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LYMPHOCYTE MEMBRANE POTENTIAL ASSESSED WITH FLUORESCENT PROBES

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Summary

The membrane potential of mouse spleen lymphocytes has been assessed with two fluorescent probes. 3,3′-Dipropylthiadicarbocyanine (diS-C_3-(5)) was used for most of the experiments. Solutions with high K^+ concentrations depolarised the cells. Valinomycin, an ionophore which adds a highly K^+-selective permeability to membranes, slightly hyperpolarised cells in standard (6 mM K^+) solution, and in 145 mM K^+ solution produced a slight additional depolarisation. These findings indicate a membrane whose permeability is relatively selective for K^+. Very small changes in potential were seen when choline replaced Na^+, or gluconate replaced Cl^-, supporting the idea of K^+ selectivity. The resting potential could be estimated from the K^+ concentration gradient at which valinomycin did not change the potential — the 'valinomycin null point' — and under the conditions used the resting potential was approx. —60 mV.

B cell-enriched suspensions were prepared either from the spleens of nu/nu mice or by selective destruction of T cells in mixed cell populations. The membrane potential of these cells was similar to that estimated for the mixed cells.

In solution with no added K^+, diS-C_3-(5) itself appeared to depolarise the lymphocytes, in a concentration dependent manner. With the 100 nM dye normally used, the membrane potential in K^+-free solution was around —45 mV, and 500 nM dye almost completely depolarised the cells. In standard solution quinine depolarised the cells. Valinomycin could still depolarise these

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Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N′*-tetraacetic acid; diS-C_3-(5), 3,3′-dipropylthiadicarbocyanine; diO-C_5-(3), 3,3′-dipentyloxacarbocyanine; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-3-propanesulfonic acid; bis-oxonol, bis-(1,3-diethylthiobarbiturate)tirmethineoxonol.

cells indicating that depolarisation had not been due to dissipation of the K^+ gradient. Since in K^+-free solution diS-C_3-(5) blocks the Ca^{2+}-activated K^+ channels in human red blood cell ghosts and quinine also blocks this K^+ channel it is suggested that the resting lymphocyte membrane may have a similar Ca^{2+}-activated K^+ permeability channel.

Because of the above mentioned effect of diS-C_3-(5) and other biological side effects, such as inhibition of B cell capping, a chemically distinct fluorescent probe of membrane potential, bis(1,3-diethylthiobarbiturate)-trimethineoxonol was used to support the diS-C_3-(5) data. This new probe proved satisfactory except that it formed complexes with valinomycin, ruling out the use of this ionophore. Results with the oxonol on both mixed lymphocytes and B cell-enriched suspensions gave confirmation of the conclusions from diS-C_3-(5) experiments and indicated that despite its biological side effects, diS-C_3-(5) could still give valid assessment of membrane potential.

Introduction

Despite the importance of membrane potential in controlling the activity of many cells and the distribution and transport of charged solutes in all cells, rather little is established about this potential, the factors which determine it or its role in cell activation, in lymphocytes. Various approaches have been used to measure lymphocyte resting potentials with rather disparate results. Two groups of workers [1,2] have used standard electrophysiological techniques on unstimulated human peripheral lymphocytes and reported quite small negative potentials: −12 mV [1] and −7 mV [2]. Somewhat more negative potentials of about −18 mV [1] and −15 mV [3] have been recorded by microelectrodes in phytohaemagglutinin transformed human lymphocytes. Iversen [4] measured the Cl^- distribution for human peripheral, rat thymus, and rat spleen lymphocytes and, assuming passive Cl^- distribution, calculated resting potentials of around −35 mV. Holian et al. [5] have measured the distribution ratios for the 'lipophilic ions' thiocyanate and triphenylmethylphosphonium in human lymphocytes suspended in Hank's medium and calculated resting potentials of −62 mV and −73 mV with the two ions. It was also found [6] that the resting potential was quite close to the K^+ equilibrium potential, −84 mV, and was reduced in magnitude when the K^+ concentration in the medium was raised. Bramhall et al. [7] reported results using the fluorescent probe 3,3′-dipentyloxacarbocyanine (diO-C_5-(3)) which showed that changes in potential of lymphocytes in suspension could be reported by the dye, but did not estimate a value for the resting potential from their data. The results shown in their Fig. 5 could, however, be interpreted as showing a membrane potential close to 0 mV.

In the present work we have sought to investigate which value, if any of these, for lymphocyte resting potentials may be correct and have used two different fluorescent probes to study mouse spleen lymphocytes. Most of the experiments were done with 3,3′-dipropylthiadicarbocyanine (diS-C_3-(5)) which is probably the most widely used potential probe [8,9,10,11]. However, this dye has certain toxic effects on lymphocytes, consequent upon blockade

of mitochondrial respiration [12] and also, under some circumstances, appears to depolarize lymphocytes (see later) and so the findings have been checked, at least qualitatively by use of a new fluorescent probe bis-(1,3-diethylthiobarbiturate)-trimethineoxonol (bis-oxonol) [13], which is chemically unrelated to the cyanines and does not seem to be toxic to lymphocytes [12]. We have been interested in the possible role of membrane potential in the capping of B lymphocytes. Splenic lymphocyte preparations, however, normally have roughly equal numbers of B and T cells, which might differ in their membrane potentials and permeabilities. We have therefore tried to prepare and examine cell suspensions containing predominantly B cells.

Methods

Cell preparation. The spleens were removed from 2-4 BALB/c mice and teased out in RPM-1-1640 culture medium. The resulting suspension was layered over a solution containing 6% Ficoll (Pharmacia) and 16.6% Triosil (Nyegaard) and then centrifuged at $1800 \times g$ for 15 min. The lymphocytes were harvested from the interface, washed twice in culture medium and kept at room temperature in a stock suspension containing $3—6 \cdot 10^7$ cells/ml. A cell count and eosin exclusion viability test (viability was always over 95%) were done on each preparation. For experiments requiring B enriched populations, cells were obtained from the spleens of outbred nu/nu mice, which genetically lack T cells, or from mixed lymphocyte suspensions treated with anti-theta anti-serum. The mixed lymphocyte stock was incubated in a 1 in 10 dilution of mouse theta AKR anti-serum (Searle) for 45 min at 4°C. The cells were then washed and resuspended in a 1 in 6 dilution of guinea pig complement (pre-absorbed against mouse spleen) for 30 min at 37°C. The cells were then again washed and the remaining intact cells harvested from a Ficoll-Triosil gradient as before.

