Biochimica et Biophysica Acta 856 (1986) 202–211
Elsevier

BBA 73031

The relationship between mitogen-induced membrane potential changes and intracellular free calcium in human T-lymphocytes

Peter E.R. Tatham [a], Kieran O'Flynn [b] and David C. Linch [c]

[a] *Department of Medical Physics and* [c] *Department of Haematology, The Middlesex Hospital Medical School, London W1P 6DB and* [b] *Department of Haematology, University College School of Medicine, Chenies Mews, London WC1E 6HX (U.K.)*

(Received November 26th, 1985)

Key words: Membrane potential; Mitogen; intracellular Ca^{2+}; Lymphocyte activation

We have investigated the effects of mitogenic lectins on human T-lymphocytes, isolated from peripheral blood, and cells from the T-cell clone, HPB-ALL, using the fluorescent dyes, bis-thiobarbiturate trimethineoxonol (bisoxonol) and quin2 to sense changes in membrane potential and intracellular free $[Ca^{2+}]$, respectively. The resting potential of both cell types is close to the K^+ equilibrium potential. Changes from the resting level occur when mitogenic concentrations of either concanavalin A or phytohaemagglutinin are added. T-lymphocytes undergo a decrease in emission, maximal at 1 to 2 min, corresponding to a small membrane hyperpolarization. This is followed by a depolarization to approximately the resting level. HPB-ALL cells, on the other hand, respond to the mitogens by a sustained increase in fluorescence, denoting a depolarization, that is maximal at 4 to 5 min and 7 to 9 min, respectively. The Ca^{2+}-dependence of these phenomena indicates that the membrane potential response, in both cell types, is the resultant of two opposing effects: a Ca^{2+}-sensitive ion movement tending to hyperpolarize the cells and a Ca^{2+}-insensitive effect that generates a depolarization. Our results suggest that Ca^{2+}-activated K^+ channels are responsible for the first effect and that an inward Na^+ movement accounts for the depolarization signal in T-lymphocytes. In HPB-ALL cells only part of the depolarization is Na^+-dependent. Although the effects elicited by phytohaemagglutinin occur more slowly than those produced by concanavalin A, similar membrane potential and $[Ca^{2+}]_i$ changes occur.

Introduction

Although the role of Ca^{2+} as a mediator of lymphocyte stimulation is now well established (see review by Lichtman et al. [1]), the significance and origin of the early changes in membrane potential that occur when cells are activated, are less well understood. To a certain extent this has been due to confusion in the literature about the nature of the membrane potential changes that occur. A membrane depolarization has been reported in human and porcine lymphocytes [2–5] and a membrane hyperpolarization in murine lymphocytes [6,7]. The latter may be explained by the existence of Ca^{2+}-activated K^+ channels [5,6,7]. Mitogens induce a rapid increase in $[Ca^{2+}]_i$ [6,8,9] and this may be sufficient to cause these K^+ channels to open in mouse lymphocytes, tending to hyperpolarize the cells. Under conditions where the activation of this channel is prevented (for instance in media containing low levels of Ca^{2+} or in the presence of an inhibiting drug), a cell depolarization is detected [5,7].

Human lymphocytes, on the other hand, have

Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; Hepes, 4-2(hydroxyethyl)-1-piperazineethanesulphonic acid.

been reported to exhibit only a cell depolarization in response to mitogenic ligands [2,3] despite the fact that there is also a substantial rise in $[Ca^{2+}]_i$ [9]. Furthermore, if human T-cells are subjected to a depolarization by treatment with ionophore or by ion substitution in the medium, both the mitogen-induced rise in $[Ca^{2+}]_i$ and DNA synthesis are inhibited [10]. In order to investigate more fully the effect of mitogens on the membrane potential of human lymphocytes and to clarify its relation ship with $[Ca^{2+}]_i$, we have monitored both these parameters in T-cells isolated from peripheral blood (E^+ cells) and in a human T-cell line, HPB-ALL. We have selected two different cell types for this study because they provide complementary data. E^+ cells are a polyclonal population of resting T-cells while the HPB-ALL line is a clone derived from an individual with a T-cell leukemia [11]. This clone proliferates in culture in the absence of IL-2 and treatment of the cells with mitogenic lectins and antibodies evokes a large increase in $[Ca^{2+}]_i$ [12]. We have used the fluorescent Ca^{2+}-indicator, quin2 [13], to monitor $[Ca^{2+}]_i$ and the fluorescent oxonol dye, bis-thiobarbiturate trimethineoxonol (bisoxonol), to sense changes in cell membrane potential [14]. Although carbocyanine dyes have been widely used to monitor potential, their positive charge causes them to accumulate within cells and to concentrate in mitochondria, affecting respiration and ATP production [15]. They also affect lymphocyte membrane potential [14] and can block Ca^{2+}-dependent K^+-channels [16]. Consequently, we have used the anionic oxonol dye as its concentration within cells is considerably less than that outside and its concentration within mitochondria is even lower. Thus the chance of toxic side-effects or other artefacts occurring is very much reduced.

Our data show that the membrane potential response of human T-cells is a composite phenomenon that is the resultant of two or more ionic events. These include an opening of Ca^{2+}-dependent K^+-channels and an inward movement of cations.

