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Permeabilization of thymocytes by photon activation of erythrosin

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Thymocytes previously loaded with quin 2 were rapidly permeabilized by the photon activation of erythrosin and the rate of permeabilization monitored by measuring fluorimetrically the increasing saturation of quin 2 with calcium. The extent of permeabilization was assessed also by the loss of [^{3}H]quin 2 from the thymocytes and penetration of the cells by eosin and trypan blue. Lactate dehydrogenase leakage from the permeabilized cells was markedly delayed compared to the rapid increase in permeability to calcium and quin 2. The rate of permeabilization was dependent upon the concentration of erythrosin, the duration of illumination, the presence of oxygen, and the temperature. These results are consistent with the rapid photochemical generation of highly reactive singlet oxygen which alters thymocyte membrane structure and permeability.

Thymocyte; Membrane permeabilization; Erythrosin; Photon activation; Quin 2; Singlet oxygen

1. INTRODUCTION

Anionic fluorescein derivatives are largely confined to the extracellular space [1]. Recently Matthews and Mesler [2] showed that photon activation of erythrosin (tetraiodofluorescein) caused a marked calcium-dependent contraction of the smooth muscle cells of the guinea-pig taenia coli superfused in vitro. It was concluded that the reaction involved the generation of singlet oxygen and that the photodynamic action of erythrosin presented a novel method for modulation of membrane calcium permeability [2]. Exploration of Ca^{2+}-dependent and other intracellular events associated with activation of thymocytes would be greatly facilitated by rapid access to the intracellular compartment of the cells without loss of intracellular macromolecules. We have therefore investigated the applicability of the technique of photodynamic membrane permeabilization for this purpose.

Changes of cellular calcium permeability in thymocytes and other cells can be measured readily by quin 2, a fluorescent derivative of EGTA introduced into intact cells in the form of the permeant acetoxymethyl ester derivative [3]. Once the ester is hydrolyzed in the cytoplasm, the tetranionic quin 2 is trapped within the cell and the proportion of quin 2 complexed with calcium can be determined by fluorescence analysis [4]. In the study reported here, thymocytes have been permeabilized by the photon activation of erythrosin and quin 2 used as a fluorescent indicator to measure the rate and extent of cell membrane permeabilization. The conditions under which thymocytes can be permeabilized to Ca^{2+} without loss of cytosolic lactate dehydrogenase have also been defined.

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2. MATERIALS AND METHODS

Thymocytes from 4- to 6-week-old mice of either sex were isolated as described [5], centrifuged for 3 min at 300 × *g*, and resuspended at 10^7 cells per ml. The medium used for all experiments unless otherwise specified was a balanced salt solution containing glucose: 140 mM NaCl, 5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 0.43 mM $CaCl_2$, 11 mM glucose, buffered with 10 mM Hepes at pH 7.3. Thymocytes were loaded with quin 2 by incubating with 3.7 μM [^{3}H]quin 2-acetoxymethylester (quin) 2 AME [6] for 35 min at 37°C. The cells were then centrifuged for 3 min at 300 × *g* and resuspended in balanced salt solution at approx. 7.5 × 10^6 cells per ml. After further incubation for a minimum of 30 min at 37°C, the required number of cells were pelleted by centrifugation and resuspended at a concentration of approx. 5 × 10^6 cells per ml.

All fluorescence measurements were made in a Perkin-Elmer 44E spectrofluorimeter equipped with a constant temperature cuvette holder and magnetic stirrer. Unless otherwise stated, 3 ml samples of cell suspensions or solutions were stirred continuously at 37°C. The excitation wavelength was 339 nm and the emission wavelength was 492 nm. The percentage of quin 2 complexed with calcium (% quin 2 saturation) was calculated from: $(I - 0.16\Delta I)/(0.84\Delta I) \times 100$, where I is the fluorescence intensity of the quin 2-calcium complex in the cell and ΔI is the fluorescence intensity of the quin 2-calcium complex after saturation with calcium, both I and ΔI being corrected for the background fluorescence in the presence of the quenching ion, Mn^{2+} [6]. Fluorescence measurements of quin 2 saturated with Ca^{2+} and subsequently fully quenched by Mn^{2+} were made at the end of each experiment by treating the suspension with 10 μl of 10% Triton X-100, which lyses the cells and allows saturation of quin 2 with extracellular calcium, followed by the addition of 10 μl manganese chloride (50 mM) to quench the quin2 fluorescence.

