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Plasma membrane depolarization and calcium influx during cell injury by photodynamic action

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The plasma membrane has been implicated as one of the critical targets of photodynamic action. We observed that plasma membrane depolarization is an early event in the photodynamic action of zinc phthalocyanine disulfonate in mouse myeloma cells, showing both photosensitizer concentration and light dose dependence. The depolarization was observed immediately upon exposure to light, while membrane integrity was retained and showed a strong correlation with cell killing. In this study the use of channel blockers and alteration of ion concentration was employed to determine the factors involved in the membrane depolarization process. A general rise in cation permeability is associated with the depolarization. Loss of intracellular potassium was detected and an increase in intracellular free calcium was also observed. Sodium was found to strongly influence the photosensitized depolarization.

Introduction

Alteration of plasma membrane structure and function has been increasingly recognized as a significant effect associated with cytotoxic damage from reactive oxygen species. Reactive oxygen species have been identified as a likely cause of cell injury in several pathophysioligical states including inflammation, aging, carcinogenesis and ischemia [1]. Endogenous as well as exogenous chemicals such as light-activated photosensitizers, have been shown to generate singlet molecular oxygen ($^1O_2$) and perhaps superoxide anion (O_2^-), two forms of activated oxygen [2–4]. Strong evidence indicates that hydrophobic sensitizers such as those in the porphyrin family which localize in membranes induce oxidation of intrinsic membrane protein [5,6] and lipids [7,8] with proteins having been found to be more susceptible to oxidation than lipids. Examination of the effect of photodynamic action on mammalian membrane systems may consequently provide important information on both the mechanism of photodynamic action itself as well as factors associated with disease states arising from reactive oxygen species.

Oxidation of either membrane lipids or proteins may affect the intrinsic properties of the membrane. Cellular ion homeostasis may be altered as a result of the oxidation of plasma membrane components, for example by the formation of membrane pores or alteration of membrane fluidity, which will subsequently alter the mobility of ions through the bilayer. In many investigations of the effect of oxidative stress on cells, by superoxide formation [9] or photodynamic action [2,5,10] leakage of intracellular potassium and increased sodium uptake have been observed as critical early events in cell injury. This alteration in ion homeostasis can be expected to produce a change in the resting membrane potential.

Abbreviations: BCECF/AM, 2',7'-bis(2-carboxyethyl)-5(and 6)-carboxyfluorescein acetoxymethyl ester; di-SBA-C_2(3), bis(1,3-diethylthiobarbiturate)trimethine oxonol; DMSO, dimethyl sulfoxide; *F*, fluorescence; fura-2, (1-[2-5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate; HPS, Hepes physiological saline; Mes, 2-*N*-morpholinoethanesulfonic acid; pH_i, intracellular pH; TEA, tetraethylammonium; Tris, tris(hydroxymethyl)aminomethane; $ZnPcS_2$, zinc phthalocyanine disulfonate; $\%\Delta\Psi$, percent change in membrane potential.

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We have previously shown that photodynamic action induced by zinc phthalocyanine disulfonate ($ZnPcS_2$) and red light (620–700 nm) results in the depolarization of the plasma membrane of mouse myeloma cells (neoplastic lymphocytes) as measured using the potential sensitive indicator di-SBA-C_2(3) [11]. The depolarization was found to be dependent on the photosensitizer concentration and light dose and was also shown to be strongly correlated with loss of clonogenic ability in mouse myeloma cells. Several other observations of membrane potential alterations by oxidative stress on non-excitable cells have also been reported [12–14].

Research examining the effect of photodynamic action on excitable cells is an ongoing process [15–18]. Kohli and Briant [15] reported that photodynamic action induced depolarization of muscle membranes. Since this effect was reduced when choline was substituted for external sodium, they attributed it to an anomalous rise in sodium permeability. Pooler [16,17] has carefully examined the photodynamic effect in lobster giant axons using voltage clamp analysis and the sucrose gap method. A fall in resting potential during illumination with no recovery was detected. It was speculated that the depolarization resulted from the increased leakage conductance which was detected but could not be accurately measured due to the lack of sensitivity of the sucrose gap method. In addition, irreversible decreases in the maximum sodium and potassium conductances were observed which were attributed to blockage of channels. A shift of some of the voltage dependent parameters for both sodium and potassium channels to more positive internal potentials and a slowing of the kinetics for both sodium and potassium, including a pronounced prolongation of sodium current was also observed in unblocked channels. More recently, Valenzeno and Tarr [18] have examined the effects of photodynamic action on frog atrial cells using the patch clamp technique. The potassium current from delayed rectifier channels was reported to decrease exponentially with illumination and did not recover. Observation of an increase in leakage current which increased non-linearly at higher sensitizer concentration and light dose was also reported. Since lymphocytes also possess activatable ion channels it is of interest to determine whether these channels are involved in the membrane depolarization process.