Materials and Methods

Cell preparation. Human T-cells were prepared as described previously [9]. Briefly, mononuclear cells from peripheral blood taken from healthy volunteers were separated by buoyant density sedimentation [17]. Monocytes were removed by overnight adherence to plastic and T-cells were separated by rosetting with sheep erythrocytes followed by Ficoll-Hypaque centrifugation to separate the rosetted and unrosetted cells. The pelleted rosettes containing the T-cells (E^+) were recovered and treated with NH_4Cl lysing reagent (Ortho Diagnostics). The remaining cells were washed and resuspended in RPMI 1640H medium (Gibco), supplemented with 1% (w/v) bovine serum albumin (RIA grade, Sigma), and then either loaded with quin2 as described below or preincubated for 1 h at 37°C prior to bisoxonol staining.

HPB-ALL cells were grown in RPMI 1640H supplemented with 5% (v/v) foetal calf serum in a fully humidified atmosphere of 5% CO_2. Cells were washed three times prior to use.

Media and materials. Fluorescence measurements were carried out in a buffered salt solution containing 145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 0.5 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM glucose and 10 mM Hepes titrated to pH 7.4 at 37°C with NaOH. In certain experiments when a low extracellular $[Ca^{2+}]$ was required, $CaCl_2$ was omitted from this medium and 1 mM EGTA added. When low extracellular $[Na^+]$ was required, NaCl was replaced by sucrose (248 mM) and the Hepes buffer was neutralized with Tris-HCl. The $[Na^+]$ of this medium was 2 mM.

Bisoxonol was obtained from Molecular Probes, quin2 from Amersham International, gramicidin D and quinine hydrochloride from Sigma, concanavalin A from Miles-Yeda and phytohaemagglutinin from Wellcome (PHA-P).

Quin2-loading and fluorescence measurement. Cells were loaded with quin2 by incubation with the acetoxymethyl ester as described previously [9]. The ester concentration in the RPMI/albumin medium was 15 μM. Between $1 \cdot 10^7$ to $1 \cdot 10^8$ cells were loaded at a time. The washed, labelled cells were kept at room temperature until used. Aliquots ($1 \cdot 10^6$ to $2 \cdot 10^6$ cells) were then centrifuged for 10 min at room temperature ($200 \times g$) and resuspended in 1 ml of a buffered salt solution (see below). Fluorescence was measured in an adapted Locarte fluorimeter with excitation at 339 nm and

emission at 500 nm [18]. The samples, in quartz tubes, were maintained at 37°C and stirred continuously during measurements. Calibration of quin2 signals was achieved by treating the cells with 0.05% (v/v) Triton X-100 (Sigma) to saturate the dye and then by quenching the fluorescence with 0.5 mM $MnCl_2$. The percentage Ca^{2+}-saturation of the intracellular dye and $[Ca^{2+}]_i$ were calculated as described previously [9].

Measurement of bisoxonol fluorescence. Bisoxonol fluorescence was measured with an Aminco-Bowman spectrofluorimeter (SPF). The sample compartment was maintained at 37°C and the excitation and emission wavelengths were set to 540 nm and 580 nm, respectively, with 2 mm slits. Immediately prior to measurement, aliquots of cell suspension were spun down and resuspended in the buffered salt solution described below. The cuvettes were quartz tubes and the samples were stirred continuously. The dye, which was dissolved in dimethyl sulphoxide, was added to the sample to a final concentration of 125 nM. The final dimethyl sulphoxide concentration was 0.25% v/v.

Results

Bisoxonol and membrane potential

When bisoxonol is added to suspensions of either E^+ lymphocytes or, as shown in Fig. 1a, HPB-ALL cells, there is an immediate increase in emission and a steady level of fluorescence is achieved after a few minutes. The negatively charged dye molecules distribute across the cell membranes in a potential-dependent fashion [14] and since the greater part of the signal from oxonol dyes comes from molecules that are bound to cytoplasmic protein [19], the suspension fluorescence varies with membrane potential, increasing as cells depolarize and decreasing as they hyperpolarize. This is confirmed by adding the ionophore gramicidin D, which forms pores that increase membrane permeability to both Na^+ and K^+. For cells with potentials already close to E_K, such as lymphocytes, this results in a depolarization [14]. Fig. 1a demonstrates the depolarizing effect of gramicidin D (37 ng/ml, approx. 20 nM) on HPB-ALL cells, producing an immediate increase in signal that peaks in 2 to 3 min and then decays slowly. In low Na^+ medium, on the other hand, the Na^+ concentration gradient is reversed and the ionophore brings about a hyperpolarization, reducing cell fluorescence (Fig. 1b and c). This is achieved within 30 s, considerably faster than the depolarization in normal medium. The intensity subsequently remains stable for several minutes (not shown). It is possible to reverse this drop in fluorescence by subsequent addition of either NaCl or KCl (Fig. 1b and c). Similar data were obtained from E^+ cells.