Controlled illumination of the solutions or cell suspensions containing erythrosin (erythrosin B, sodium salt; Sigma) was by a Schott KL 150 quartz-halogen light source equipped with a heat filter (KG1) and producing a maximum intensity of 14 × 10^4 lux at 640 nm. A fibre optic probe provided a 60° illumination field of uniform intensity that could be directed into the top of a fluorimeter cuvette (1 cm square) containing 3 ml of solution. The top of the fibre optic probe was positioned 1 cm above the surface of the stirred solution and generated a light output > 10^5 lux at the surface. The excitation and the emission slits on the fluorimeter were closed during the illumination process. All experiments were carried out at 37°C unless otherwise indicated. The time course of changes in fluorescence intensity is reported from the beginning of illumination.

To deplete thymocyte suspensions of oxygen, cells were pelleted by centrifugation and resuspended in balanced salt solution that had been purged with N_2 for at least 5 min. In permeabilization experiments, these cell suspensions were stirred in the fluorimeter under an atmosphere of N_2.

The extracellular LDH activity of cell suspensions was assayed by adding 45 μl suspension to 955 μl isotonic assay solution (1 mM pyruvate and 10 mM NADH in 136 nm NaCl, 18.5 mM Hepes, pH 7.2) and following the change of absorbance at 340 mM in a Perkin-Elmer 557 spectrophotometer. The total LDH activity of the same cell suspension was measured following the addition of 0.1% (v/v) Triton X-100. Balanced salts solution, erythrosin and quin 2 had no measurable absorbance effect on the assay at the concentrations used.

3. RESULTS

The use of quin 2 for the measurement of Ca^{2+} concentration after the illumination of thymocytes in the presence of erythrosin is illustrated in fig.1. In fig.1a, spectrum I is that of thymocytes loaded with quin 2 only. On addition of erythrosin, 5 μM (in the absence of illumination), the quin 2 emission peak at 492 nm is lowered slightly and a second emission peak, that of erythrosin itself appears at 555 nm (spectrum II). The small decrease in quin 2 fluorescence upon addition of erythrosin is explained by the fact that the erythrosin absorption spectrum, which peaks at 538 nm, has a slight shoulder at 492 nm.

When the quin 2 loaded cells exposed to erythrosin were illuminated, the Ca^{2+} level detected by the quin 2 increased markedly (spec-

