THE USE OF A FLUORESCENT PROBE TO MONITOR ALTERATIONS IN TRANS-MEMBRANE POTENTIAL IN SINGLE CELL SUSPENSIONS

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SUMMARY. When single cell suspensions of thymic lymphocytes, splenic lymphocytes and platelets are equilibrated with a carbocyanine dye, 3,3'-dipentyloxacarbocyanine iodide the fluorescence emission intensity is related to the trans-membrane potential. Addition of valinomycin to these cells provokes a net potassium efflux, and membrane hyperpolarisation, which is reflected as a decrease in fluorescence intensity. Increasing the extracellular potassium concentration, and thus decreasing the concentration gradient of that ion across the membrane, reduces the change in fluorescence intensity induced by valinomycin.

The ionophore A23187 induces a calcium influx into thymic lymphocytes and a concomitant increase in fluorescence intensity indicative of hypo-polarisation. The movements of several ionic species may contribute to this altered membrane potential since Na, K, PO4 and Ca are all induced to move across the cell membrane following the application of A23187. Removal of extracellular calcium markedly reduced both the hypopolarisation and ion redistribution elicited by the ionophore. Thus when calcium enters the cell it initiates a chain of events involving ionic rearrangement and altered transmembrane potential which can be monitored using the fluorescent probe system described here.

INTRODUCTION

The initiation and propagation of nerve impulses depends upon alterations in ionic equilibria across the cell membrane (1,2). In cells of non-neural origin triggered responses such as stimulus-secretion coupling and the activation of cells by hormones and mitogens may also depend on changes in ion permeability (3 - 7). These vital ion movements may well result in changes in the trans-membrane potential which, if they could be measured, would provide some means of investigating the biochemical and biophysical events associated with cell activation. The use of microelectrodes for the measurement of the trans-membrane potential of many cells in

suspension is precluded on both morphological and manipulative grounds. The present study describes the use of a carbocyanine dye for this purpose. When equilibrated with a variety of cell types the intensity of fluorescence is related to the trans-membrane potential and changes very rapidly in response to induced ion movements.

METHODS:

1. CELL PREPARATIONS:

All the cell types used were maintained in suspension culture in a modified Earle's salts solution, at a cell density of $5 \times 10^7 - 10^8$ cells per ml. The salts solution contained sodium bicarbonate 7.7 mM, and was buffered with 20 mM HEPES at pH 7.3. Thymic lymphocytes were prepared as previously described (8). To obtain splenic lymphocytes spleens were extruded through a fine steel filter, and contaminating erythrocytes were removed from this cell suspension by hypotonic shock in 0.76% ammonium chloride buffered to pH 7.3 with Tris-HCl (9). Following two washes, the cells were suspended in medium at the appropriate cell density.

For the preparation of platelets, human venous blood was diluted 1:1 with phosphate buffer (pH 7.4) and aliquots of this solution were layered over a column which had been prepared previously by mixing 9% aqueous Ficoll 400 (24 parts) with 34% aqueous sodium metrizoate (3 parts). The column was spun at 400 g for 20 minutes, and the platelet layer was aspirated away and resuspended in salts solution. This solution was spun at 400 g for 5 minutes, and the supernatant, containing purified platelets, was diluted with salts solution and used in the subsequent experiments.

2. FLUORESCENCE:

All fluorescence experiments were performed on a specially modified spectrofluorimeter (Aminco-Bowman) equipped with an EMI 9781B photomultiplier tube. The excitation source was a 250W Xenon arc (Englehard Hanovia Inc.) focussed at the cathode spot. Emission outputs were fed directly to recorders or an oscilloscope display. Emission spectra were corrected using a computer programme which calculated the energy transfer efficiencies of the apparatus at differing wavelengths. Typically, 3 ml aliquots of cells, prepared as previously described, were stirred and maintained at 37° in a quartz 1 cm light path cuvette within the instrument housing. The system was allowed to attain equilibrium after the addition of 3,3'-dipentyloxacarbocyanine iodide (CC5) to a final concentration of 2 μM, and the fluorescence emission intensity was recorded continuously during and after the addition of test reagents (normally in 30 μl aliquots) to the cuvette. Using this amount of dye, further changes in CC5 concentration do not affect the observed fluorescence quantum yield in cell-free conditions. Ionophores were presented as a solution in ethanol which itself has no detectable effect on the fluorescence intensity.