The focus of the research presented in this paper was to examine the factors involved in the membrane depolarization process. In this study the concentration of various cations or anions was altered and specific channel blockers were added in order to determine the effect on the rate of plasma membrane depolarization induced by 10 nM $ZnPcS_2$. The study of the disruption of ion homeostasis was extended to examine the effect of photodynamic action on the intracellular free calcium concentration, $[Ca^{2+}]_i$.

This work was presented in part at the annual meeting of the American Society for Photobiology, June, 1990.

Materials and Methods

Solutions. Tissue culture reagents, salts, dimethylsulfoxide (DMSO), amiloride, tetrodotoxin, 4-aminopyridine, quinine and gramicidin, were obtained from Sigma Chemical Co., St. Louis, MO. The potential-sensitive probe, bis(1,3-diethylthiobarbiturate)trimethine oxonol (di-SBA-C_2(3)), the pH_i indicator, 2′,7′-bis(2-carboxyethyl)-5(and 6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM) and the acetoxymethyl ester of the calcium indicator, fura-2, (1-[2–5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid were obtained from Molecular Probes, Eugene, OR.

A mixture of mono- and disulfonated zinc phthalocyanine ($ZnPcS_2$) enriched in the amphiphilic isomers was a gift from Dr. J. van Lier, University of Sherbrooke, Sherbrooke, QC Canada. Concentrations of stock solutions were based on the molar absorption of the dye in methanol and buffer solutions [19]. Dilutions of aqueous stock solutions were made into Hepes physiological saline for cell studies.

Cell preparation. Mouse myeloma cells (strain NS1) were maintained in suspension at 37 °C, 5% CO_2 in Dulbecco's modified Eagle medium supplemented with 10% horse serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.25 μg/ml fungizone, 50 units/ml penicillin and 50 mg/ml streptomycin.

The standard Hepes Physiological Saline (HPS) solution was made to approximate the ion composition of the culture medium. It contained 20 mM Hepes (*N*-2-hydroxyethyl-4-piperazine-*N*′-2-ethanesulfonate), 110 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.9 mM NaH_2PO_4, 25 mM glucose and 44 mM $NaHCO_3$, pH 7.4, 37 °C.

Photosensitization / membrane potential effects. Membrane potential measurements and photodynamic effects were described previously [11]. Briefly, mouse myeloma cells were washed twice and resuspended in HPS containing 10 nM $ZnPcS_2$. Cells were incubated for 1 h at 37 °C in the dark, then washed and resuspended in HPS or the appropriate buffer at 10^6/ml. Three milliliters were added to quartz fluorescence cuvettes and the potential-sensitive indicator, di-SBA-C_2(3) in DMSO, was added to a final concentration of 75 nM. Suspensions were incubated in the fluorometer for 10 min. to equilibrate.

Fluorescence measurements were recorded on a SPEX Fluorolog spectrofluorometer, equipped with a temperature controlled 4-cell holder maintained at 37 °C. Fluorescence was recorded using the front-face arrangement with excitation at 540 nm, 2 mm slitwidth