When KCl is added to E^+ cells in normal medium without ionophore, a rapid depolarization takes place, confirming that the resting membrane potential is close to E_K (which is approximately -85 mV). An example is shown in Fig. 2a, where it can be seen that successively increasing $[K^+]_o$ causes successive depolarizations. Under the same conditions increasing $[Na^+]_o$ by 35 mM has no effect. Similar data were obtained from HPB-ALL cells.

As a further check on the validity of using bisoxonol as a membrane potential probe, we have investigated the dependence of the fluorescence signal upon intracellular pH by adding either 5 mM NH_4Cl or 10 mM sodium acetate to stained cells. These compounds, which bring about an almost instantaneous increase and decrease, respectively, in intracellular pH [20] have only slight effects on the emission (Fig. 2b and c).

Although changes in suspension fluorescence reflect changes in cell membrane potential, there is a difficulty in calibrating the dye response. A simple method that has been used with carbocyanine dyes, involves hyperpolarizing cells by treating them with the K^+-ionophore valinomycin and then adding KCl to the suspension until the emission is restored to its original level [14]. At this null point, $E_m = E_K$ which can be calculated from the Nernst equation. We cannot use this method because valinomycin interacts with bisoxonol, preventing its fluorescence [14]. Nor can be use the null point data of Fig. 1b and 1c, because gramicidin has increased cell permeability to both Na^+ and K^+. However, an approximate calibration of changes in cell emission can be achieved if the relationship between fluorescence intensity and membrane potential is sufficiently linear. When cells are treated with gramicidin D

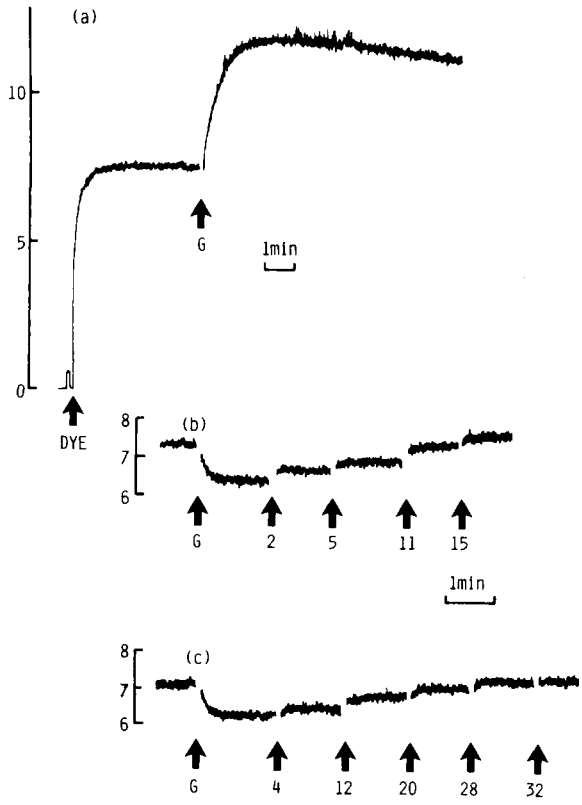

Fig. 1. Effect of gramicidin D on bisoxonol fluorescence. Emission was recorded from HPB-ALL cells ($2 \cdot 10^6$/ml) in 1 ml of buffered salt solution (see Materials and Methods). The vertical axes in this and in subsequent figures represent fluorescence intensity in arbitrary units and the gaps in the traces indicate where the shutter was closed during each addition. Panel a shows the effect of adding dye to the cuvette (125 nM). The shutter was opened and closed briefly beforehand to indicate the light scatter contribution. Gramicidin D was added to a final concentration of 37 ng/ml (G). Panels b and c show the effect of gramicidin on cells in low-Na^+ medium. KCl (b) or NaCl (c) were then added to the final concentrations indicated (mM).

the increased permeability permits K^+ and Na^+ to exchange rapidly down their concentration gradients, depleting cell K^+ and increasing cell Na^+. However, in low Na^+ medium this rapid exchange is prevented, enabling us to investigate the relationship between $[K^+]_o$, membrane potential and fluorescence. Under these conditions a modified form of the Goldmann equation may be used to calculate membrane potential as a function of $[K^+]_o$:

$$E_m = 61.5 \log_{10} \frac{[Na^+]_o + (P_K/P_{Na}) \cdot [K^+]_o}{[Na^+]_i + (P_K/P_{Na}) \cdot [K^+]_i}$$

Fig. 3a is a plot of membrane potential, calculated from this equation, against external $[K^+]$ for various values of P_K/P_{Na} and with $[Na^+]_i = 18$ mM and $[K^+]_i = 153$ mM [21]. The relationship approaches linearity and is quite insensitive to the permeability ratio. Fig. 3b shows the results of experiments in which KCl was added to gramicidin-treated E^+ and HPB-ALL cells in low Na^+ medium. The relationship between fluorescence intensity and membrane potential may then be deduced from the above equation and is shown in Fig. 3c. The data are reasonably linear enabling us to obtain calibration factors from the slopes. Using