Dyes of the carbocyanine family are known to reflect transmembrane potential difference (10) and CC5 in particular has been used most effectively in erythrocytes (11). Their mechanism of action however remains unclear. It has been suggested that the formation of non-fluorescent aggregates in the membrane occurs when the association of dye with membrane is promoted by the existence of a potential across the plasma membrane. Because

of the delocalised positive charge on the dye molecule, hyperpolarisation of the cell will encourage movement of the dye into the membrane where aggregation reactions will tend to decrease the observed fluorescence emission. We have confirmed the observation that the dye 3,3'-dipropylthiocarbocyanine iodide can form aggregates (10) but under a wide range of conditions the related CC5 molecule used in these studies does not. Clearly CC5 has an affinity for the hydrophobic environment of the plasma membrane in which it fluoresces more intensely than in an aqueous milieu (10). Thus the net fluorescence observed will be the resultant of a partitioning of dye between the extra- and intra-cellular compartments and the plasma membrane. This partitioning will be altered by potential difference changes.

3. ION TRANSPORT IN THYMIC LYMPHOCYTES:

To assess the effect of valinomycin and A23187 on ion uptakes, 45-Ca, 42-K, 22-Na, 32-PO4 were added to cell cultures to a final radioactivity of 1μCi.ml^{-1} at time zero, together with the agent under investigation. Aliquots of this mixture (0.2 ml) were immediately dispensed into microfuge tubes containing 50 μl formic acid overlaid with 100 μl silicone oil (DC550). After the appropriate incubation period at 37°C the tubes were spun in a Beckman Microfuge for 2 minutes and subsequently treated as described by Freedman et al. (12). Radioactivity of tube fractions was determined on either a Tracerlab Gamma set 500 gamma emission spectrometer, or on a Beckman LS 230 liquid scintillation spectrometer using 5 ml NE 260 (Nuclear Enterprises) as scintillant fluor. For efflux studies, cells were incubated for 50 min in the presence of 1 μCi.ml^{-1} of the different isotopes. After this period the cells were spun from suspension and washed three times in unlabelled medium to remove excess isotope. Subsequently cells were subjected to the agent under investigation as described above.

4. REAGENTS:

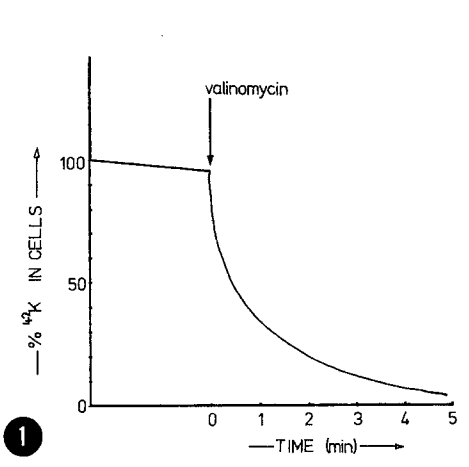
Initially CC5 was the generous gift of Dr. Alan Waggoner (Amherst College). Subsequent supplies of the dye were synthesised by one of us (JSB) using an established method (13). Valinomycin was obtained from the Sigma Chemical Co. The ionophore A23187 was the generous gift of the Lilly Research Centre Ltd. HEPES was obtained as a sterile solution from Flow Laboratories. Radioisotopes were purchased from the Radiochemical Centre, Amersham and silicone fluid DC550 from Hopkin and Williams.

RESULTS AND DISCUSSION

The potential which exists across biological (plasma) membranes is adequately described by the Goldman equation (14)

$$E = \frac{RT}{F} \text{ Ln } \frac{P_k(K_o) + P_{Na}(Na_o) + P_{Cl}(Cl_i)}{P_k(K_i) + P_{Na}(Na_i) + P_{Cl}(Cl_o)}$$

Thus alterations in the gradient for specific ions, or alterations in the permeability coefficient (P) for an individual ion, will alter the transmembrane potential (E_m). Our approach has been to apply the agents valinomycin and A23187, which have been shown to alter ion fluxes in defined cell



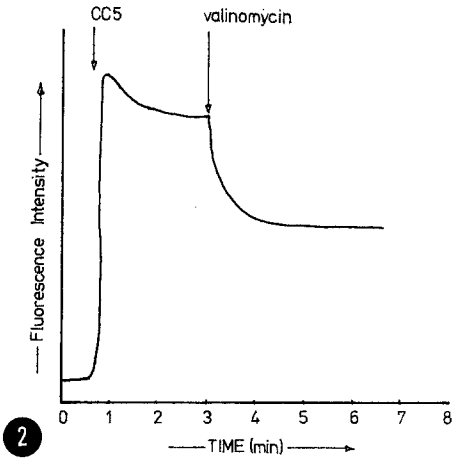


Fig. 1

Effect of adding valinomycin (10^{-5}M), to a suspension of thymocytes pre-loaded with ^{42}KCl, on the rate of efflux of potassium.

