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# DICARBOCYANINE FLUORESCENT PROBES OF MEMBRANE POTENTIAL BLOCK LYMPHOCYTE CAPPING, DEPLETE CELLULAR ATP AND INHIBIT RESPIRATION OF ISOLATED MITOCHONDRIA

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## Summary

3,3′-Dipropylthiodicarbocyanine iodide, a widely used fluorescent probe of membrane potential, was found to inhibit anti-Ig antibody, induced capping of mouse lymphocytes. The dye also lowered the cell ATP content. Experiments with isolated mitochondria revealed that the probe had a potent inhibitory action at site I of the respiratory chain. This mitochondrial blockade helps to explain the ATP depletion and blockade of capping, and gives cause for caution in the use of this dye as a probe of cell membrane potential.

Three related dicarbocyanine dyes had similar toxic effects, but two cyanine dyes with much longer alkyl side chains, which have been used as probes of membrane fluidity, did not.

Certain dicarbocyanine dyes have been widely used to assess the membrane potential of cells or organelles that are difficult or, at present, impossible to study with microelectrodes [1,2]. One of the most widely used and best understood of these dyes is 3,3′-dipropylthiadicarbocyanine iodide (diS-$C_3$-(5)) [3,4]. However, diS-$C_3$-(5) may exert biological side effects at rather low concentrations. For example the dye blocks the $Ca^{2+}$-activated $K^+$ channel in human red blood cells [5] and the closely related compound Dithiazine (diS-$C_2$-(5)) has been used as an antihelminthic drug [6] which indicates some powerful toxic properties. During the examination

Abbreviation: diS-$C_3$-(5), 3,3′-dipropylthiadicarbocyanine iodide.

of the membrane potential of mouse spleen lymphocytes (Rink, T.J. and Montecucco, C., unpublished data) we observed that diS-$C_3$-(5) powerfully inhibited anti-Ig antibody induced capping of B lymphocytes [7—10]. This effect seems to be due to a depletion of lymphocyte ATP which is in turn the result of a blockade of mitochondrial respiration.

Lymphocytes were teased from the spleens of balb/C mice in Hank's minimal essential medium (Flow Laboratories) buffered with 20 mM Hepes to pH 7.3, and then purified on a Ficoll-Metrizamide gradient. After two washings the cells were suspended at $2—3 \cdot 10^7$ cells/ml. Aliquots of suspension were preincubated at 37°C for 5 min with the dyes added from dimethyl sulphoxide stock before either: (a) fluorescein-labelled rabbit, anti-mouse Ig antibody (Miles-Yeda) was added and incubation continued for 5 min, when the cells were fixed with formaldehyde and the percentage of stained cells showing capping measured, or (b) the suspensions were treated with 10% perchloric acid, then neutralised with NaOH and $NaHCO_3$ and the supernatant assayed for ATP by the luciferase method [11]. Rat liver mitochondria were prepared as described by Massari et al. [12]. The usual suspending medium contained: 200 mM sucrose, 20 mM Tris-HCl and 2 mM Tris phosphate, pH 7.2. Sometimes 4 mM Tris glutamate and 2 mM Tris malate were present in the medium and on occasion 100 mM KCl replaced the sucrose. Oxygen consumption was measured with a Clark electrode, at 20°C in a continuously stirred, thermostatted cuvette.

Fig. 1 shows the inhibitory effect of diS-$C_3$-(5) on lymphocyte capping. 25 nM dye reduced the number of capped cells to less than half the control value and capping was virtually abolished by 100 nM dye. Three other dicarbocyanine dyes (diS-$C_2$-(5), diI-$C_6$-(5) and diO-$C_2$-(3)) very similarly inhibited capping (see Table I). Two dyes with much longer alkyl side chains diI-$C_{14}$-(3) and diI-$C_{18}$-(3) which have been used as probes of membrane fluidity [13,14] did not affect lymphocyte capping at concentrations up to 0.5 $\mu$M and neither did a chemically unrelated fluorescent probe of potential, bis-(1,3-diethyl-thiobarbiturate)-trimethineoxonol (bis oxonol) [15]. The inhibitory dyes did not affect antibody binding, but halted the capping