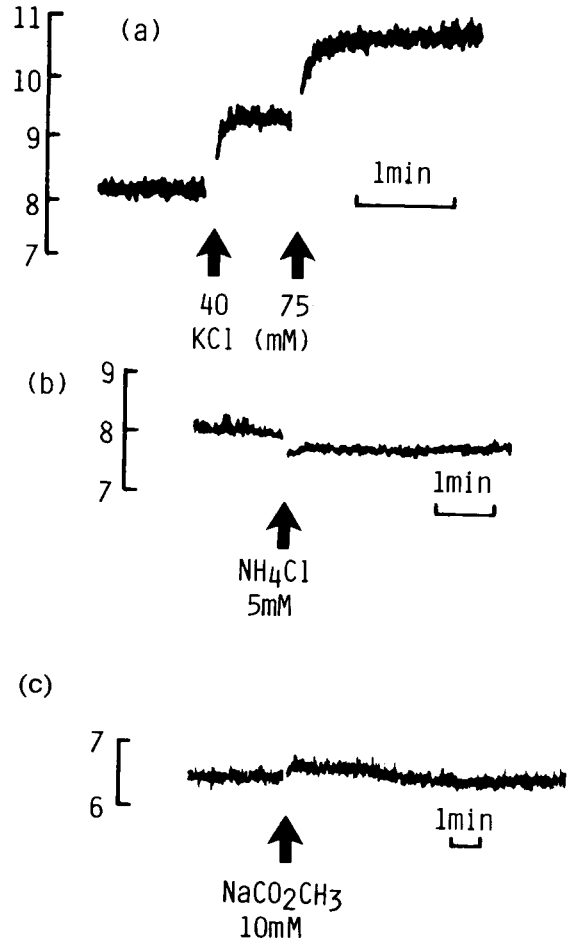

Fig. 2. Effect of extracellular K^+ and intracellular pH on bisoxonol fluorescence. Panel a shows the effect on E^+ cells ($5 \cdot 10^6$/ml) of increasing $[K^+]_o$ by adding KCl as indicated. Panels b and c show the effect of adding NH_4Cl (5 mM) and sodium acetate (10 mM), respectively, to HPB-ALL cells.

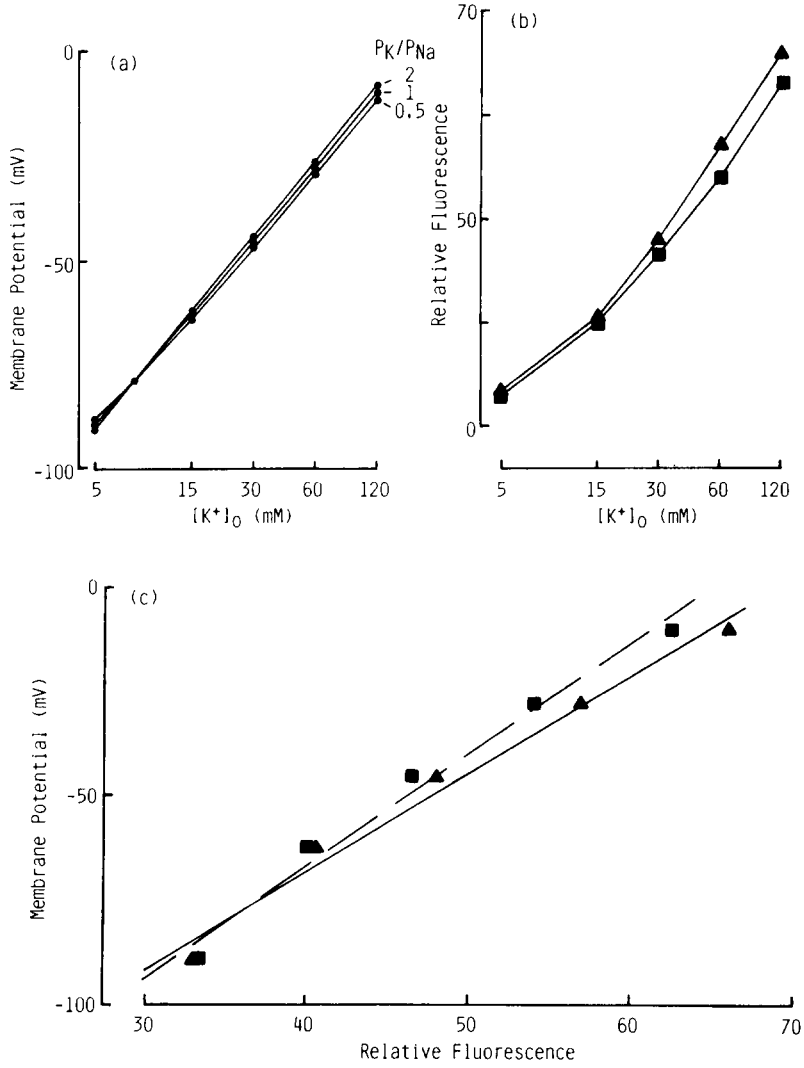

Fig. 3. Calibration of fluorescence signals. Panel a is a plot of cell membrane potential against external $[K^+]$ for human lymphocytes suspended in a low-Na^+ medium and made permeable to both Na^+ and K^+. Each curve was calculated from the equation given in the text, for different values of P_K/P_{Na} as indicated. Panel b shows experimental data from gramicidin-treated E^+ (■) and HPB-ALL (▲) cells in low-Na^+ medium. Panel c shows the relationship between membrane potential and cell fluorescence obtained from the data in (a) and (b). The straight lines are regression lines (■— — —■, E^+ cells; ▲———▲, HPB-ALL cells).

these factors and allowing for the deviations from linearity, we estimate that, in normal medium, gramicidin D depolarizes E^+ cells by 20 to 30 mV and HPB-ALL cells by 60 to 90 mV. However, it should be noted that in calibrating the signals from HPB-ALL cells, we have assumed that $[Na^+]_i$ and $[K^+]_i$ are the same as in resting lymphocytes.