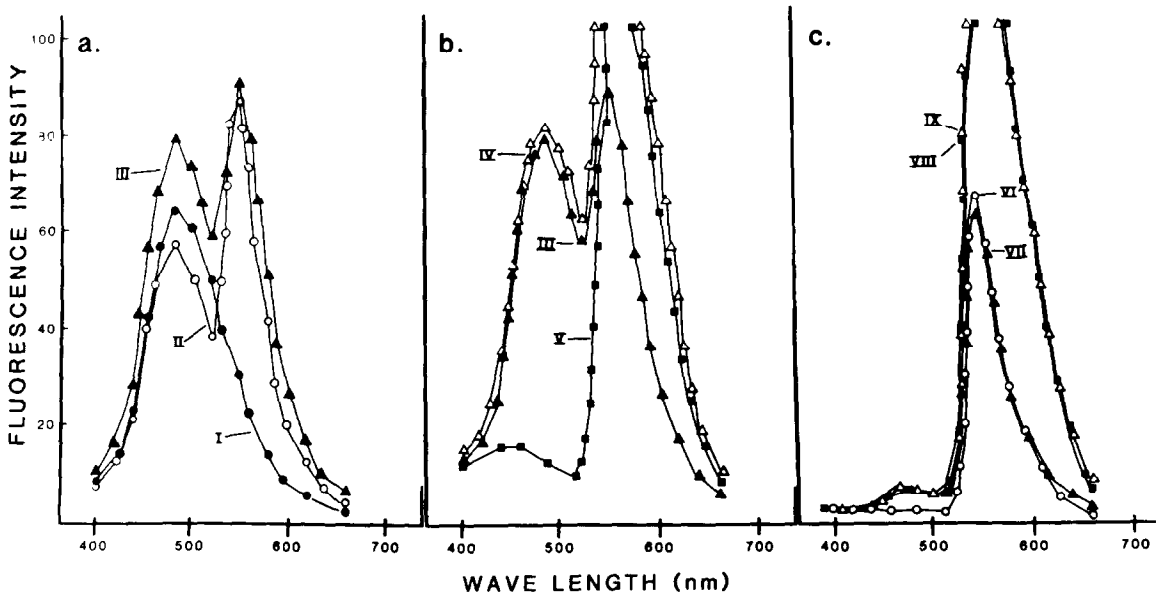

Fig.1. Fluorescence from quin 2-loaded thymocytes before and after the addition and photon activation of erythrosin. (a) Spectrum I, quin 2-loaded thymocytes in balanced salts solution (●); spectrum II, after the addition of erythrosin, 5 μM (○); spectrum III, 1 h after illumination for 2 min (▲). (b) Spectrum III, as in (a); spectrum IV, after the addition of Triton X-100, 0.03% (△); spectrum V, after the addition of Mn^{2+}, 166 nM (■). (c) Control experiments using non-quin-2-loaded thymocytes. Spectrum VI, 5 μM erythrosin in balanced salts solution (○); spectrum VII, after illumination for 2 min (▲); spectrum VIII, after the addition of Triton X-100, 0.03% (■); spectrum IX, after the addition of Mn^{2+}, 166 nM (△).

trum III). That this was due to almost total permeabilization of the thymocytes is confirmed in fig.1b since spectrum IV, which follows the addition of Triton (0.03%) to lyse the cells, shows little difference from spectrum III. The specificity of the quin 2 reaction is demonstrated by spectrum V taken after the addition of Mn^{2+} to completely quench the quin 2 fluorescence by displacement of Ca^{2+}. These interpretations are substantiated by the spectra of fig.1c (i.e., spectra VI, VII, VIII and IX) which illustrate the results obtained in parallel experiments with thymocyte suspensions containing no quin 2.

These experiments indicate that thymocytes are permeabilized by illumination in the presence of erythrosin but the increase in quin 2 fluorescence alone does not allow a distinction to be made between the entry of extracellular calcium into the permeabilized cells and the efflux of quin 2 into the calcium containing medium. Further experiments were therefore carried out using thymocytes loaded with [^{3}H]quin 2. The fluorescence of the [^{3}H]quin 2 was monitored in both illuminated and non-illuminated thymocyte suspensions containing erythrosin. An increase in quin 2 fluorescence occurred only in the illuminated sample as the cells were permeabilized (fig.2a). The relative intracellular/extracellular distribution of the [^{3}H]quin 2 before and after illumination is illustrated in fig.2b. Permeabilized cells lost 80–90% of the [^{3}H]quin 2 from the intracellular compartment whereas $<5\%$ of labelled quin 2 was lost from the control, non-illuminated, cells during the same time interval (fig.2b). Other experiments under comparable conditions with unlabelled quin 2 (not shown) confirmed that any quin 2 remaining associated with the cells after permeabilization (i.e. 10–20% of the total) was accessible to extracellular Ca^{2+}.