and emission at 580 nm, 4 mm slitwidth. The initial fluorescence was recorded. Cell suspensions were then illuminated for incremental periods of time using a slide projector equipped with a 750 watt tungsten-halogen bulb, a >620 nm cut-on filter and 700 nm cut-off heat filter. The light fluence over the region 620–700 nm was determined using a GE type 213 light meter. Cells were illuminated at room temperature then returned to the fluorometer and the fluorescence recorded at 37 °C once the temperature was stabilized and a steady reading was observed. The cells were again illuminated and the fluorescence recorded. The maximum total illumination time was 10 min corresponding to a total light fluence over the phthalocyanine absorbance band of 7.2 kJ/m^2. Following the last illumination, the cells were totally depolarized by addition of KCl to give a final concentration of 150 mM KCl in the medium or 10 μl gramicidin (0.2 mg/ml in DMSO for K^+ channel blocking experiments. We previously showed that similar changes in fluorescence were observed by either method. The % change in potential (%$\Delta\psi$) was determined from the difference in fluorescence (F) using Eqn. 1 as shown previously:

$$\%\Delta\psi = \left(\frac{F_{\text{treatment}} - F_{\text{initial}}}{F_{\text{final}} - F_{\text{initial}}}\right)100. \quad (1)$$

Ion substitution or channel blocker effects. For studies on the contribution of a specific ion to the membrane potential effect, buffers were made by isosmotic substitution of the ion of interest. Choline chloride was substituted for sodium chloride in the reduced sodium buffers. Sodium gluconate and potassium gluconate were used in place of NaCl and KCl for chloride free buffers. Calcium chloride was replaced by sodium chloride. Bicarbonate free buffers were made by replacement with NaCl.

In general, for channel blocking experiments, appropriate amounts of the channel blocker were added from concentrated stock solutions in DMSO or ethanol. A few blockers slightly hyperpolarized the cells as explained in Results. Tetrodotoxin was stored as an acidic aqueous stock solution (pH 4.0). In the case of the potassium channel blocker, tetraethylammonium chloride (TEACl), 40 mM TEA was substituted for 40 mM NaCl. Vehicle controls performed at the highest dose of DMSO or ethanol were the same as untreated controls. Dark controls were performed for each channel blocker and only those which did not modify the probe performance were used for further experiments.

Intracellular potassium. Intracellular K^+ was determined on mouse myeloma cells treated with 10 nM photosensitizer, as above. Volumes were scaled up so that a total of 15 ml was irradiated. Three milliliters were removed for membrane potential measurements. Following irradiation, 10^7 cells, as determined by hemocytometer count, were centrifuged then washed in cold 150 mM choline chloride, 25 mM glucose (pH 7.4) lysed in 100 μl double-deionized water, then assayed for intracellular K^+ by flame photometry. The amount of total protein (mg) was also determined for each sample by the standard Lowry colorimetric assay (Sigma Chemical Co.). Dark controls treated with photosensitizer, but without irradiation were run in each experiment. Intracellular potassium levels were determined based on the total protein (e.g. mequiv. K/mg protein).

Internal pH measurements. Determination of the internal pH was performed according to the method of Rink, et al. [20]. The cells were loaded with the pH-sensitive indicator BCECF by incubating with the acetoxymethyl ester derivative (0.5 μM) in HPS at 10^6 cells/ml for 1 h at 37 °C. For photosensitization experiments, 10 nM $ZnPcS_2$ was also present during the incubation period. The cells were then washed twice in bicarbonate free HPS and resuspended at 10^6/ml for fluorescence measurements, using 460 nm and 500 nm excitation (e.g. scan from 460 to 500 nm using a 40 nm increment) with emission at 530 nm using 1 mm and 3 mm slitwidths, respectively. Measurements were made using the front face mode and cell holder thermostated at 37 °C. The ratio of 500/460 nm was used to determine the intracellular pH (pH_i).

The nigericin/potassium method of Thomas et al. [21] was used to calibrate the pH_i. Briefly, cells were suspended in high K^+ Hepes buffer (135 mM KCl, 20 mM NaCl). Nigericin, a H^+/K^+ exchanger, was added from an ethanol stock solution to give a final concentration of 3 μM. The external pH was measured using a semi-micro pH electrode. The pH of the medium was changed by addition of either Tris base (tris(hydroxymethyl)aminomethane) or Mes (2-*N*-morpholinoethanesulfonic acid) from 1 M stock solutions. The fluorescence was recorded after a 1–3-min incubation period to allow for equilibrium of pH_i with pH_o.

For effects due to photodynamic action, cells were loaded with both $ZnPcS_2$ and BCECF as described above. The initial fluorescence ratio was recorded and the cells were than illuminated as before with the steady-state BCECF fluorescence recorded between light doses. A dark control was also run. Potassium chloride and nigericin were added to the cell suspension following the last irradiation. pH_i was calibrated and compared to untreated calibration controls.