Effect of mitogenic lectins

The effect of mitogenic lectins on bisoxonol fluorescence in E^+ lymphocytes is illustrated in Fig. 4. When a mitogenic concentration of concanavalin A is added to a suspension of E^+ cells there is a decrease in fluorescence to a minimum value that is reached after 1 to 2 min. This is followed by an increase in intensity to approximately the resting level or just above it. This pattern has been observed in five experiments (five different cell preparations). A typical trace is shown in Fig. 4a. Using the calibration method described above, we estimate that these changes are equivalent to an initial hyperpolarization of 5.4 ± 1.1 mV (S.E.) with a subsequent depolarization of similar size. This assumes that all the cells in the preparation are responding identically.

To determine the Ca^{2+}-dependence of these signals, experiments were conducted in low-Ca^{2+} medium. A typical trace from one of four experi-

ments is shown in Fig. 4b where it can be seen that reduction of $[Ca^{2+}]_o$ to the nM range by EGTA abolished the early hyperpolarization, revealing a depolarization signal of between 5 and 10 mV that is maximal at 4 to 5 min and steady thereafter. Phytohaemagglutinin acts on E^+ cells in a similar fashion to concanavalin A (initial hyperpolarization of 3.7 ± 0.7 mV (S.E., $n = 3$)), but the changes are slower (Fig. 4c and d). Thus in normal medium, both mitogens bring about a small, transient hyperpolarization that is Ca^{2+}-dependent, suggesting that the increase in $[Ca^{2+}]_i$ is causing plasma membrane K^+ channels to open. In low-Ca^{2+} medium this does not occur. Instead a depolarization takes place that is maximal at 4 to 5 min in concanavalin A-treated cells or 7 to 9 min in phytohaemagglutinin-treated cells.

In HPB-ALL cells, in normal medium, there is no apparent hyperpolarization phase in the response to concanavalin A, merely a depolarization of 10 to 20 mV reaching a steady level as shown in Fig. 5a (a representative example from 10 experiments). The effect of removing Ca^{2+} from the medium (Fig. 5b) is to enhance the fluorescence increase induced by the lectin, especially at the early stage of the response (four experiments). This suggests that the event that gives rise to the hyperpolarization in E^+ cells also occurs in HPB-ALL cells, but is concealed by the larger depolarization. Similar effects are produced by phytohaemagglutinin but, once, again, the responses are slower (not shown).

Thus, the membrane potential response of both cell types to the mitogens can be described in terms of two components, (1) a Ca^{2+}-sensitive effect that tends to generate a hyperpolarization and (2) a Ca^{2+}-insensitive depolarization. The Ca^{2+}-sensitivity of the hyperpolarizing effect suggests once again that K^+ channels are opening in response to the mitogen-induced increase in

