. 2 shows the result of one of those experiments where junctional conductance was probed during a hyperpolarizing current pulse. Note that the coupling coefficient of approximately 0.3 applies both to the long and short pulses.

In similar experiments using depolarizing pulses no changes of the transjunctional conductance were detected. These findings support the view that the inter-lymphocyte junction behaves like an ohmic conductor.

When current is used to drive calcium ions into the cytoplasm a striking decrease of the junctional permeability is observed after a latent period of 2 to 4 min.

Fig. 3 shows a progressive increase in V_1, a simultaneous decrease in V_3 and consequently a gradual reduction of the coupling coefficient to zero, indicating sealing of the communication. The effect is not due to Tris that is injected together with the Ca^{2+} because in other experiments with pipettes containing 0.2 M of $CaCl_2$ and 0.2 M of KCl the same results were reproduced.

Both the constant junctional conductance during and after current flow

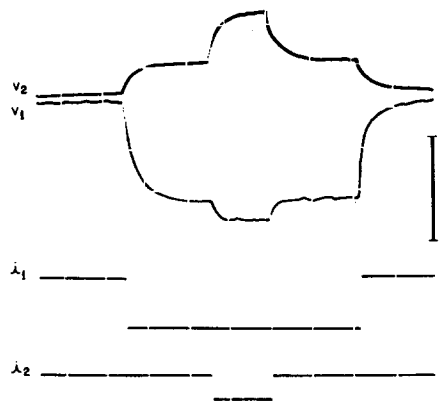

Fig. 2. Persistence of coupling ratio during inward current flow through the junction. In this experiment the current pulse i_1 (80 ms) was injected into Cell 1 causing the voltage drops V_1 and V_2 (recorded with an inverted scale in Cell 2). During the application of the pulse i_1 another probing current (i_2 of 20 ms) was injected into cell 2; this flows across the junction in the opposite direction. For both pulses the coupling ratio is about 0.3. Calibration bar 20 mV and $5 \cdot 10^{-9}$ A.

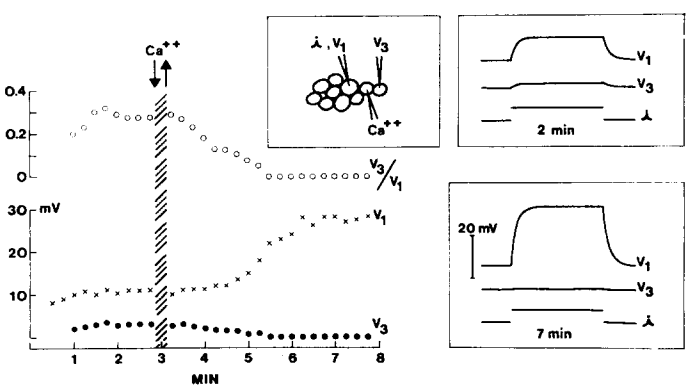

Fig. 3. Effect of calcium injection on junctional conductance. Outward pulses of 50 ms, $2.5 \cdot 10^{-8}$ A at a rate of 10 pulses/s during 10 s were applied with Ca^{2+}-filled electrode in Cell 2. Note progressive uncoupling with simultaneous increase of V_1 and decrease of V_3. Insets: Samples of oscilloscope records (retouched) of the voltage drops (V_1, V_3) and current (i) before (2 min) and after (7 min) calcium injection. Current pulse duration 50 ms and amplitude 10^{-8} A. Schematic drawing of the cell clump with position of the electrodes (see text for details).

(12 cases) and the effect of calcium application (6 cases) are seen consistently in different preparations.

Current flow through the junction has a variety of effects in different structures:

(a) In *Chironomus* salivary glands depolarizing (outward) pulses depress the junctional conductance [8], whereas the application of hyperpolarizing current into previously uncoupled cells produces junctional recoupling [9]; (b) the retinula cells in the lateral eye of the horseshoe crab uncouple when either depolarizing or hyperpolarizing current is passed [10]; (c) in the touch cells of the leech, Baylor and Nicholls [11] have shown that if either of the coupled cells is depolarized no current crosses

the junctional membrane. This is not a permanent effect and coupling returns as the membrane potential drops. These results illustrate the differences of behaviour in the transjunctional conductance when current flows through the junctions. No single explanation seems possible for the different situations described above. The fact that current flow itself has no effect in the lymphocyte junction suggests that in this respect the preparation is hardly comparable with those previously analyzed. The findings described here are in perfect agreement with the previously stated linear behaviour of the current–voltage relationship in the present lymphocyte junctions (see Fig. 2 of ref. 4).

The role of calcium as a membrane "stabilizer" in excitable tissues is well known. There is also strong evidence that free calcium in the bathing medium is a basic requirement for cell aggregation and for the establishment of communicating junctions in other cells [12] as well as for lymphocyte transformation [13].

In insect salivary gland cells the intracellular calcium concentration is probably one of the main factors controlling junctional permeability. The most direct evidence of the calcium ion involvement in junctional uncoupling is its release from some intracellular storage site during the cyanide-induced uncoupling, as detected with the specific calcium indicator aequorin [3, 14]. In these experiments Loewenstein reports a transient rise in aequorin's light output after inhibition of the energy metabolism on which Ca^{2+} removal from the cytoplasm depends [15, 16]. Previous experiments with the squid giant axon [17] suggested that the calcium release is sustained and not transient under these conditions. It is not advisable to attempt to explain the difference, especially after the recent demonstration by Endo and Blinks [18] of a dissociation between aequorin luminescence and calcium release in skinned muscle fibres.

A higher Ca^{2+} uptake by stimulated lymphocytes has been described [19]. However, this observation has not been confirmed in work with rabbit lymphnode lymphocytes in our laboratory (Salek, M., de Meis, L., Oliveira-Castro, G. M. and Barcinski, M. A., unpublished). If calcium uptake is really increased in phytohemagglutinin-stimulated cells the ions must remain tightly bound, otherwise junctional conductance would be impaired. Work in progress in our laboratory has revealed that the only kind of specialized junctions found in transformed lymphocytes are gap junctions (Gaziri, I. F., Oliveira-Castro, G. M., Machado, R. D. and Barcinski, M.A., unpublished). The possibility exists that in these contact regions an increase in the free calcium triggers conformational and/or structural changes that seal off the junctions after a lag-time of a few minutes. We have no explanation for the fact that the time course of the calcium effect is about the same in the very large salivary gland cells of insects [1] and in the small lymphocytes where one would expect a faster effect.

It has been previously proposed [8] that in the uncoupling produced by outward pulses in salivary gland cells a current-induced release of Ca^{2+} ions would be the mediator of the process. Again, the next step would be the uptake of the divalent cation by the membrane itself (including its junctional portions), resulting in the decrease of transjunctional conductance. In this respect the most interesting result reported here is the failure of current to induce uncoupling. One may think that in stimulated lymphocytes either current flow does not release calcium or there are very limited storage sites for this ion.

Since in epithelial cells calcium-dependent intercellular communication allows the direct flow of large molecules [20], these same mechanism operating in stimulated lymphocytes could act as a pathway for transfer of specific immunologic information or some other form of metabolic cooperation.

Considering that some specific immune reactions require cellular interactions [21], the demonstration that free-floating peripheral blood lymphocytes are capable of establishing epithelial cell-like communicating systems allows us to suggest this mechanism as the one involved in situations of specific stimulation.

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