TABLE I

THE EFFECT OF DIFFERENT DYES ON LYMPHOCYTE CAPPING, LYMPHOCYTE ATP LEVELS AND GRAMICIDIN-STIMULATED MITOCHONDRIAL RESPIRATION

DiS-$C_3$-(5), diI-$C_{14}$-(3) and diI-$C_{18}$-(3) were synthesized by Dr. Alan Waggoner, Amherst, MA. DiI-$C_6$-(5) and bis-oxonol were synthesized by Dr. Roger Tsien, Cambridge, U.K. DiS-$C_2$-(5) and diO-$C_2$-(3) were obtained from Koch-Light, U.K. For a discussion of nomenclature and structure of the cyanine dyes see Ref. 1.

| Dye | Inhibition of lymphocyte capping with 100 nM dye | Lymphocyte ATP, % control (mean ± S.E.) with 100 nM dye | Effect on gramicidin-stimulated mitochondrial respiration with 1 $\mu$M dye |
|---|---|---|---|
| DiS-$C_3$-(5) | + | 40 ± 7 | Inhibitory |
| DiS-$C_2$-(5) | + | 36 ± 8 | Inhibitory |
| DiO-$C_2$-(3) | + | 35 ± 10 | Inhibitory |
| DiI-$C_6$-(5) | + | 43 ± 12 | Inhibitory |
| DiI-$C_{14}$-(3) | — | No ATP depletion | No effect |
| DiI-$C_{19}$-(3) | — | No ATP depletion | No effect |
| Bis-oxonol | — | No ATP depletion | No effect |

process at the stage of patching [10], nor did they affect cell viability as judged by eosin red exclusion.

Many agents inhibit lymphocyte capping either by interfering with cytoskeletal function, as do the cytochalasins, or by acting as metabolic poisons [9,10]. DiS-$C_3$-(5) is known to bind strongly to protein and to form bound dimers on F-actin [4], as indicated by a new absorption peak at 595 nm. Such an absorption peak was observed when 1 $\mu$M diS-$C_3$-(5) was added to lymphocytes, and could have indicated dye binding to an actin-like protein. However, no such peak could be detected with 100 nM dye and unlike cytochalasin D [16], diS-$C_3$-(5) did not produce cap reversal and so an effect of the dye on some actomyosin-like function could not be supported. We therefore investigated the possibility that the inhibitory dyes were acting as metabolic poisons, by measuring lymphocyte ATP levels. Fig. 1 shows that diS-$C_3$-(5) reduced lymphocyte ATP levels. The maximum depletion seen was some 60%, similar to that observed with inhibitors of mitochondrial function such as rotenone or oligomycin [17]. The concentration dependence of the ATP depleting effect of the dye paralleled quite closely the effect on capping. As indicated in Table I, diS-$C_2$-(5), diO-$C_2$-(3) and diI-$C_6$-(5) also depleted lymphocyte ATP; the long chain cyanine dyes and bis oxonol did not. Why only 50—70% depletion of cellular ATP should be associated with almost total abolition of capping is not clear. Possibly the explanation lies in compartmentalisation of ATP within lymphocytes or in different populations of the mixed lymphocyte preparation.

Next, the basis of this ATP depletion was examined by assessing the effect of these dyes on the respiration of isolated mitochondria. Rat liver mitochondria were used, as a well characterised preparation and because mitochondria could not be isolated from mouse spleen lymphocytes in sufficient amounts. Fig. 2A shows the oxygen consumption of a mitochondrial suspension (protein content 1 mg/ml) in a buffered sucrose medium with

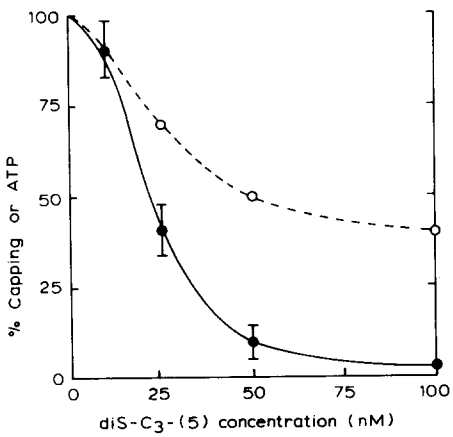


Fig.1. Effect of diS-$C_3$-(5) on lymphocyte capping and ATP content. Number of cells capping, •, is expressed as a percentage of the number capping after preincubation in dimethyl sulphoxide only. The bars indicate ± S.E. of 3 or more determinations. ATP content, ○, is expressed as the percentage of that in cells incubated with dimethyl sulphoxide only (approx. 5 mmol/l cells). The values are the means of 2 or 3 measurements.