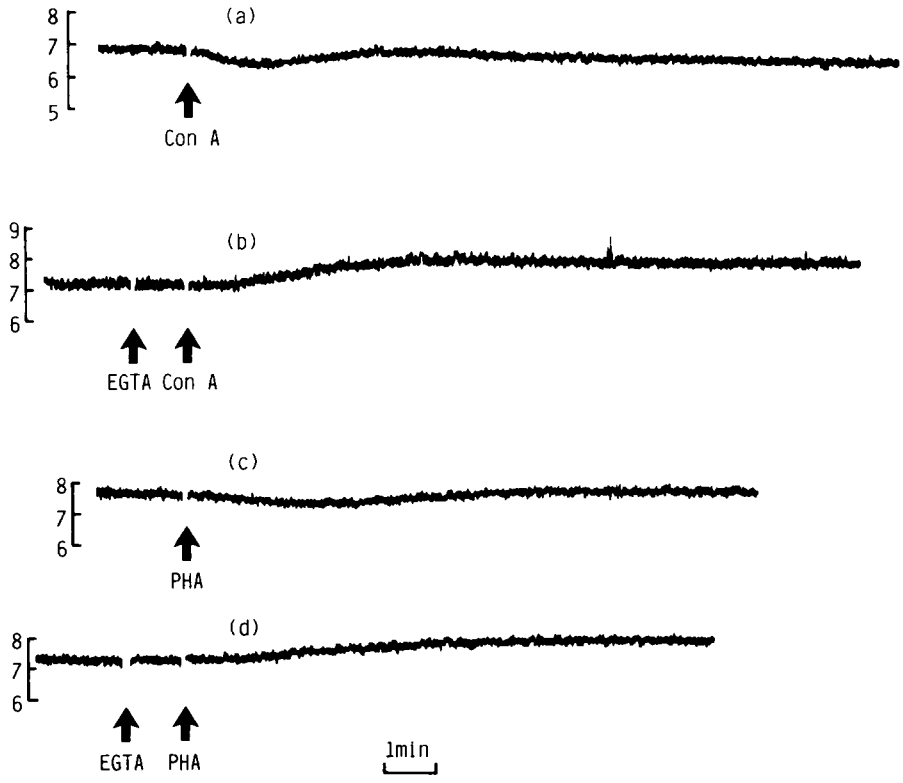

Fig. 4. Effect of concanavalin A and phytohaemagglutinin on E^+ lymphocytes. For panels a and c bisoxonol-stained cells ($5 \cdot 10^6$/ml) were treated with concanavalin A (10 μg/ml) or phytohaemagglutinin (4 μg/ml) in normal medium ($[Ca^{2+}] = 1$ mM). For panels b and d Ca^{2+} was omitted from the medium and EGTA (1 mM) was added prior to the lectins.

$[Ca^{2+}]_i$. This is supported by measurements with quin2, which show that E^+ cells in low-Ca^{2+} medium (1 mM EGTA) have an intracellular $[Ca^{2+}]$ below 30 nM. Treatment with either concanavalin A or phytohaemagglutinin increases this by only 15 to 20%, which is insufficient to activate Ca^{2+}-dependent K^+ channels. However, adding $CaCl_2$ (2 mM) to the medium at this stage causes an immediate, substantial rise in $[Ca^{2+}]_i$ together with a rapid hyperpolarization (data not shown).

We have investigated the effect of quinine, a known inhibitor of Ca^{2+}-activated K^+ channels [22–24], on the lectin-induced potential changes in E^+ cells. When 0.1 mM quinine is added to stained cells there is no change in cell fluorescence and subsequent addition of concanavalin A still induces a hyperpolarization. At a concentration of 1 mM the drug itself depolarizes the cells, suggesting a blockade of K^+ channels [14] and the lectin-induced hyperpolarization is abolished (data not shown).

To show that the membrane potential changes induced by concanavalin A require the binding of the lectin to sites that have a specific affinity for it, we have investigated the effect of the blocking sugar, α-methyl mannoside [25,26]. In the presence of this compound (50 mM), concanavalin A does not depolarize HPB-ALL cells although the same concentration of fucose, a sugar which does not bind to the lectin, does not prevent a depolarization (not shown). α-Methyl mannoside also abolishes the concanavalin A-induced increase in $[Ca^{2+}]_i$ reported by quin2 while fucose does not.

Timing of the changes in $[Ca^{2+}]_i$ and membrane potential

In order to make a direct comparison between the time-courses of membrane potential and $[Ca^{2+}]_i$ after mitogen treatment, the bisoxonol fluorescence of quin2-loaded cells was studied. Using two fluorimeters, simultaneous measurements of the emission from each probe were made during stimulation. (The cells used for the measurement of quin2 fluorescence were not stained with bisoxonol). The effect of the lectins on the quin2 emission from E^+ cells was identical to that previously reported by us [9] with an approximately 2-fold increase in $[Ca^{2+}]_i$ (not shown). Fig. 6 shows the effect of the lectins on HPB-ALL cells.

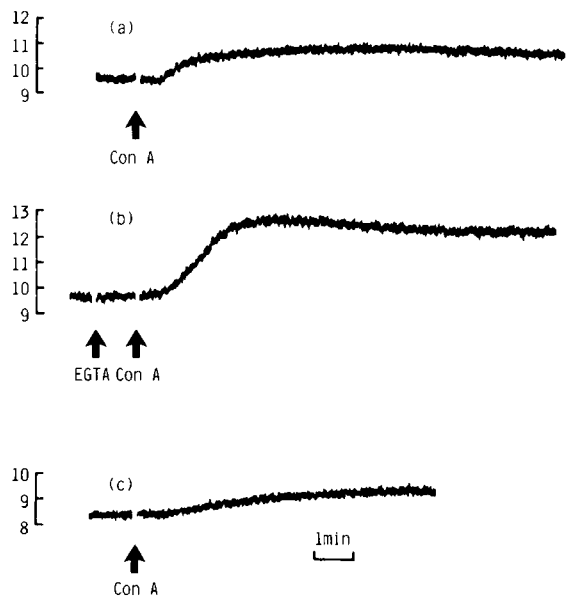

Fig. 5. Effect of concanavalin A on HPB-ALL cells. In each experiment bisoxonol-stained cells ($2 \cdot 10^6$/ml) were treated with concanavalin A (10 μg/ml) as indicated. In panel a normal medium containing 1 mM Ca^{2+} was used. In panel b Ca^{2+} was omitted and EGTA (1 mM) added prior to concanavalin A. In panel c low-Na^+ medium was used ($[Ca^{2+}] = 1$ mM; see Materials and Methods).