Patching and capping of B lymphocyte suspensions was assessed using fluorescein-labelled anti-IgG antibody (Miles-Yeda) as previously described [14]. The spleen of nu/nu mice did not always yield cells with a satisfactory proportion of capped cells. Two preparations were achieved in which over 85% of the cells did cap normally. The T depletion procedure yielded variable and somewhat unsatisfactory preparations. Only once in 6 trials were more than 80% of the cells in final suspension stained by anti-IgG antibody and only in that preparation did more than 80% of the stained cells go on to cap normally.

Solutions. The standard experimental solution was made to approximate the ion composition of the culture medium and contained 139 mM NaCl, 6 mM KCl, 1.25 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM Na_2HPO_4, 10 mM Na Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonate), 5.6 mM dextrose; pH 7.2; temperature 37°C. When required the K^+ was altered by isotonic substitution of KCl for NaCl or, on occasion, by addition of 1 M KCl to the cuvette. Ca^{2+} and Mg^{2+} concentrations were changed by simply adding or omitting $CaCl_2$ or $MgSO_4$. Low Na^+ solutions were prepared by isotonic substitution of choline Cl^- or sucrose for NaCl. Low Cl^- solutions were prepared by isotonically

replacing NaCl and KCl with sodium gluconate and potassium gluconate or sodium methylsulphate and K_2SO_4.

Procedure for fluorescence experiments. All the experiments were done in a Perkin-Elmer MPF 44A spectrophotometer whose output was connected to a Y-t pen recorder. 2 ml of solution was added to a chromic-sulphuric-acid-washed glass test tube in a specially constructed holder, maintained at 37°C. Next, dye was added from a Me_2SO stock solution and the solution briefly stirred by means of small glass covered magnetic followers. Usually 2 μl or 4 μl of 0.1 mM diS-C_3-(5) or 2 μl of 0.15 mM bis-oxonol was used. When different dye concentrations were required different stocks were prepared to limit the addition of Me_2SO to 4 μl. The fluorescence signal of the solution was then recorded with excitation and emission at 620 nm and 660 nm for diS-C_3-(5) and 540 nm and 580 nm for the oxonol and 10 nm slit widths. After one or more repeated stirrings to achieve a reasonably steady 'initial' signal (for a full discussion of stirring artefacts with diS-C_3-(5) see references [9,10]) an aliquot of the cell suspension was added and the suspension briefly stirred. In a series of experiments with any one cell preparation the same number of cells was added in every tube used. The change in fluorescence was then recorded and when a new steady level was reached various reagents could be added and the changes in signal recorded. Valinomycin (Sigma), gramicidin D (Koch Light, U.K.) and A23187 (Eli Lilly) were added from stock solutions in Me_2SO. These stocks were prepared so that the final Me_2SO concentration in the suspension never exceeded 0.9% (v/v). Ouabain or quinine were dissolved in the solution prior to the addition of dye and cells. Flame emission photometry was done with an EEL flame photometer.

Reagents. DiS-C_3-(5) was synthesised in the laboratory of Dr. Alan Waggonner. Bis-oxonol was synthesised as described previously [13]. Sodium and potassium gluconate stock solutions were extracted twice with activated charcoal before use. Choline chloride was recrystallised from ethanol.

Use of diS-C_3-(5) and bis-oxonol to assess membrane potential. The mechanism by which diS-C_3-(5) reports membrane potential has been previously described in detail [9,10]. The dye is a membrane permeant cation and the distribution of the free dye in a cell suspension therefore varies with membrane potential, dye uptake into the cells increasing as the membrane potential becomes more negative. When diS-C_3-(5) binds to lymphocytes the fluorescent signal with excitation at 620 nm and emission at 660 nm is diminished. This is because binding to some cell components red-shifts the excitation and emission spectra away from the wavelength used, while part of the binding suppresses the fluorescence altogether. These changes are similar to those seen with red blood cells [9,10]. For a given number of cells and initial dye fluorescence in a suspension, a smaller fluorescence signal represents increased binding and a more negative potential, and a larger signal indicates depolarization. Thus, one has an indicator of potential whose response is not linear [9,10] and which in almost all preparations requires calibrating under the conditions used. This is most often done by using solutions of different K^+ concentrations and observing the changes in signal evoked by addition of valinomycin which confers a large increase in K^+ permeability in membranes, and this is the major approach used here.

Bis-oxonol also has physico-chemical properties which make its fluorescence change with membrane potential [13]. This dye is a lipophilic anion. When it moves from aqueous solution into a non-polar environment, as when it binds to membranes or proteins, its fluorescence quantum efficiency increases by up to 20-fold. This marked enhancement of fluorescence by binding is not accompanied by any significant change in the wavelengths of the absorption and emission maxima. Depolarization of the membrane transfers dye anions from the external solution onto binding sites inside the cell, thus increasing the net fluorescence. Bis-oxonol and diS-C_3(5) both give increased overall fluorescence on cell depolarization because the opposite signs of their charges counteract the opposite effects of binding on fluorescence efficiency. The utility of the oxonol as a potential probe was confirmed by trials in liposomes and guinea-pig spermatozoa (Tsien, R.Y. and Rink, T.J., unpublished observations).

