An electrophysiological study of calcium entry during normal human T-lymphocyte activation

Riccardo Ricci[a], Mario Buffelli[b], Anna P. Riviera[c], Alberto Cangiano[b,*]

[a]*Istituto di Anatomia Patologica, Universita' Cattolica, Largo F. Vito 1, 00168 Roma, Italy*
[b]*Dipartimento di Scienze Neurologiche e della Visione, Sezione di Fisiologia Umana, Universita' di Verona, Strada le Grazie 8, 37134 Verona, Italy*
[c]*Istituto di Immunologia e Malattie Infettive, Ospedale BorgoRoma, 37134 Verona, Italy*

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Abstract Our aim was to observe whether normal human T-cells respond to mitogenic stimulation with large whole-cell inward currents (composed of identifiable single-channel contributions) when $[Ca^{2+}]_i$ is not markedly lowered but instead kept normal or moderately low, as has been reported in human leukaemic Jurkat T-cell line and T-cell clones [Kuno et al. (1986) Nature 323, 269–73; Kuno and Gardner (1987) Nature 326, 301–304; Gardner (1990) Annu. Rev. Immunol. 8, 231–252]. Whole-cell patch recordings showed no such currents in cells otherwise normally responding to depolarisation with the macroscopic I_K described in T-lymphocytes and thus deemed viable, in agreement with the notion that Ca^{2+} influx in normal T-cells enterily depends on depletion of internal stores [Putney (1986) Cell Calcium 7, 1–12; Putney (1990) Cell Calcium 11, 611–624].

Key words: T-lymphocyte; Calcium influx; Patch-clamp technique; Signal transduction; Immune system; Whole-cell recording

1. Introduction

Calcium ions participate in many basic aspects of cell signalling in both electrically excitable and non-excitable cells. In the latter group, T-lymphocyte proliferation in response to antigen is a process of fundamental biological value, forming the basis of the immune defense mechanism. Ligand binding to surface receptors leads to intracellular production of inositol 1,4,5-triphosphate (IP_3) which in turn releases calcium from internal stores, thus raising its cytosolic concentration ($[Ca^{2+}]_i$) transiently [6–8]. Continuous influx from the cell exterior is, however, necessary for maintaining the plateau of such a response, a prerequisite for the final mitogenic effect [7,9,10].

Demonstration of the route and mechanism of calcium entry proved difficult in leukocytes in general. In human T-cell clones and the leukaemic Jurkat T-cell line, discrete ion channel and macroscopic whole-cell inward currents have been recorded by one group of investigators in response to mitogenic lectins or IP_3 [1–3], but ion exchange mechanisms by Ca^{2+} transporters have also been claimed [11,12]. On the other hand, a model of Ca^{2+} influx which is dependent on previous emptying of intracellular calcium stores has long been suggested [4,5]. In agreement with this model, it has recently been found that intracellular calcium depletion by markedly lowering $[Ca^{2+}]_i$ with excess doses of the Ca^{2+} chelator EGTA [13], or by emptying the endoplasmic reticulum stores with IP_3, calcium ionophores ([13]; mastocytes) or thapsygargin ([14]; Jurkat T-cells) leads to the opening of a membrane low calcium conductance [15–18] in which discrete ion channel contributions cannot be resolved.

Our study was directed to examine further these points using normal, freshly isolated peripheral blood human T-lymphocytes. Our preliminary question is whether activation of such normal cells with mitogenic doses of lectins or anti-CD3 antibodies is followed by discrete single-channel or macroscopic Ca^{2+} currents, when $[Ca^{2+}]_i$ is not markedly lowered but instead kept normal or moderately low as was the case in the study reported above [1,2]. We used the whole-cell variation of the patch-clamp technique which is the configuration of choice for detecting either macroscopic currents or single-channel fluctuations when using very small, high impedance and thus low noise cells such as T-lymphocytes.

2. Materials and methods

2.1. Preparation of cells

Peripheral blood mononuclear cells were isolated on a Ficoll-Hypaque density gradient and T-cells further enriched by macrophage and B lymphocyte subtraction through a Sephadex G-10 (Pharmacia) and nylon-wool columns [19]. $CD2^+$ cells routinely exceeded 90% (flow cytometric analysis). Further T-cell enrichment through E-rosetting methods (see [17]) was not performed, to avoid interfering with CD2 antigen which could lead to T-cell activation [20] prior to mitogen treatment. Patch recording was performed on cells with apparent diameter $< \sim 8$ μm, to minimise the probability of studying macrophages and large granular lymphocytes. About 100 μl of cell suspension (RPMI-1640 with 2 mM L-glutamine and 1% FCS, 3–5 cells $\times 10^6$/ml) were added to recording chambers containing 200 μl of medium whose bottom was a glass coverslip coated with poly-L-lysine (Sigma). Chambers were kept at 4°C for up to a few hours until patch recording.