ATP determination. Cellular ATP levels were determined using the standard luciferase assay which is commercially available (ATP Bioluminescent Assay Kit from Sigma Chemical Co.) Photosensitized cells were compared to dark controls.

Determination of internal calcium concentrations. Mouse myeloma cells (10^6/ml) were loaded with the

acetoxymethyl ester of fura-2 by incubating with a 0.5 μM solution in buffer for 1 h at 37 °C in the dark. For photosensitization experiments, the cells were incubated with both the calcium indicator and photosensitizer. The dye loaded cells were then washed twice in buffer and resuspended in HPS at 10^6/ml. In some experiments calcium-free buffers were used. Three milliliters of the cell suspension were added to a fluorescence cuvette and fluorescence measurements were made using 340 nm and 380 nm excitation (40 nm increment scan) and 510 nm emission, using the front face mode in a 37 °C thermostated cuvette holder on the Spex fluorolog. Measurements were taken after each irradiation time point. Relative calcium concentrations were calculated based on previously published methods [22,23]. (Note: concentrations are determined as relative as opposed to actual values because of the approximations and thus potential errors which must be made in order to do the calculations, as described in the above references. Even so, these methods are more than adequate to determine basic trends.) Briefly, F_{max} was determined by lysis of cells by Triton-X 100 into the extracellular buffer containing 1.8 mM calcium. F_{min} was determined by adding an excess of EDTA (10 mM) and adjusting the pH with Tris base. Intrinsic leakage of the dye from the cells over the time period of the light treatment was determined as an increase in fluorescence in sensitizer-free dark controls. This was subtracted from the photosensitizer treated samples. To confirm that the resulting fluorescence increase was due to an increase in Ca^{2+} uptake and not leakage of fura-2 from the cell 1 mM $MnCl_2$ was added to both dark controls and photosensitized cells to quench fluorescence of external fura-2. Cells were also examined under a Zeiss fluorescence microscope with 365 nm excitation, and observing emission above 395 nm. This indicated a diffuse fura-2 fluorescence throughout the cells which was still present following the highest light treatment in the 10 nM $ZnPcS_2$ treated samples, indicating fura-2 retention in the cytoplasm.

Results

We previously reported that mouse myeloma cell suspensions treated with 10 nM $ZnPcS_2$ and red light were observed to depolarize, reaching 80% depolarization with 7.2 kJ/m^2 light dose. It was also shown that ouabain, which inactivates the Na^+/K^+-ATPase, did not affect the depolarization. In the experiments presented here the cells were treated with the same sensitizer and light dose in the presence of channel blockers or altered ion concentrations and the effect compared to the photosensitized cells alone.

Membrane potential effects

Sodium. The degree of membrane depolarization produced by $ZnPcS_2$ photosensitization of mouse myeloma cells was found to be strongly dependent on the concentration of extracellular sodium. These results are shown in Fig. 1. Decreasing the $[Na^+]_o$ by equimolar substitution with choline, a large organic cation, decreased the rate of depolarization, although a complete inhibition was not achieved. When the $[Na^+]_o$ was nominally zero, the depolarization rate was approximately half that of the control, reaching 44% depolarization with 7.2 kJ/m^2 light dose.