Results

Mixed lymphocytes assessed with diS-C_3-(5)

Fig. 1 shows fluorescence signals from diS-C_3-(5) in high K^+ and standard solutions after addition of mixed lymphocytes. In the standard 6 mM K^+ solution there was a fall in signal to 42% of the initial level. This fall had a fast component, which is attributed to virtually instantaneous binding of the dye to the outside of intact cells and to any cell debris, and a slow component due to the slow entry of dye into the cells followed by binding of the dye to internal cell constituents. In high K^+ solution there was again a rapid fall in signal followed by a slow decline, but here the new level was 70% of the initial signal. The much higher fluorescence in the high K^+ solution indicates a substantial K^+ depolarization of the cells. This difference in fluorescence level from suspension in normal and high K^+ media was consistently seen and other examples are shown in Figs. 2 and 6. Addition of the potassium ionophore, valinomycin,

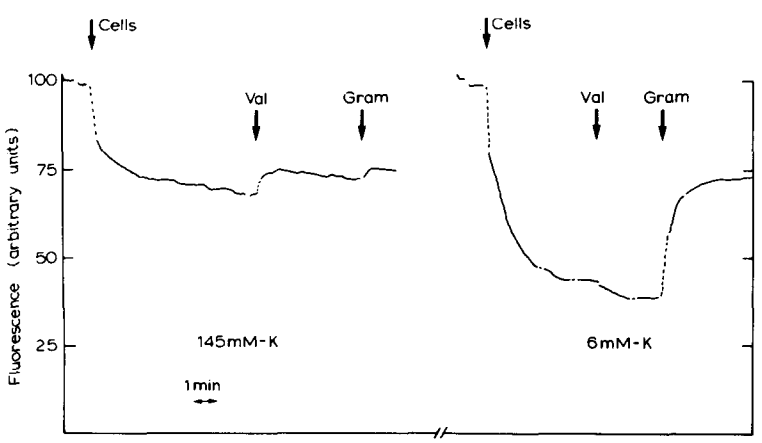

Fig. 1. Fluorescence signals from 200 nM diS-C_3-(5) on addition of $1.4 \cdot 10^6$ mixed lymphocytes/ml. 1 μM valinomycin and 20 nM gramicidin were added as indicated, first in 145 mM K^+ solution and then standard (6 mM K^+) solution. The initial dye fluorescence has been normalised to 100 arbitrary units in all diS-C_3-(5) traces shown. The dotted lines indicate periods of additions to the tube or stirring. Excitation and emission wavelengths were 620 nm and 660 nm.

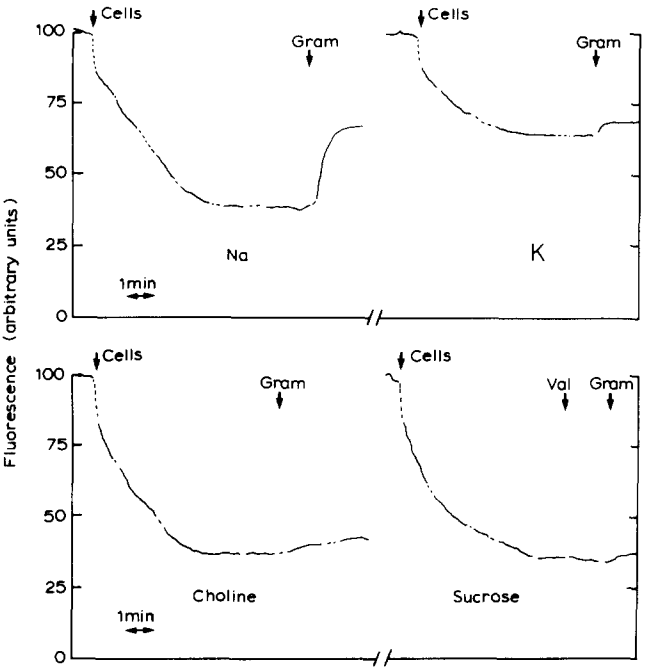

Fig. 2. Fluorescence signals from 100 nM diS-C_3-(5) on addition of $8 \cdot 10^5$ mixed lymphocytes/ml in standard Na^+ solution, 145 mM K^+ solution, low Na^+, choline solution, and low Na^+, sucrose solution. 20 nM gramicidin or 1 μM valinomycin were added as indicated.

evoked a small fall in signal in standard solution indicating a small hyperpolarization and a small depolarization was evoked in the high K^+ solution. The small effects of valinomycin imply that in both solutions the membrane potential is close to the potassium equilibrium potential (E_K).

Gramicidin forms pores in lipid membranes which confer a large permeability to K^+ and Na^+. The effect of this antibiotic (Fig. 1) was to increase markedly the fluorescence when added in standard solution, but there was little change in the high K^+ solution. The same changes were seen when gramicidin was added without prior addition of valinomycin, as in Fig. 2. This depolarization by gramicidin in standard solution is expected for cells whose membrane potential is close to E_K and remote from E_{Na}, from both the large increase in Na^+ permeability and an exchange of internal K^+ for Na^+.

So these results suggest that lymphocytes, like most cells, have a K^+-selective membrane and strongly negative resting potential. This inference was tested by replacing the Na^+ or Cl^- or both with larger, presumably impermeant, molecules. Fig. 2 shows two typical records obtained with low Na^+ solutions; in one case choline chloride replaced NaCl and in the other sucrose was used. Similar falls in signal were seen in the standard and in the low Na^+ solutions indicating that the potential was not much different with widely different Na^+ concentrations and supporting the idea that the K^+ permeability was much higher than the Na^+ permeability. The fluorescence level was actually a little lower in the choline and sucrose solutions suggesting that Na^+ was making some contribution to the resting potential, and that its removal could produce a slight hyper-

polarization. Also shown in Fig. 2 is the small change evoked by gramicidin in the choline solution (in this experiment there was actually a small depolarization but in other tests a small hyperpolarization was seen, e.g. that seen in Fig. 5A). Since choline does not permeate gramicidin channels no great change in potential would be expected and the significance of the small changes seen is difficult to assess. There was still 6 mM Na^+ in the choline solution, added with the Hepes and phosphate, and this may have prevented gramicidin evoking any hyperpolarization from the resting potential. In a solution containing only sucrose, Tris-Hepes and $MgSO_4$, gramicidin did produce quite a marked hyperpolarization. In the final panel of Fig. 2 the effect of adding valinomycin in the sucrose solution is seen to be very small, suggesting that membrane potential here may be rather close to E_K. Subsequent addition of gramicidin produced only a small change in signal. In other experiments replacing Cl^- with gluconate or methylsulphate and sulphate had little effect on the fluorescence levels seen in either 6 mM K^+ or 145 mM K^+ solutions. There was a slightly lower decrement in fluorescence in 6 mM K^+, low Cl^- solutions indicating a small depolarization and suggesting some small contribution of Cl^- to the determination of the membrane potential. These ion substitution results support the idea that the lymphocyte membrane potential depends mainly, but not entirely, on the K^+ gradient.