2.2. Electrophysiological recordings

Chambers were placed on the stage of a Zeiss Axiovert 35M inverted microscope and washed out of medium using an extracellular type of fluid with the following composition (mM): NaCl, 142; KCl, 5; $CaCl_2$, 10; $MgCl_2$, 1; HEPES Na^+, 10; pH $\sim$ 7.3 (a small proportion of cells were instead studied using a 110 mM $BaCl_2$ and 10 mM HEPES K^+ bathing fluid). Electrophysiological recordings were taken at room temperature (21–24°C). Cells adhering to the glass coverslip were patch-clamped through a List EPC-7 amplifier and 3–6 MΩ sylgard coated pipettes, using a conventional whole-cell configuration [21]. The current output was low-pass filtered at 10 or 3 kHz (8-pole Bessel filter), monitored on a Tektronix 2211 storage CRO and recorded on digital tape. Pipettes were filled with 140 mM K Asp, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES K^+, 5 mM EGTA K^+: $[Ca^{2+}]$ was calculated to be $\sim 5 \times 10^{-8}$ M, using an apparent dissociation constant of 10^{-7} (estimated from [22]). The holding potential of cells was −80 mV throughout. Phytohaemagglutinin (PHA) and an anti-CD3 antibody (OKT3, OrthoPharmaceutical) were used at final concentrations of 6 and 12.5 μg/ml, respectively, after having been tested for their full mitogenic effect using the [^{3}H]thymidine incorporation procedure.

*Corresponding author. Fax: (39) (45) 580881.
E-mail: cangia@borgoroma.univr.it

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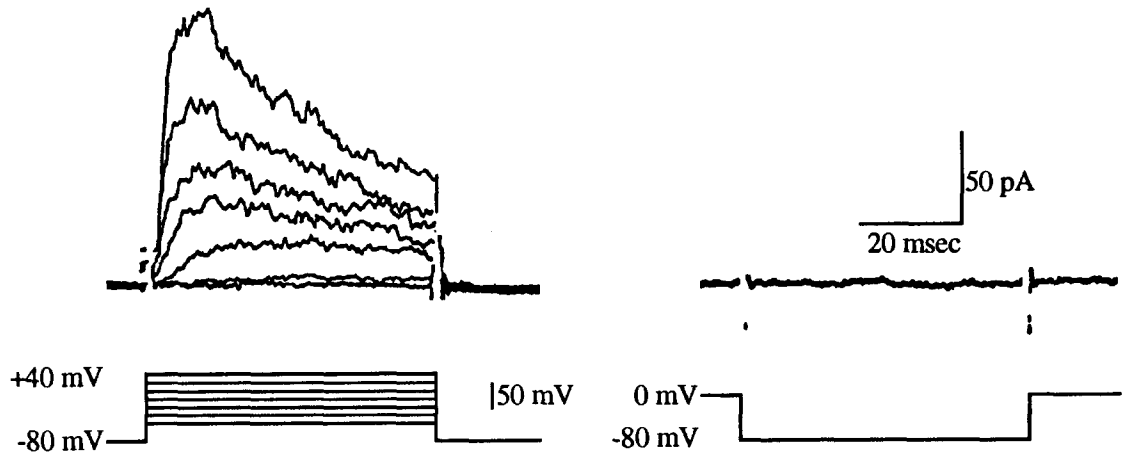

Fig. 1. Typical inactivating outward currents evoked in the human peripheral blood T-cells included in the present study, by depolarising voltage steps from −80 mV holding potential (left) and recorded with the patch-clamp technique from one such cell in whole-cell configuration (superimposed CRO traces). (Right) A hyperpolarising step evokes no current. Presence and substantial stability of the current, known to be due to the activation of a K^+ conductance [23–25], provided in all cells of this study (but four, bathed in $BaCl_2$ and thus with blocked K^+ conductances) evidence of viable conditions throughout the recording period.

3. Results and discussion

To ascertain whether cells remained in viable condition throughout the entire recording period (up to ~10 min), after rupturing the patch membrane and establishing the whole-cell mode, a series of depolarising steps was applied in order to observe the well characterised macroscopic voltage-dependent outward current of T-cells. This consists of an inactivating current, usually a few hundred pA in peak amplitude, due to the opening of a selective K^+ conductance [23–26]. All cells included in our study presented this current with typical characteristics, including an activation threshold close to −45 mV, a highly variable (from cell to cell) maximal conductance ranging between 0.5 and 4.5 nS similar to published values (see, for example, [25]) and a more or less pronounced inactivation during prolonged depolarisation (example in Fig. 1). Furthermore, the current underwent only marginal changes throughout the recording period, both before and after PHA application. Thus, applying infrequently (~1/min or less) a command voltage step to 0 mV and of short duration, to avoid excessive depolarisation and resultant cumulative inactivation, we obtained in the cells shown in Fig. 2 a peak current of 141.57 ± 20.63 pA (mean ± S.E.M.) and 150.0 ± 18.7, before and during PHA application, respectively.