The onset of the increase in $[Ca^{2+}]_i$ and the onset of the membrane depolarization occur at the same time (approx. 30 s after addition of lectin). However, the presence of quin2 has an effect on the membrane potential response in that there is an increase in the magnitude of the depolarization signal in the early stages. This effect is very similar to that of removing Ca^{2+} from the external solution (Fig. 5b). In the case of E^+ cells the presence of quin2 abolishes the hyperpolarization signal (not shown).

The Ca^{2+} signals in these experiments also provide further evidence that there is a difference in the timing of the early events in the activation of the cells by phytohaemagglutinin compared with concanavalin A. For both cell types the maximum quin2 signal occurs 2 to 3 min after concanavalin A addition and 5 to 10 min after addition of phytohaemagglutinin. Thereafter, there is a fall in quin2 emission in both cases, but the decay is slower for phytohaemagglutinin-stimulated cells (not shown).

Na^+-dependence of membrane potential changes

Since an increase in plasma membrane Na^+ permeability tends to depolarize cells and since it has been shown that concanavalin A induces such an increase in porcine lymphocytes [27], we have investigated the dependence of the mitogen-induced depolarizations that we observe on external $[Na^+]$. When E^+ cells are treated with the lectins in low-Na^+ medium containing 1 mM Ca^{2+} ($[Na^+]_o = 2$ mM), there is no detectable change in bisoxonol fluorescence (not shown). Since in normal medium larger depolarizations are observed when external Ca^{2+} is removed, E^+ cells were also treated with lectins in low-Na^+ medium without Ca^{2+} (1 mM EGTA). Once again, under these conditions there is no detectable movement from the resting membrane potential. However, when HPB-ALL cells are subjected to the same conditions, a depolarization signal is still observed, although of reduced magnitude. This is illustrated in Fig. 5 where cells from the same preparation have been treated with concanavalin A in normal medium (panel a), low-Ca^{2+} medium (panel b) and low-Na^+ medium)($[Ca^{2+}]_o = 1$ mM, panel c). Note

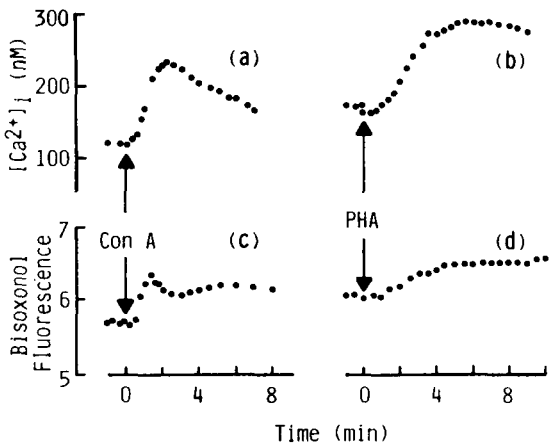

Fig. 6. Effect of mitogens on $[Ca^{2+}]_i$ and membrane potential of HPB-ALL cells. After loading cells with quin2, each suspension was divided into two aliquots so that simultaneous measurements of quin2 and bisoxonol fluorescence could be made during treatment with concanavalin A (Con A) (10 μg/ml, a and c) and phytohaemagglutinin (PHA) (4 μg/ml, b and d). The upper curves (a and b) show the time-courses of $[Ca^{2+}]_i$ and the lower curves (c and d) show the bisoxonol fluorescence. Quin2 loading and calibration are described in Materials and Methods.

that in low-Na^+ medium the cells undergo a normal increase in $[Ca^{2+}]_i$ (not shown) and in medium that is low in both Na^+ and Ca^{2+} (1 mM EGTA), a virtually identical response to that shown in panel c is obtained. It is unlikely that this residual depolarization in HPB-ALL cells is caused by a reduction in K^+ permeability, such as that which accompanies glucose-stimulated insulin secretion by pancreatic β-cells [28,29], since addition of KCl (40 mM), 4 to 5 min after concanavalin A, still causes a marked depolarization both in normal and Ca^{2+}-free medium (data not shown).

Discussion

Our data confirm that bisoxonol senses changes in membrane potential in human T-lymphocytes and HPB-ALL cells. The rapid depolarization caused by increasing the external $[K^+]$ demonstrates the significant permeability to this ion in both cell types and shows that the resting membrane potentials are close to E_K, in accordance with previous animal data [6,14,30]. The relatively show onset of the depolarization induced in normal medium by gramicidin D may be explained by changes in intracellular $[Na^+]$ and $[K^+]$ brought about as these species exchange rapidly across the membrane. The stoichiometry of this process appears to make it electrogenic, to a small extent, slowing the rate of depolarization and generating a subsequent slow repolarization (Fig. 1a). In low-Na^+ medium, on the other hand, the direction of the concentration gradient for both ions is the same, but both species cannot leave the cell without creating an osmotic imbalance or without some other compensating ion movement. Consequently there is no immediate effect on $[Na^+]_i$ or $[K^+]_i$.

The small sensitivity to intracellular pH that we have detected should not interfere significantly with the assessment of membrane potential. The pH changes that accompany mitogenic stimulation of murine lymphocytes are in the alkaline direction and take about 5 min to occur [31]. Similar effects take place in human lymphocytes (our unpublished observations). We would expect such changes to cause a correspondingly slow reduction in bisoxonol emission. Such a component may be present in our traces but cannot by itself explain the effects that we observe.