One way to attempt calibration of the dye response is to find the external K^+ concentration at which valinomycin does not alter the signal, and presumably therefore does not alter the potential. This null point method has been used, for example, in red blood cells [15] and spermatozoa [16]. With those cells it is relatively easy as the membrane potential is normally remote from E_K and valinomycin induces large changes in signal. The approach used in the present work was to make additions of 1 M-KCl to the suspension after application of valinomycin and to see what external K^+ concentration, $[K]_o$, was needed to return the signal to the resting value. An example of this kind of experiment is shown in Fig. 3 where, in standard solution, some 15 mM K^+ was required to achieve this 'null point'. In other experiments just 12 mM K^+ was required. At the 'null point' it can be taken that the membrane potential (E_m) equals E_K and

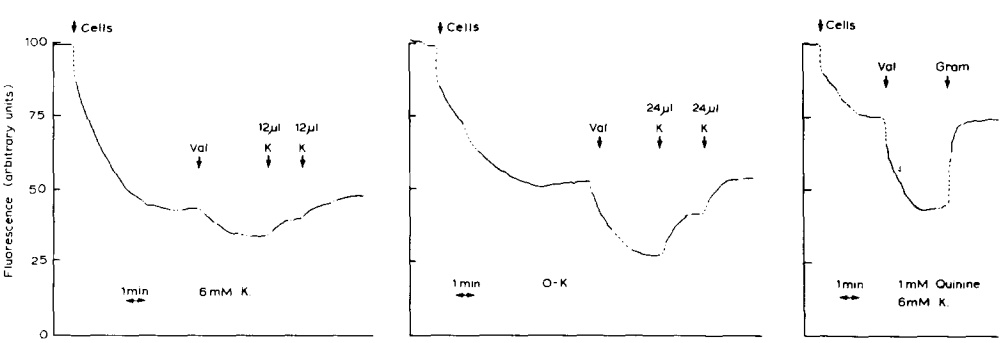

Fig. 3. Fluorescence signals from 200 nM diS-C_3-(5) on addition of $8 \cdot 10^5$ mixed lymphocytes/ml then 1 µM valinomycin was added and then the indicated amounts of 1 M KCl as indicated, firstly in standard 6 mM K^+ solution, secondly in K^+-free (O-K) solution and thirdly in 6 mM K^+ solution with 1 mM Quinine.

from the Nernst equation

$$E_m = \frac{RT}{F} \ln \frac{[K]_o^n}{[K]_i}$$

when $[K]_i$ is the intracellular K^+ concentration and $[K]_o^n$ is the external K^+ concentration at the null point. A value for $[K]_i$ is therefore needed. In all reported determinations of which we are aware, lymphocytes have been found to have high $[K]_i$, values ranging from 125 to 160 mmol/kg cell water [4,6,17]. In rat thymic and splenic lymphocytes, for example, values of 136 mmol/kg cell water were reported [17]. The mouse spleen lymphocytes used in the present work were certainly high K^+ cells. A K^+/Na^+ ratio of 8 : 1 was found in cell pellets after centrifugation through Na^+ and K^+-free isotonic choline solutions. The exact value of $[K]_i$ proved difficult to determine due to problems we cannot explain in obtaining reliable measurements of the trapped extracellular, and the intracellular water content of the cell pellets. Similar difficulties have been noted by other investigators in trying to measure those quantities with very small amounts of cells, like the 1—2 μl packed cells which were produced in our preparations [17]. The cells were found to contain some 100 mmol K^+/l cell volume (calculated from cell count and mean cell diameter), which suggests that the value of 136 mmol/kg cell water, as found for rat spleen lymphocytes, is a very reasonable figure for mouse spleen cells. Using this figure for $[K]_i$ and 12 mM for $[K]_o^n$, one obtains a value of —62 mV for the resting potential of mouse spleen lymphocytes in the conditions used here. With $[K]_o^n$ = 15 mM, E_m would be —57 mV. We tend to favour the more negative estimate since in vitro damage to the cells will tend to depolarize them. Obviously these estimates depend on the reliability of the value for $[K]_i$ but in fact an error of 20% gives only a 5 mV error in the potential and, anyway, the most accurate stoichiometric determination of cell K^+ cannot reveal the actual concentration of K^+ just beneath the membrane nor its activity coefficient, which are the values actually required for a more rigorous calculation of the potential.