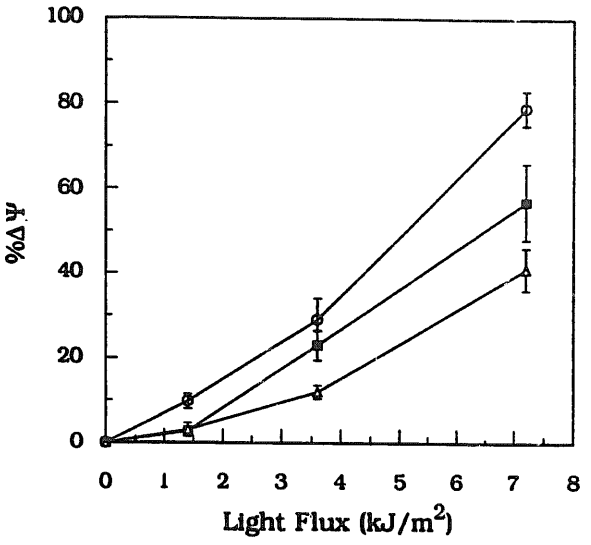

Fig. 1. The dependence of the photodynamically induced membrane potential change (%$\Delta\psi$) on the external sodium concentration. Mouse myeloma cells were incubated with 10 nM $ZnPcS_2$ for 1 h, then washed and irradiated (10^6/ml) in a Hepes buffer with the NaCl concentration adjusted by isotonic substitution with choline chloride. The change in membrane potential was assessed after each light treatment using the fluorescent potentiometric indicator di-SBA-C_2(3). Each point represents the average of at least three trials. Error bars represent the standard error. ○, Normal (155 mM) NaCl; ■, 85 mM NaCl; △, 0 mM NaCl.

The sodium channel blocker amiloride was also found to exhibit a protective effect, although no protection was afforded by tetrodotoxin. Addition of 1 mM amiloride to the cells suspended in normal physiological saline prior to the irradiation decreased the depolarization rate to half that of the control, similar to the effect observed with removal of external sodium. These results are shown in Fig. 2. Addition of 20 μM tetrodotoxin, which blocks some conducting sodium channels, did not affect the rate of membrane depolarization.

In order to rule out effects on Na^+/H^+ antiport involvement in the depolarization process, intracellular pH was determined using the fluorescent indicator BCECF/AM. The cells were treated in a bicarbonate-free solution to eliminate Cl^-/HCO_3^- exchange. Under these conditions the HCO_3^--dependent pH_i regulatory mechanisms are inoperative. Results with the bis-oxonol probe indicated that removal of HCO_3^- from the buffer did not alter the rate of membrane depolarization induced by $ZnPcS_2$.

Under these conditions the cells were observed to acidify upon photodynamic treatment by approximately 0.3 pH units. Addition of 1 mM amiloride, which blocks the Na^+/H^+ exchanger, did not prevent the intracellular acidification. If the acidification was due to an effect resulting from the action of the Na^+/H^+ exchanger then addition of amiloride should have affected the acidification. Most likely the acidification results from the uptake of H^+ which occurs in exchange for K^+ which is lost during the depolarization. The same treatment in HCO_3^- containing buffer did not alter the internal pH, since the accumulated H^+ can be exchanged for HCO_3^-. From these results it is concluded that the Na^+/H^+ anti-port is not directly involved in the depolarization process.

Potassium. Although K^+ leakage by photodynamic action has been reported by many other investigators, intracellular K^+ levels were also evaluated in these preparations to determine whether potassium was also affected under these conditions. Potassium levels were determined as described above. Non-treated and dark photosensitizer controls were run for each sample. Cell count and total protein were also assayed for each sample. Total protein levels remained the same in treated samples as in controls verifying that cells had not lysed during treatment. Potassium, determined on the basis of total protein per sample, was found to decrease in photosensitized cells, indicating that cells were losing intracellular potassium. (In separate controls, cells were given much higher light doses which reduced the membrane integrity, as determined by Trypan blue exclusion. In such samples the total cellular protein was reduced by 60% and potassium was below detectable limits indicating lysis had occurred.) These results are summarized in Table I.

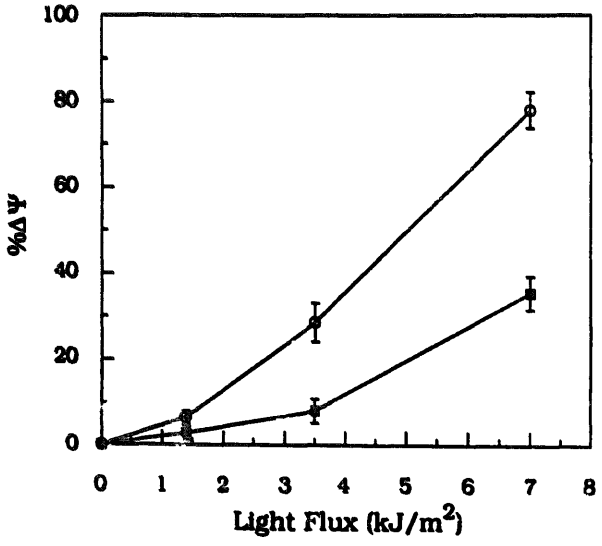

Fig. 2. The effect of amiloride on the $\%\Delta\psi$ induced by 10 nM $ZnPcS_2$ and light. Addition of 1 mM amiloride prior to the irradiation protected the mouse myeloma cells from the photodynamically induced depolarization. See Fig. 1 for details. ○, Control; ■, 1 mM amiloride.