In order to stimulate the T-cell receptor complex and observe the possible activation of an inward current attributable to Ca^{2+} influx through the surface membrane, mitogenic doses of PHA or, in fewer instances, OKT3 were applied to the bath. In no instance was a sustained inward current observed for several minutes (up to 10 min in many cells) following the stimulus onset. To provide statistical evidence of this negativity, computer averages were obtained of the current traces of 20 cells, recorded over 5 min following PHA or OKT3 bath application (19 and 1 cell, respectively, isolated from peripheral blood of several normal subjects). The result is shown in Fig. 2.

The current traces summated in Fig. 2 were also examined individually at CRO gains and sweep speeds adequate for single-channel current resolution. Our recordings did not show consistent discrete current fluctuations in response to the mitogenic lectin. Only occasionally was an inward single-channel current activity observed in some of the cells, but it was difficult to quantify due to its rare occurrence. An example is shown in Fig. 3. The lack of blockade by cadmium, reversal potential near 0 mV when tried, raised doubts as to the calcium selectivity, if any, of this activity. Even if conducting calcium, these sporadic single-channel openings would hardly be relevant for the problem under study, being recorded from the entire cell (see also [16]).

In conclusion we observed a lack of response to mitogens of

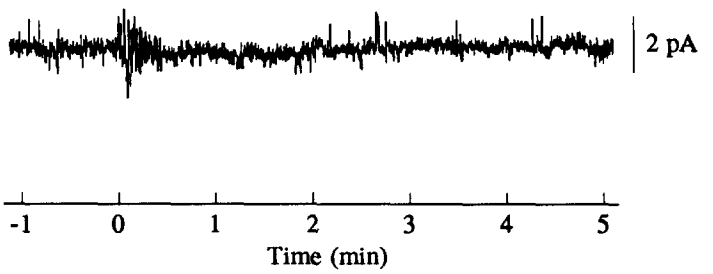

Fig. 2. Computer averages of total membrane current traces recorded in whole-cell mode from 20 peripheral blood human T-lymphocytes, during a period of several minutes following mitogenic stimulation starting at time zero. Current traces are acquired through storage CRO and summated on MacIntosh Quadra 950 at 125 ms sampling interval, using Microsoft Excel software. In 4 cells, after establishing the whole-cell condition and observing the depolarisation-induced transient outward current (Fig. 1), the regular bathing fluid containing NaCl and $CaCl_2$ was switched to one in which $BaCl_2$ was the main salt (see Section 2), to increase detectability of calcium channel currents [3].

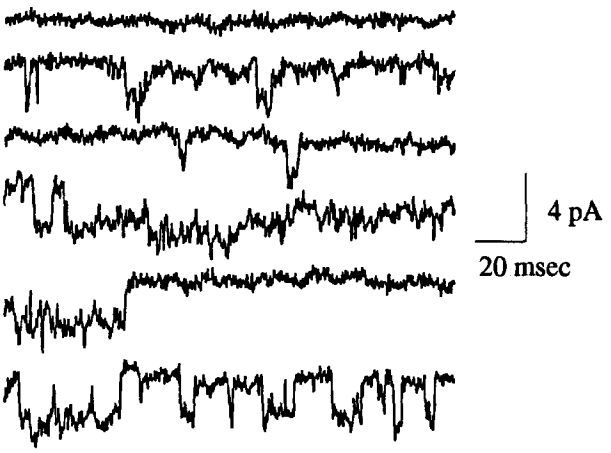

Fig. 3. Single-channel inward current recorded in whole-cell mode after PHA stimulation, from one of the cells whose current traces are summated in Fig. 2. Consecutive traces are shown relative to a sporadic burst of activity.

normal T-lymphocytes under experimental conditions appropriate for the detection of calcium currents and similar to those used in a previous study [1,2]. The only simple explanation for the positive results obtained in that study, under the form of both single-channel and macroscopic whole-cell currents, is the use of T-cell clones, while in our work normal, freshly isolated peripheral blood lymphocytes were used. On the other hand, a barely detectable smooth inward current has been reported to occur inconstantly in activated mastocytes without deliberate calcium depletion [16,18] and to be increased by previous emptying of intracellular calcium stores (see [23] and Section 1, also for references). Our negative results in activated normal T-cells without calcium depletion are therefore in substantial agreement with these studies and are consistent with the notion that the main mechanism of T-cell Ca^{2+} influx is that dependent on previous depletion of internal calcium stores [4,5].

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