B cells assessed with diS-C_3-(5)

Cells from the spleens of nu/nu mice gave rather less reproducible results than the normal mixed lymphocyte population. Fig. 4 shows fluorescence records with these cells from one of the best preparations, in standard, high K^+ and low Na^+ solutions. The responses were similar to those seen with mixed lymphocyte; the fluorescence signal being much larger in the high K^+ solution than in standard solution indicating K^+ depolarization and there was evidence of slight hyperpolarization in the choline solution. The responses to valinomycin indicated that the resting potential of these cells was fairly close to E_K. These cells seemed to have therefore a K^+-dominated negative resting potential. T-depleted cell suspensions obtained by treating mixed lymphocytes with anti-T serum did not prove very satisfactory. When the best of these preparations was examined with diS-C_3(5), the cells were clearly depolarized by high K^+ solutions and only slightly hyperpolarized by low sodium choline solutions, and generally confirmed the conclusion that B cells do not seem to differ im-

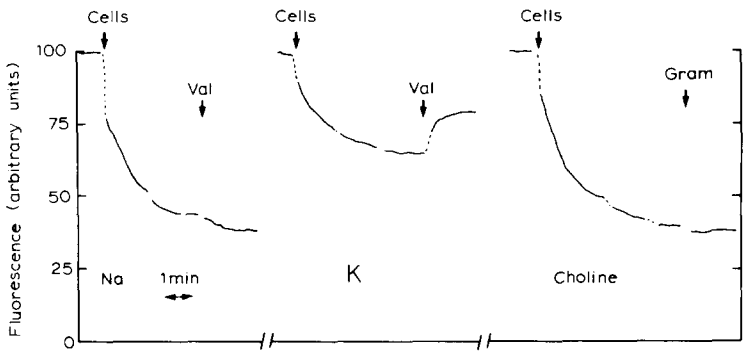

Fig. 4. Fluorescence signals from 100 nM diS-C_3-(5) on addition of $5 \cdot 10^5$ B lymphocytes/ml with addition of 1 μM valinomycin or 20 nM gramicidin in standard solution, 145 mM K^+ solution or low Na^+, choline solution as indicated. These lymphocytes were prepared from the spleens of nu/nu mice.

portantly, in terms of membrane potentials and permeability, from the mixed cell population.

Effects of diS-C_3-(5) on the lymphocyte membrane

During experiments done initially to see if there might be any contribution of an electrogenic Na^+ pump to the lymphocyte membrane potential, an effect of the K^+-free solution was indeed observed, as shown in Fig. 3. In this experiment there was a smaller fluorescence signal after addition of cells in 6 mM K^+ than in the K^+-free solution indicating some depolarization in the latter one. After addition of valinomycin the fluorescence level as lower in the K^+-free solution was expected because of the much more negative E_K. Using the KCl addition method to find a valinomycin null point, $[K]_o^n$ was about 15 mM K^+ in the standard solution and 24 mM K^+ in the K^+-free solution. This indicates that the cell-potential was depolarized some 10 mV in the K^+-free solution, compared to the standard solution. This could have been due to inhibition of a powerful electrogenic Na^+ pump, but no effect could be seen with 1 or

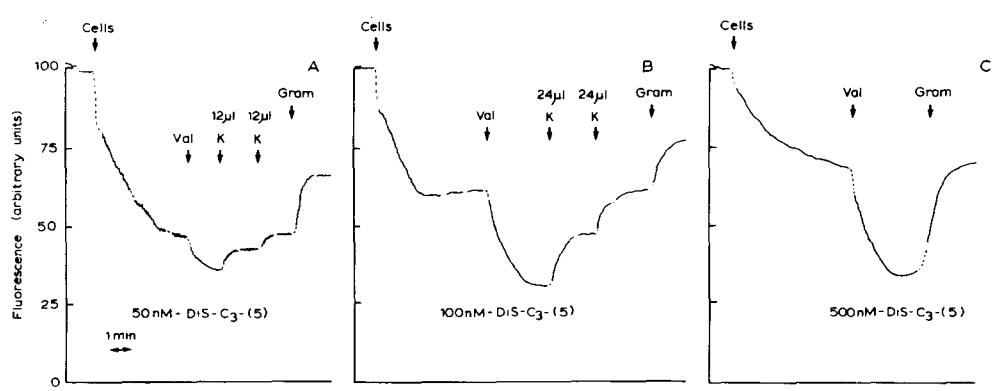

Fig. 5. Fluorescence signals from first 50 mM then 100 nM, then 500 nM diS-C_3-(5) in K^+-free solution on addition of $8 \cdot 10^5$ mixed lymphocytes, followed by 1 μM valinomycin or 20 nM gramicidin. The initial fluorescence levels varied with the different dye concentrations, but they have been normalised to 100 for purposes of comparison.

2 mM ouabain which suggests that there may have been some other cause for the depolarization in the K^+-free solution.

Fig. 5 shows an experiment where, in the K^+-free solution, increasing concentrations of diS-C_3-(5), 50 nM, 100 nM and 500 nM, appeared to increasingly depolarize the cells. The data have been normalised to give initial dye fluorescence at 100 nM so that the traces may be compared, although one cannot assume that a given percentage-fall in signal denotes an identical potential at different dye concentrations. It is probable that the higher the dye concentrations, the larger the decrement in signal for a given potential will be (see e.g., Fig. 12 of Ref. 10). This effect makes the depolarization in the higher dye concentrations if anything underestimated by the records. From the null points determined by KCl addition, the membrane potential in 50 nM diS-C_3-(5) can be estimated at −62 mV and in 100 nM diS-C_3-(5) it was ~−45 mV. The rather larger subsequent depolarizing effects of gramicidin with the lower dye concentration further supports the idea that the cells were indeed initially depolarized by the 100 nM dye. The very large hyperpolarization produced by valinomycin in the presence of 500 nM dye and the fact that gramicidin depolarized the cells just to the initial level suggests that here the cells had been very substantially depolarized by the high dye concentration. Much smaller depolarizing actions of diS-C_3-(5), at concentrations up to 600 nM, were seen in the presence of 6 mM K^+.

This concentration-dependent depolarizing action of diS-C_3-(5), apparently inhibited by external K^+, is strikingly reminiscent of the effect of this dye reported in Ca^{2+}-containing human red cell ghosts [18]. In these ghosts diS-C_3-(5) blocked the Ca^{2+}-activated K^+ channel, and this blockade was powerfully inhibited by external K^+. It is tempting to suppose that the K^+ permeability of lymphocytes may be due to the presence of a similar K^+ channel. This idea was tested by exposing lymphocytes to quinine, an agent also known to powerfully block Ca^{2+}-activated K^+ channels in red blood cells [19]. The third panel of Fig. 3 illustrates the effect of 1 mM quinine in standard solution. The cells appeared to be completely depolarized, consistent with blockade of a K^+ channel, and valinomycin produced a strong hyperpolarization, showing that the ionic gradients had not been dissipated.