Experiments similar to those of fig.2 were carried out to assess the cellular penetration of eosin and trypan blue as a further measure of permeabilization. It was found that illumination in the presence of erythrosin (5 μM) caused $>99\%$

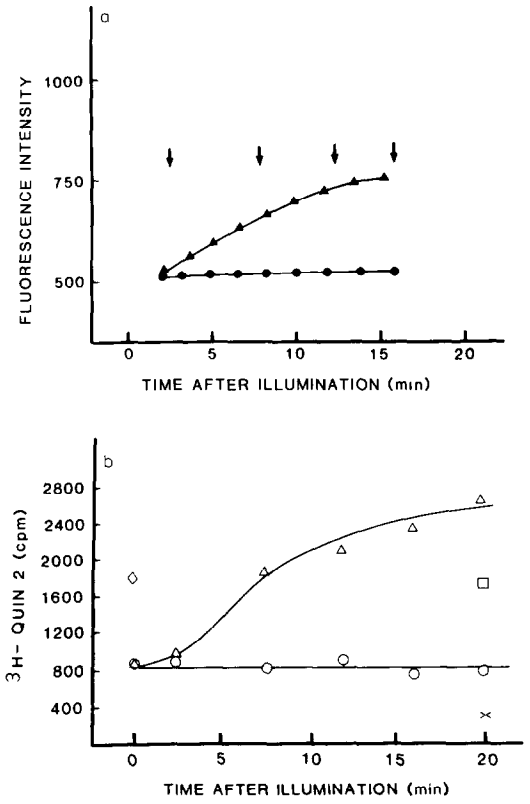

Fig.2. The permeabilization of quin 2 loaded thymocytes by the photon activation of erythrosin. (a) Thymocytes in balanced salts solution containing 5 μM erythrosin were illuminated for 2 min beginning at time zero (▲). The control sample was treated in the same way but without illumination (●). (b) Samples were taken at the times indicated (↓) in (a) and assayed for leakage of [^{3}H]quin-2 in (b). Extracellular [^{3}H]quin 2 is plotted for illuminated (△) and non-illuminated (○) cells, and was determined by scintillation counting of the supernatant after centrifugation of the cells at the time points indicated in (a). Intracellular [^{3}H]quin 2, determined by either the difference between total and extracellular [^{3}H]quin 2 or the resuspension of the cell pellet after centrifugation, is plotted at the beginning (◇) and end of the experiment for illuminated (×) and non-illuminated cells (□).

thymocytes to become permeable to eosin and trypan blue, but only 13% and 6%, respectively, of non-illuminated cells were permeable to these dyes.

The experiments described above demonstrate that the thymocyte permeabilization process depends upon the photon activation of erythrosin by illumination; light alone or erythrosin alone are ineffective. The rate of the permeabilization reaction upon illumination increased as a function of erythrosin concentration and was also dependent on the duration of illumination. If oxygen was removed (by purgation with N_2) the photon-induced permeabilization of thymocytes was inhibited but could still be elicited on the same sample of cells once O_2 was restored to the system. Other experiments demonstrated that the rate of permeabilization in normal medium was much decreased below 37°C (fig.3).

To assess the extent to which the permeabilization process led to loss of molecules larger than quin 2 and Ca^{2+}, the amount of cytosolic LDH released into the extracellular space was determined in parallel with the change in quin 2 fluorescence in both illuminated and non-illuminated cells. The results are illustrated in fig.4 from which it can be seen that there was a rapid permeabilization to quin 2 and Ca^{2+} with an effect 50% of the maximum after 30 min. In contrast, there was little or no leakage of LDH from the cells over the same time period after illumination. Control experiments established that illumination in the presence of erythrosin did not cause inactivation of LDH within the cells.