TABLE I

Comparison of the change in membrane potential ($\%\psi$) and intracellular potassium levels ($\%K^+$) for mouse myeloma cells treated with 10 nM $ZnPcS_2$ with and without light

Treatment	$\%\psi$ [a]	$\%K^+$ [b]
Initial	100	100
4.5 kJ/m²	63±4	75+3
6.5 kJ/m²	32±4	53±4
Dark control [c]	100	100

[a] Membrane potential was determined using di-SBA-C_2(3) on aliquots removed from the treated sample. Values represent the average ± S.E. for four samples.

[b] Potassium was determined by flame photometry following lysis of 10^7 cells. Intracellular potassium levels were determined based on the total amount of protein per sample. Values represent the average ± S.E. for four samples.

[c] The dark control consisted of cells treated with sensitizer and kept in the dark in parallel with the longest light treatment.

This loss of intracellular potassium could be explained by photosensitized damage to plasma membrane K^+ channels which would alter the normal K^+ permeability. In an attempt to elucidate any involvement of K^+ channels on the observed depolarization by photosensitization several different K^+ channel blockers were analyzed using identical photosensitization treatment. One drawback to this technique is that this is an indirect method which is limited by the fact that channel blockers which depolarize the cell prior to photodynamic treatment will not be useful for measuring changes in the rates of membrane depolarization. Therefore it was necessary to use concentrations of channel blockers which produced minimal dark effects in the hopes that they would still show some effect on the rate of membrane depolarization if potassium channels were involved in the photosensitization effect.

In these experiments K^+ channel blockers were added to the cell suspension prior to light treatment. Compounds were selected on the basis of prior reports of modulation of K^+ channel conductances in the literature. An extensive range of different lymphocyte types including almost all murine T cell and B cell lines investigated by patch clamp techniques have been found to contain voltage activated K channels [24–26]. Although slight variations in K channel behavior are observed, in general these channels display similar characteristics eliciting an outward current detectable at about −40 mV which is modified by extracellular potassium. In B lymphocytes, channel conductances are almost completely blocked by 10 mM tetraethylammonium chloride (TEA) or 1 mM 4-aminopyridine (4-AP) [26]. Similar channels were also shown to be blocked by 15–30 μM quinine and 6–7 μM verapamil in other lymphocytes [24]. Cesium and barium have

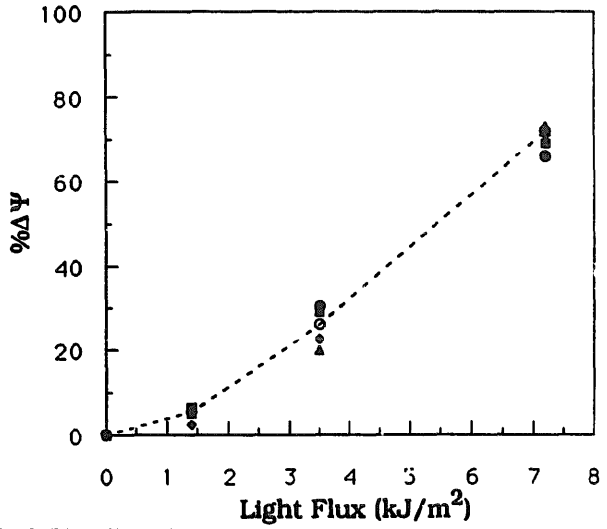

Fig. 3. The effect of potassium channel blockers on the%$\Delta\psi$ induced by 10 nM $ZnFcS_2$ and light. Following the $ZnPcS_2$ incubation the mouse myeloma cells were resuspended in HPS and channel blockers were added prior to irradiation. The fluorescence intensity immediately before the first irradiation point was used to assess the%$\Delta\psi$. See Fig. 1 for details. ○, Control; ■, 40 mM TEA; ◆, 1 mM 4-aminopyridine; ▲, 1 μM quinine; ●, 1 mM Ba^{2+}.

also been shown to block resting and other K channels in many cell types [27]. Although the mouse myeloma cell strain NS-1 (derived form B lymphocytes) has not been extensively characterized by patch clamp technique it seems most probable that similar K channel characteristics could be expected.