Effects of Ca^{2+} on suspension fluorescence were examined but these experiments failed to support or contradict the idea of a Ca^{2+}-activated K^+ channel. Variations in external Ca^{2+} produced only small changes. There was no discernible difference in fluorescence levels when Mg^{2+} replaced Ca^{2+} in the solution. A slight hyperpolarization could be obtained on addition of 5 or 10 mM $CaCl_2$ to suspensions lacking any divalent cation. Since the activation of Ca^{2+} on a K^+ channel like that described in red blood cells [18,19] would be at an internal site it was, perhaps, not surprising to find that alterations of external Ca^{2+} had so little effect. Application of 2 μM A23187 (a concentration known to promote Ca^{2+} entry into lymphocytes [14]) in the standard 1.25 mM Ca^{2+} solution, produced a marked depolarization of the cells, which could not be reversed by addition of excess EGTA to chelate the external calcium. Since valinomycin could not now hyperpolarize the cells it seems likely that the K^+ gradient had become dissipated under these conditions. A rapid loss of cell K^+ and uptake of Na^+ has been reported in lymphocytes exposed to A23187 in the

presence of Ca^{2+} [8] and a similar effect has been seen in isolated chromaffin cells (Rink, T.J., unpublished observations).

Lymphocytes assessed with bis-oxonol

The depolarizing effect of diS-C_3-(5) and its inhibitory action on mitochondrial respiration must make one cautious about interpreting results obtained with this dye. Confirmation of the results with another probe was desirable and bis-oxonol was used as it had no discernible toxic properties on lymphocytes assessed by studies of viability, ATP levels or capping in response to anti-IgG antibodies [12]. Fig. 6 shows fluorescent records from one preparation of mixed lymphocytes in standard, high K^+ and low Na^+, choline solutions examined firstly with diS-C_3-(5) and then with bis-oxonol. The diS-C_3-(5) traces are typical of those seen in other experiments. The cells were depolarized by high K^+ solution and the effects of gramicidin in the two solutions were essentially as described already, as were the responses in choline solution. Fig. 6B shows the responses seen from these cells with bis-oxonol. On addition of the cells the fluorescence signal rapidly increased to a new, relatively steady level. The increased signal is expected from the increased quantum yield resulting from dye binding to hydrophobic cell components. A simple demonstration of dye binding was to centrifuge the suspension and note the bright pink staining of the pelleted cells. The rapid change in signal is expected since this dye is much more permeant than diS-C_3-(5) and should therefore distribute into the cells much more rapidly. The permeability of glycerol mono-oleate bilayers to bis-oxonol, calculated from the data of Tsien [13], is approx. $2 \cdot 10^{-1}$ cm/s

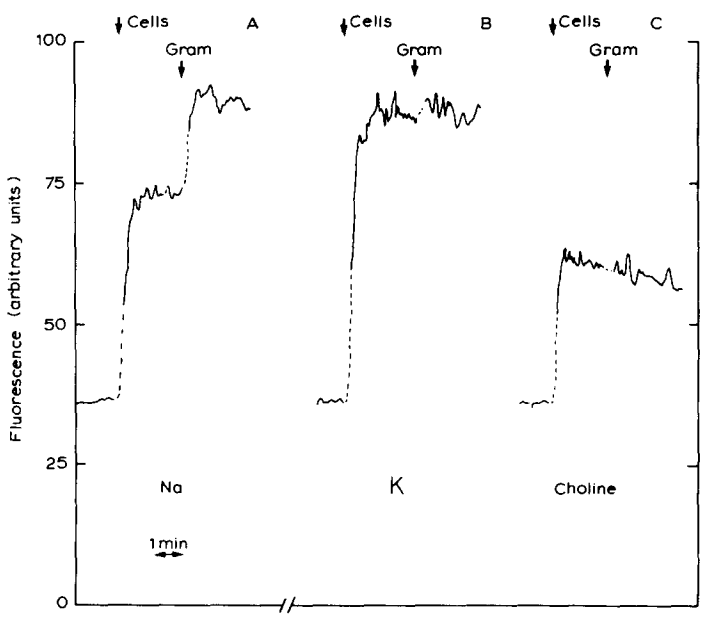

Fig. 6. Fluorescence signals from 100 nM diS-C_3-(5); A, and 150 nM bis-oxonol; B, on addition of $8 \cdot 10^5$ mixed lymphocytes in standard Na^+ solution, 145 mM K^+ solution, and low Na^+, choline solution. 1 μM valinomycin or 20 nM gramicidin were added as indicated. Excitation and emission were 620 and 660 nm for diS-C_3-(5) were 540 and 580 nm for bis-oxonol.

whereas the permeability of red blood cell membranes to diS-C_3-(5) is approx. $5 \cdot 10^{-4}$ cm/s [9].

The actual levels of fluorescence reached were different in the different media. In standard medium the fluorescence increased some 50% on addition of cells while in the high K^+ medium it almost doubled. Since a less negative potential should be reported by a larger signal (as described in the Methods section) this result indicates a K^+ depolarization of the cells. Subsequent addition of gramicidin brought the fluorescence in the standard solution close to that in the high K^+ solution showing that the dye reported the expected gramicidin induced depolarization. In high K^+ solution gramicidin evoked little change in signal. These results therefore qualitatively support those obtained with diS-C_3-(5). The fluorescent level seen with choline replacing Na^+ was somewhat lower than that seen in a standard solution, suggesting a somewhat more negative resting potential. A small hyperpolarization in choline was seen in some of the experiments with diS-C_3-(5). The small hyperpolarization induced by gramicidin in the choline solution was in line with the effect seen in these cells with diS-C_3-(5). Results like these were obtained with several other mixed cell preparations. Fig. 7 shows that rather similar results were also seen with bis-oxonol assessment of B cells from nu/nu mice. The responses were essentially those just described for mixed cells and suggest, as do the diS-C_3-(5) data,