Fig. 2.

Effect of adding valinomycin (10^{-5}M) to a stirred suspension of thymocytes, equilibrated with the dye CC5, on the fluorescence emission from the system.

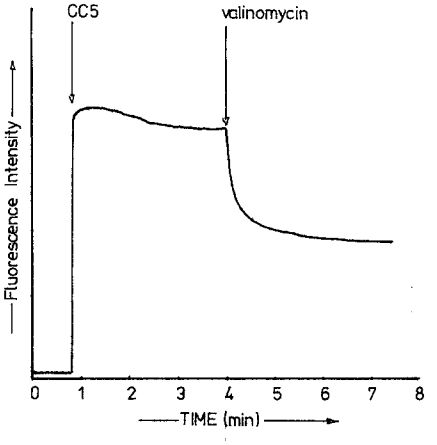


Fig. 3.

Effect of adding valinomycin (10^{-5}M) to a stirred suspension of splenic lymphocytes, equilibrated with the dye CC5, on the fluorescence emission from the system.

systems (15,16) and to relate the effects of these agents to changes in fluorescence in a previously equilibrated cell/dye system.

When valinomycin was added to thymic lymphocytes there was a marked efflux of ^{42}K from the cells which previously had been equilibrated with the isotope (Fig.1). Clearly, the membrane permeability to potassium is enhanced by valinomycin, so the predominant effect will be the movement of potassium down its concentration gradient, which, if unaccompanied by anion efflux, or an opposing cation influx, will cause membrane hyperpolarisation as it does in other systems (15).

When valinomycin was added to thymocytes which had been equilibrated with carbocyanine dye there was a significant decrease in fluorescence emission intensity (Fig.2) and similar changes were also observed when this procedure was repeated with other cell types (Figs.3,4.) These changes in fluorescence intensity were reduced when the extracellular potassium concentration was raised, and increased when it was lowered (Fig.5).

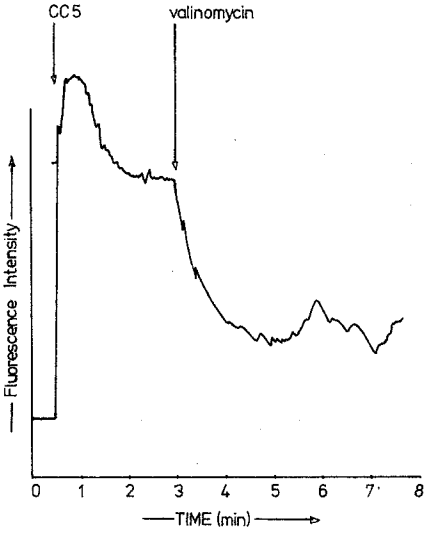


Fig.4

Effect of adding valinomycin (10^{-5}M) to a stirred suspension of human platelets, equilibrated with the dye CC5 on the fluorescence emission from the system.

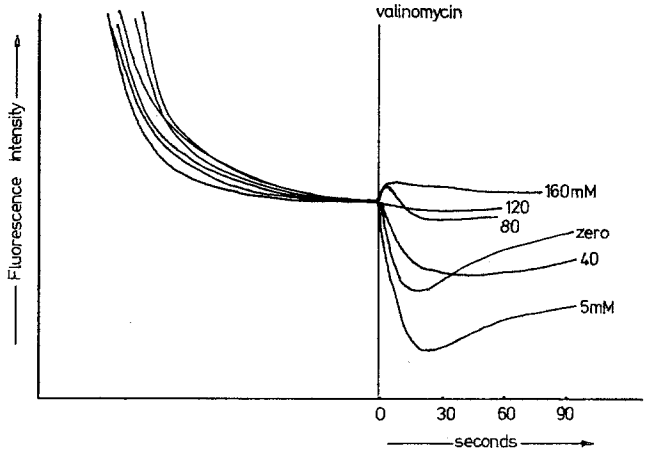


Fig.5.