The stability of thymocyte cell structure after permeabilization was determined by cell counts performed by optical microscopy on parallel samples of thymocyte suspensions over 7 h after illumination. One sample was illuminated in the presence of erythrosin (5 μM) for 2 min to

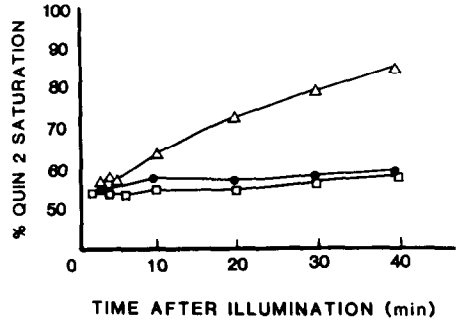

Fig.3. Dependence of the permeabilization reaction on temperature. Thymocytes in balanced salts solution containing 5 μM erythrosin were illuminated for 80 s, at the following temperatures: (□) 25°C, (●) 31°C, (△) 37°C.

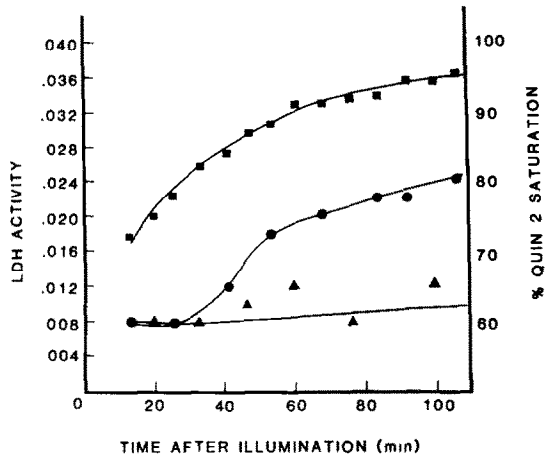

Fig.4. The leakage of LDH from thymocytes following permeabilization. Thymocytes in balanced salts solution containing 5 μM erythrosin were illuminated for 75 s. Control cells were not illuminated. The cells were centrifuged and resuspended in the same volume. The percent saturation of quin 2 in the permeabilized sample (■) and the extracellular LDH activity of both the illuminated sample (●) and the control sample (▲) were followed as a function of time after the beginning of illumination.

permeabilize the cells; the other sample was not illuminated. The number of cells in both samples remained constant over the 7 h period demonstrating that permeabilized cells retain a defined matrix and do not disintegrate.

4. DISCUSSION

The results described demonstrate that, using quin 2 fluorescence as an indicator, permeabilization of thymocytes occurs on the photon activation of erythrosin. The distribution of ^{3}H-labelled and of unlabelled quin 2 between the intracellular and extracellular compartments and the penetration of the cells by eosin and trypan blue all indicate a rapid permeabilization process. The magnitude of the permeabilization reaction of thymocytes is dependent upon the concentration of erythrosin, the intensity and duration of the incident light, and the presence of oxygen, i.e., exactly those factors found previously to determine the magnitude of erythrosin-induced contraction of smooth muscle cells [2]. Additionally we have shown that the rate of permeabilization of thymocytes by erythrosin is responsive to temperature. All these findings are consistent with the photon activation of erythrosin initiating the generation of highly reactive singlet oxygen, O_2 ($^1\Delta_g$) which interacts with a component of membrane ternary structure and alters permeability (see [2]).

Quin 2 fluorescence facilitates a continuous study of the kinetics of the permeabilization process following the virtually instantaneous photon activation of erythrosin and generation of singlet oxygen. For example, the results of quin 2 and LDH measurements in parallel indicate that the membrane is rapidly permeabilized to small molecules whereas the leakage of cytosolic macromolecules such as LDH is considerably delayed. With these observations as a starting point it may be possible to develop precise control of molecular access to the thymocyte intracellular compartment by a photochemical process. Once exogenous molecules of interest have been permitted to enter the permeabilization 'window' it may be possible also to re-establish the cell membrane barrier by annealing the cell membrane. These are both reasonable possibilities because our experiments establish that the gross structural features of the thymocytes are retained for several hours after membrane permeabilization.

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