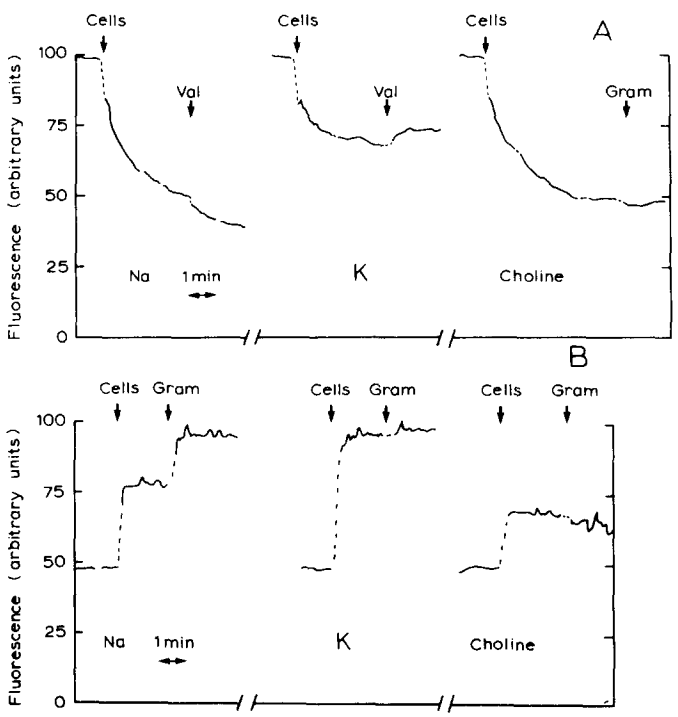

Fig. 7. Fluorescence signal from 150 nM bis-oxonol on addition of $8.5 \cdot 10^5$ B lymphocytes, from nu/nu mice, and 20 nM gramicidin as indicated, in standard Na^+ solution, 145 mM K^+ solution and low Na^+, choline solution.

that B cells have similar membrane potentials and properties to those seen in the mixed cell populations.

Discussion

The results with diS-C_3-(5) indicate that mouse spleen lymphocytes, in physiological saline, have a resting potential of about −60 mV and that the resting membrane permeability is predominantly K^+-selective. The mechanism by which diS-C_3-(5) reports potential [9,10] and the action of the ionophores used in the experiments are well enough established to allow some confidence in these conclusions. However, the biological side effects of the dye and the ionophores have to be considered with the possibility that spurious changes in fluorescent signal might be interpreted as changes in potential. One may accept that an inhibition of mitochondrial respiration by diS-C_3-(5) [12] would not greatly affect the results of these experiments. But the finding that the dye can itself depolarize the cells gives cause for caution. This action was much more obvious in K^+-free solution than in 6 mM K^+ but it may have led to an underestimate of the true resting potential. The estimation of the membrane potential depended on the use of valinomycin to find the 'valinomycin null point' and on the value taken for the intracellular K^+ concentration. The problems of the latter determination have been previously mentioned and the use of valinomycin could introduce other errors. If, for instance, application of valinomycin altered the dye binding properties of the cell interior then a systematic spurious change in signal would be produced which would give an error in the null point. Evidence that any such effect is small is the observation that, in high K^+ solution, for example, where valinomycin and gramicidin would be expected to have similar small effects on the membrane potential, similar small effects on diS-C_3-(5) fluorescence are seen. Gramicidin appears not to enter the cytoplasm of lymphocytes in suspension, judged by its failure to deplete cell ATP [12], despite its being a potent mitochondrial uncoupler. The applied gramicidin presumably remains bound to the plasma membrane.

In the face of these problems with diS-C_3-(5) it was felt important to use some other probe of potential, and to assess the present results in the light of those previously published. The experiments with bis-oxonol offered qualitative support for the results with diS-C_3-(5). The dye behaved as would be predicted from its physicochemical properties and revealed a substantial depolarization of the lymphocytes in high K^+ solution and on application of gramicidin. The apparent hyperpolarization in choline solution was perhaps more marked than that seen with diS-C_3-(5) but a similar small additional hyperpolarization was seen after addition of gramicidin with the two dyes. More work will be needed to develop fully the use of bis-oxonol as a probe of potential but we are encouraged by this trial. Its speed and apparent lack of toxicity are valuable attributes, the noisiness of the traces and the formation of complexes with valinomycin are disadantages.

Our conclusions on the lymphocyte resting potential agree with those obtained by measurement of lipophilic ion distribution [5,6]. Those values were −62 and −73 mV, obtained with thiocyanate and triphenylmethyl phosphonium, respectively, and the potential was found to vary with alteration in

external K^+ concentration. There are, of course, possible artefacts with the ion distribution technique, for instance, ion binding inside the cells will lead to an over-estimate of the internal free ion concentration and it is not unlikely that lipophilic ions could alter membrane properties. However, the reasonable agreement between the results with one anion and one cation suggests that there were no serious problems with these artefacts. The estimation of potential based on chloride distribution requires the assumption that no active transport of chloride occurs. If the −62 mV resting potential seen in the present work is right then the finding of a chloride potential of −35 mV [4] in human lymphocytes would imply the existence of an inwardly directed pump. The values for lymphocyte resting potential obtained with microelectrodes [1,2] are very different from those in the present work and those of Holian et al. [4]. Very small (around −10 mV) potentials were seen which could have reflected a dissipation of the true potential due to leaks around the electrodes. This can be a serious problem with small cells and further investigation of this point, perhaps using ultrafine electrodes, may be required to assess this possibility. The discrepancy between the present results with fluorescent probes and those seen in a previous study [7] also requires explanation. The valinomycin null point for rat thymic cells in Fig. 5 of Bramhall et al. [7] occurs with 120 mM K^+, and in standard (5 mM K^+) solution a very marked hyperpolarization developed after addition of valinomycin. The cells appear therefore to have had a very small resting potential. One reason for this could have been the high concentration of the probe which was used; 2 μM diO-C_5-(2). This dye may be more toxic to red cells than diS-C_3-(5) [8,15], and if diO-C_5(3) had the same effect in depolarizing lymphocytes as diS-C_3-(5) then 2 μmol/l might have depolarized the thymic lymphocytes. It seems important to use fluorescent probes at as low a concentration as will give adequate signals.