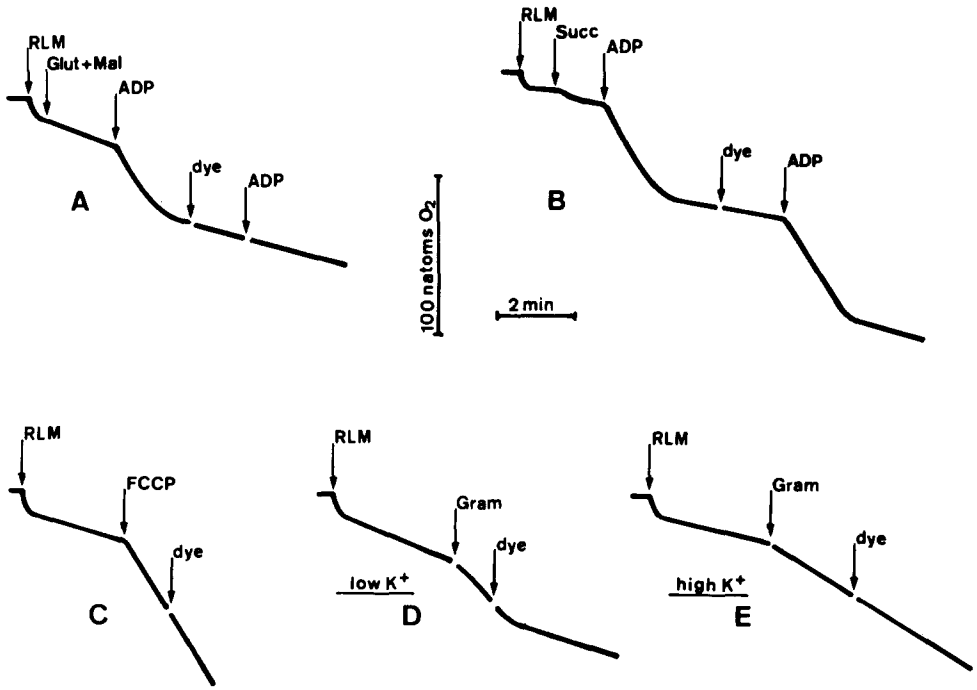


Fig.2. Effect of diS-$C_3$-(5) on the oxygen consumption of isolated mitochondria. In A, B and C the standard sucrose medium was initially in the cuvette. In D and E 4 mM Tris glutamate and 2 mM Tris malate were present from the start and in E 100 mM KCl replaced the 200 mM sucrose. Additions of mitochondria (RLM), 1 mg/ml protein; Tris glutamate and Tris malate 4 mM and 2 mM respectively; succinate, 2 mM; ADP, 100 $\mu$M; diS-$C_3$-(5), 1 $\mu$M; FCCP, 1 $\mu$M; or gramicidin, 500 nM; were made as indicated.