Effect of adding valinomycin (10^{-5}M) to a stirred suspension of rat thymocytes, equilibrated with CC5, and with differing concentrations of potassium in the supporting medium, on the fluorescence emission of the system.

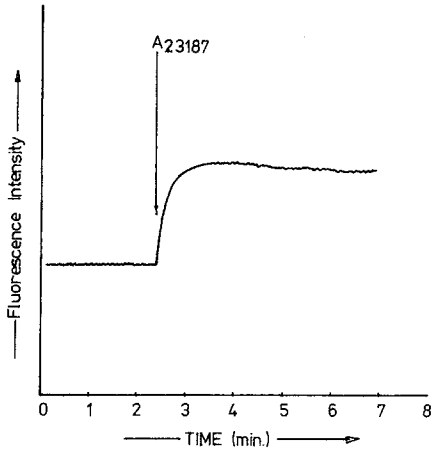


Fig.6.

Effect of adding A23187 (10^{-5}M) to rat thymocytes suspended in medium containing (0.6 mM) Ca., and equilibrated with CC5, on the fluorescence emission from the system.

When the calcium ionophore A23187 was used to increase membrane permeability to calcium ions, its addition caused a change in the fluorescence emission intensity (and hence a change in the trans-membrane potential)

Table 1.

To show the effect of adding Con A (5 μg ml^{-1}), A23187 (10^{-5}M) or Calcium (1.8 mM) to stirred suspension of lymphocytes on the movements in and out of the cell of several ionic species.

ION		Con.A.	A_{23187}	Ca^{+}
Na^{+}	IN	+	+	O
	OUT	N.T.	N.T.	N.T.
K^{+}	IN	+	+	O
	OUT	+	+	O
$PO_4^{///}$	IN	+	−	N.T.
	OUT	O	+	N.T.
Ca^{++}	IN	+	+	+
	OUT	+	+	N.T.

which was dependent, in magnitude and direction on the extracellular calcium concentration. This response is directly analogous to that observed with valinomycin, except that calcium is now the principal permeant ion. Fig. 6 illustrates this result, and Table 1 shows that A23187 not only facilitated calcium movements but also dramatically stimulated the efflux of K and PO_4, as well as the uptake of Na and K. In the absence of extracellular calcium A23187 promoted only trivial ion movements. Thus the resultant transmembrane potential is clearly a function of the concerted movements of several ions, and these movements are probably triggered by the action of calcium on the cell membranes. Fig. 7 shows that these events can be modified by the subsequent addition of EGTA. This has the effect of decreasing the extracellular concentration of ionised calcium, and lends more support to the assertion that these events are, at least in their initial stages,

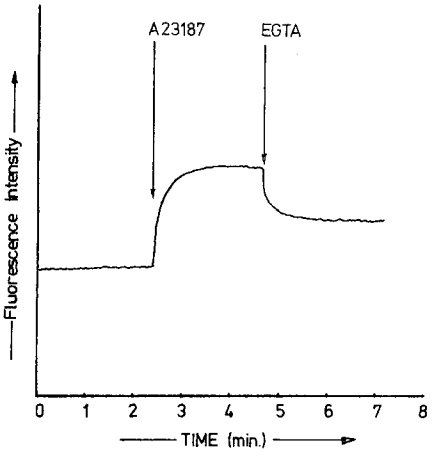


Fig.7.

Effect of the addition of A23187 (10^{-5}M) and the subsequent addition of EGTA on the fluorescence emission from a stirred suspension of rat thymocytes, equilibrated with CC5, in a medium containing 0.6 mM Ca.

largely calcium-dependent. These responses are common to all the cell types studied but do not apply to aged or lysed cells. In sonicated preparations containing membrane fragments neither valinomycin nor A23187 induced any alteration in fluorescence intensity. Furthermore in moribund cell cultures in which transmembrane ion gradients have degenerated no fluorescence changes could be elicited by the two ionophores. Therefore we believe that these changes in transmembrane ion balance may be involved in the cellular response to several humoral stimuli, and the technique described here permits the dissection of hitherto inaccessible membrane processes. It may be equally applicable to whole cells, liposomes and sub-cellular organelles. Thus even with the simplest instrumentation, valuable qualitative information can be gained about the responses of a variety of somatic cells to given stimuli. It is to be expected that the use of these techniques will gather momentum as the dependence of cell function on membrane responsiveness becomes more clearly recognised.

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