Our data show that although the membrane potential responses to concanavalin A or phytohaemagglutinin appear to be different for the two cell types, similar underlying events are taking place. In each case the membrane potential change is the resultant effect of two or more ionic events which have different timings. An early, transient effect that occurs is a small membrane hyperpolarization seen in E^+ cells. The timing of this phenomenon is identical to that of the rise in $[Ca^{2+}]_i$. Also it is abolished either when low Ca^{2+} medium is used, so that $[Ca^{2+}]_i$ does not rise above 50 nM, or in the presence of 1 mM quinine. This suggests that it is caused by the opening of Ca^{2+}-activated K^+ channels. A similar electrogenic phenomenon takes place in HPB-ALL cells, but its effect is concealed because of the large depolarization. However, when the external $[Ca^{2+}]$ is very low, the activation of these channels is limited or prevented altogether and only the depolarizing component of the response is observed. Moreover, when cells are loaded with quin2 its intracellular concentration is likely to approach 1 mM [13] and it may be expected to exert a buffering action on Ca^{2+} transients, blunting the mitogen-induced rise in $[Ca^{2+}]_i$. Since the presence of this indicator either prevents the hyperpolarization of E^+ cells or enhances the depolarization of HPB-ALL cells, we conclude that it is preventing $[Ca^{2+}]_i$ from rising to a level that will fully activate Ca^{2+}-dependent K^+ channels. By the same token, it is clear that quin2 underestimates the extent of the Ca^{2+} increase that occurs.

While there is a clear link, in both types of cell, between the Ca^{2+} response and the opening of K^+ channels, the depolarization signals that we observe appear to be unrelated to $[Ca^{2+}]_i$. In the first place, the timing of the potential changes is quite different from that of the Ca^{2+} responses. For instance, the depolarization signal from HPB-ALL cells does not decay significantly between 3 and 15 min. This is in contrast to $[Ca^{2+}]_i$, which peaks at 1 to 2 min and then decays, though it does remain above the resting level for a considerable period thereafter. Secondly although the onset of the depolarization coincides with the Ca^{2+} movement, there is no requirement for extracellular Ca^{2+}.

While the ionic events that give rise to the cell depolarization appear to be independent of Ca^{2+}, there is an effect on this response when the extracellular $[Na^+]$ is reduced. In E^+ cells the depolarization signal is abolished, suggesting that it is caused by an increase in Na^+ permeability, similar to that detected in porcine lymphocytes [27]. In the cell line, however, reversing the $[Na^+]$ gradient reduces, but does not abolish the signal. The remaining depolarization must therefore be caused by a different electrogenic effect. Since HPB-ALL cells depolarize in response to KCl given after concanavalin A stimulation, it is unlikely that a decrease in K^+ permeability accounts for this effect. Other alternatives include a decrease in Na^+/K^+-pump activity or an increase in Cl^- permeability.

Although the membrane potential changes induced by the mitogens in E^+ and HPB-ALL cells appear to be quite different, our data show that similar ionic events are taking place. Ca^{2+}-dependent K^+-channels appear to be activated in both cases and an increase in Na^+ permeability causes a depolarization in both cell types. The principal difference between the responses lies in the Na^+-insensitive component of the depolarization. This is only observed in the cell line and may reflect a different state of activation of these cells compared with E^+ lymphocytes. The latter are a quiescent population that do not enter the mitotic cycle until several hours after activation. Cells from the line, by contrast, are already a dividing population.

Our data suggest that the opposing effects of the opening of Ca^{2+}-activated K^+-channels and an increase in Na^+ permeability are responsible for the rather small membrane potential changes that we observe in E^+ cells. Similarly, variations in the activity of Ca^{2+}-dependent K^+-channels and the balance between the two effects, probably accounts for the differences in the reported membrane potential responses of other lymphocytes.

The existence of voltage-gated K^+-channels in human T-cells has been demonstrated recently [32,33]. Moreover, phytohaemagglutinin modulates these channels by reducing, within 1 min, the voltage threshold for opening [32]. During this period we would expect the cells to hyperpolarize through the Ca^{2+}-activated K^+-channels. An alternative explanation of this hyperpolarization would be that it is caused by the voltage-dependent chan-

nels opening spontaneously as their threshold falls, under the influence of the mitogen.

There are two major pathways by which human T-cells are activated, defined by the interaction of ligands with different cell surface receptors. The T3-Ti receptor complex recognises, and is triggered by, a combination of antigen and major histocompatibility determinants. It is also activated by antibodies directed against elements of the complex [18]. An alternative mechanism is available through the T11 molecule, which is the sheep red blood cell receptor [34]. Phytohaemagglutinin stimualtes lymphocytes by interacting with the T11 molecule while concanavalin A does not act through this pathway [35]. Our data show that phytohaemagglutinin evokes similar intracellular Ca^{2+} and membrane potential responses to concanavalin A, but more slowly. We therefore conclude that although the ligands interact initially with different molecules, the subsequent early events that occur are similar.

Acknowledgements

This work was supported in part by the Wellcome Trust. We would like to thank Russul Saib for technical assistance.

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