malate and glutamate added as substrates. Respiration showed a typical state 4-state 3-state 4 transition as added ADP was phosphorylated. 1 $\mu$M diS-$C_3$-(5) completely inhibited the stimulation of oxygen consumption normally evoked by addition of ADP, and in most experiments there was a 20—30% inhibition of state 4 respiration as well. In this medium, with malate and glutamate as substrates, respiration could also be stimulated by addition of 500 nM gramicidin (Koch-Light), 1 $\mu$M valinomycin (Sigma) with 1 mM $K^+$, or 200 $\mu$M $Ca^{2+}$, and 1 $\mu$M diS-$C_3$-(5) completely inhibited stimulation by these agents too, as shown for gramicidin in Fig. 2D. In most of the experiments 1 $\mu$M diS-$C_3$-(5) was used but 150 nM dye produced 50% inhibition of gramicidin-stimulated respiration. It should be noted here that although addition of only 100 nM of diS-$C_3$-(5) abolished capping in intact cells, the existence of a substantial negative membrane potential (Rink, T.J. and Montecucco, C., unpublished data) results in intracellular accumulation of these cationic, membrane permeant dyes [3], and so intracellular concentrations were probably substantially inhibitory on mitochondrial respiration. However, with succinate as substrate the inhibitory effect of diS-$C_3$-(5) was completely lost, as shown in Fig. 2B for ADP stimulation. The inhibition was again apparent in $\beta$-hydroxybutyrate medium. Since this substrate and malate and glutamate can cross the inner mitochondrial membrane [18, 19] and fuel the respiratory chain at site 1, whereas succinate activates at

site 2, diS-$C_3$-(5) must inhibit at site 1. This conclusion is in agreement with an unpublished observation cited by Waggoner [2]. Site 1 inhibition would not have vitiated the experiments of Laris et al. [20] who used diS-$C_3$-(5) as a fluorescent probe of mitochondrial membrane potential mainly in succinate media.

An interesting result was that shown in Fig. 2C where, in malate and glutamate medium, 1 $\mu$M diS-$C_3$-(5) failed to inhibit the stimulation of respiration evoked by the proton translocating uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). The same was seen with 20 $\mu$M dinitrophenol. It is known that these dyes will complex with FCCP and dinitrophenol and this could have prevented the inhibitory action of diS-$C_3$-(5). But examination of the absorption spectra of mixtures of dye and uncoupler in mitochondrial suspensions indicated no significant complexation, at the concentrations used. One major difference between the action of the uncouplers and the other stimulating agents is that the former collapse the mitochondrial membrane potential (see e.g. Ref. 20) whereas in the standard (0-K) medium the latter do not. This suggests that the dye is inhibitory only when a large negative potential results in its accumulation inside the inner compartment where site 1 is accessible. When the uncouplers collapse the potential much of the dye will redistribute into the external medium, onto the outer face of the inner membrane, and onto the outer membrane, greatly reducing the amount of dye available for interaction with site 1. A consequence of this idea is that if gramicidin or valinomycin are used to stimulate respiration in a high-$K^+$ medium the inhibitory effect of 1 $\mu$M diS-$C_3$-(5) should be lost, since in that medium these ionophores will collapse the potential [20]. This is just what was found; Fig. 2E shows that in 100 mM KCl medium gramicidin stimulation of respiration was no longer blocked by the dye — in contrast to the total inhibition seen in the 0-K sucrose medium in Fig. 2D. The same loss of the inhibitory action was seen with valinomycin-stimulated respiration in 100 mM KCl medium. So the effective inhibitory action of diS-$C_3$-(5) seems to depend on the mitochondrial membrane potential, because that potential governs its distribution.

Table I summarizes the effects of the other dyes on gramicidin-stimulated mitochondrial respiration. DiS-$C_2$-(5), diO-$C_2$-(3) and diI-$C_6$-(5) were inhibitory at site 1 of the respiratory chain, but the long chain cyanines and bis oxonol were not. It may be that the long chain carbocyanines partition so strongly into lipid membranes that they do not actually enter the cytoplasm of intact cells, or the inner compartment of intact isolated mitochondria. None of the dyes appeared to have any uncoupling action.

These results show a potent and specific inhibitory action of diS-$C_3$-(5) and related dyes at site 1 of the mitochondrial respiratory chain. Those used as probes of membrane potential readily permeate biological membranes and therefore, at the concentrations usually employed, 0.2—2.0 $\mu$M are likely to depress mitochondrial function. Clearly caution must be applied to the interpretation of results of studies using dicarbocyanine dyes as probes of cell membrane potential, since mitochondrial blockade could produce a rapid alteration in the intracellular environment. Perhaps dicarbocyanine dyes will prove to be a useful addition to the range of mitochondrial inhi-

bitors. They are potent and very membrane-permeable, and should not themselves dissipate electrical or ionic gradients.

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