Some of the discrepancies might be due to use of lymphocytes from different sources but the studies of ion distribution and those with microelectrodes were done on human peripheral lymphocytes and disagree strongly, while the present work on mouse spleen cells agrees well with the former results on human cells. We feel that the balance of the evidence favours the conclusion that lymphocytes have a K^+ dominated negative resting potential, like most animal cells, especially since most technical problems in measuring membrane potential lead to underestimates.

One disadvantage of fluorescent probe or ion distribution techniques which use cell suspensions is that some sort of average potential from all the cells is obtained and differences between sub-groups in the cell population would be missed. However, experiments with B cell populations obtained from nu/nu mice gave results similar to those seen with mixed cell populations and suggest that there is little difference between mouse spleen B and T cells in terms of membrane potential and permeability properties.

Two observations gave a clue to the nature of the K^+ permeability of the lymphocyte membrane. The cells were strongly depolarized by diS-C_3-(5) in the absence of external K^+, in a concentration dependent manner. Virtually complete depolarization was seen with 500 nM dye. Much smaller effects were seen in the presence of 6 mM K^+. The cells were also strongly depolarized by 1 mM quinine. In both cases valinomycin was able to hyperpolarize the cells

showing that the K^+ gradient had not been dissipated. The depolarizing effect of these two agents seemed therefore to be due to their altering membrane permeability. A blockade of K^+ permeability could have produced these results. Since both diS-C_3-(5) [18] and quinine [19], at the concentrations used, are known to block the Ca^{2+}-activated K^+ channel of red blood cells it is possible that the K^+ permeability of the lymphocyte membrane is due to the same kind of channel. Many cells have Ca^{2+}-activated K^+ channels [20] but lymphocytes would be unusual in having the channel open under resting conditions. In most cells this channel is activated only when the intracellular free Ca^{2+} rises above the resting level. Clearly the evidence for a Ca^{2+}-activated K^+ channel in lymphocytes is very indirect. Indeed the depolarization produced by diS-C_3-(5) and quinine could have been due to generalised leakiness of the membrane, though diS-C_3-(5) does not seem to induce ion leaks in other cells (see, e.g. Ref. 8) and quinine is generally thought to inhibit transmembrane ion fluxes. The depolarizing action of diS-C_3-(5) requires further elucidation by some means other than its own ability to report potential changes. Using a fluorescent probe to assess its own toxic effect has an admirable economy of procedure but requires independent substantiation. It was impossible to check the depolarizing effect of diS-C_3-(5) with bis-oxonol since the two dyes form an insoluble complex.

The biological significance of the lymphocyte membrane potential awaits investigation. In the subsequent paper experiments on the role of membrane potential in the initiation of anti-IgG antibody induced capping are reported. [21]. The role of this potential in the stimulation and transformation of lymphocytes remains to be examined. As noted in the introduction, the membrane potential affects the transport and distribution of any charged solute. A large negative potential and a large inward concentration gradient for Na^+ could allow coupled uphill transport of glucose and amino acids into the cells and Ca^{2+} and H^+ out of the cells. Such systems, which have been extensively studied elsewhere, might prove fruitful for investigation in lymphocytes.

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References

1 Taki, M. (1970) Mie Med. J. 29, 245–262
2 Kowal, E., Malofiejew, M. and Kostrzewska, A. (1975) Acta Biol. Med. Ger. 34, 1007–1011
3 Oliveira-Castro, G.M. and Barcinski, M.A. (1974) Biochim. Biophys. Acta 352, 338–343
4 Iversen, J.G. (1976) J. Cell Physiol. 89, 267–276
5 Holian, A., Deutsch, G.J., Holian, S.K. and Daniel, R.P. (1977) Fed. Proc. 36, 826
6 Holian, S.K., Holian, A. and Daniel, R.P. (1977) Fed. Proc. 36, 1223
7 Bramhall, J.S., Morgan, J.I., Perris, A.D. and Britten, A.Z. (1976) Biochem. Biophys. Res. Commun. 72, 654–662
8 Sims, P.J., Waggoner, A.S., Wang, C.H. and Hoffman, J.F. (1974) Biochemistry 13, 3315–3330
9 Hladky, S.B. and Rink, T.J. (1976) J. Physiol. 263, 287–319

10 Tsien, R.Y. and Hladky, S.B. (1978) J. Membrane Biol. 38, 73—97
11 Waggoner, A.S. (1976) J. Membrane Biol. 27, 317—334
12 Montecucco, C., Pozzan, T. and Rink, T. (1979) Biochim. Biophys. Acta 552, 552—557
13 Tsien, R.Y. (1976) Ph.D. Thesis, University of Cambridge
14 Hesketh, T.R., Smith, G.A., Houslay, M.D., Warren, G.B. and Metcalfe, J.C. (1977) Nature 267, 490—494
15 Hoffman, J.F. and Laris, P.C. (1974) J. Physiol. Lond. 239, 519—552
16 Rink, T.J. (1977) J. Reprod. Fert. 51, 155—157
17 Lichtman, M.A., Jackson, A.H. and Peck, W.A. (1972) J. Cell Physiol. 80, 383—396
18 Simons, T.J.B. (1976) Nature 264, 467—469
19 Armando-Hardy, M., Ellory, J.C., Ferreira, H.G., Fleminger, S. and Lew, V.L. (1975) J. Physiol. Lond. 250, 32—33P
20 Lew, V.L. and Ferreira, H.G. (1978) Curr. Top. Membranes Transp. 10, 217—277
21 Montecucco, C., Rink, T.J., Pozzan, T. and Metcalfe, J.C. (1979) Biochim. Biophys. Acta 595, 65—70