Addition of 20 mM CsCl or 1 mM $BaCl_2$ had no effect on the rate of photodynamically induced membrane depolarization. These results are shown in Fig. 3. It should be noted that 10 mM $BaCl_2$ was also examined, but immediate depolarization and ultimate dark toxicity resulted. Addition of 1 mM 4-AP, 40 mM TEA or 3 μM quinine, did not affect the rate of membrane depolarization. Initially, addition of 4-AP caused a small hyperpolarization of approx. 10% in the resting membrane potential, but the rate of change of membrane potential upon light treatment did not differ significantly from that of the control. Although a rather low concentration of quinine was used in this preparation, an approx. 10% initial depolarization was observed. In addition, 10 μM valinomycin was found to induce immediate depolarization and dark toxicity so was not pursued further.

Overall, it is difficult to draw any firm conclusions from this data since without conductance measurements there is some uncertainty as to the actual effectiveness of these blockers on K^+ channels under these conditions. While the lack of an observable effect may indicate that the blockers were ineffective under these conditions, it would also support the conclusion that K^+ channels were not involved in the photosensitized depolarization. Further electrophysiological experiments will be necessary to clarify these results.

Calcium. In order to determine whether external calcium was involved in the depolarization process the same experiments were performed in Ca^{2+} free, 5 mM EGTA Hepes-buffered saline. The same rate of depolarization occurred with light treatment, indicating that calcium was not necessary for the depolarization effect.

Chloride. It is also possible that alteration of chloride permeability could have an effect on the plasma membrane potential. The influence of chloride was determined by photodynamic treatment of cells in which the internal and external chloride concentrations were reduced. The cells were incubated in chloride free buffer for 1 h prior to light treatment in order to reduce the $[Cl^-]_i$ levels [28]. Photodynamic treatment of these cells suspended in Cl^- free HPS did not significantly affect the rate of membrane depolarization, suggesting that alteration of Cl^- permeability is not involved in the membrane depolarization process.

ATP. Since some photosensitizers appear to target the mitochondria, or alter intracellular ATP levels, the intracellular ATP levels were measured under these experimental conditions by the standard luciferase assay. No significant changes in intracellular ATP levels were observed with this treatment, indicating that the depolarization is occurring before any significant alterations to the cellular metabolic pathways.

Cytosolic calcium concentrations, $[Ca^{2+}]_i$

The fluorescent probe, fura-2, was used to evaluate changes in cytosolic calcium induced by photodynamic action. Although removal of external calcium did not alter the rate of membrane depolarization, the concentration of free internal calcium, $[Ca^{2+}]_i$ was found to increase both as a function of photosensitizer concentration and light dose. These results are shown in Fig. 4. Since the 50 nM $ZnPcS_2$ treatment resulted in complete loss of membrane integrity at the highest light dose, only two light treatments were used at this loading. A greater than 4-fold increase in cytosolic calcium levels were observed following the final light dose for the 50 nM and 10 nM $ZnPcS_2$ treatments. Control samples of cells without phthalocyanine, (light only) or 10 nM $ZnPcS_2$ without light (dark controls) showed only slightly increases in $[Ca^{2+}]_i$. As shown in Fig. 5, no significant increase in $[Ca^{2+}]_i$ was observed for cells treated with 10 nM $ZnPcS_2$ when irradiated in a Ca^{2+}-free buffer. This suggests that the increase in $[Ca^{2+}]_i$ observed under physiological conditions is due to an influx of external Ca^{2+} rather than release from internal stores.

A similar experiment was conducted by irradiating 10 nM $ZnPcS_2$ in a Na^+-free buffer. Under these conditions, the rate of membrane depolarization is about half that of the cells irradiated in physiological saline. The results presented in Fig. 6 show that removal of external Na^+ resulted in an approx. 50%

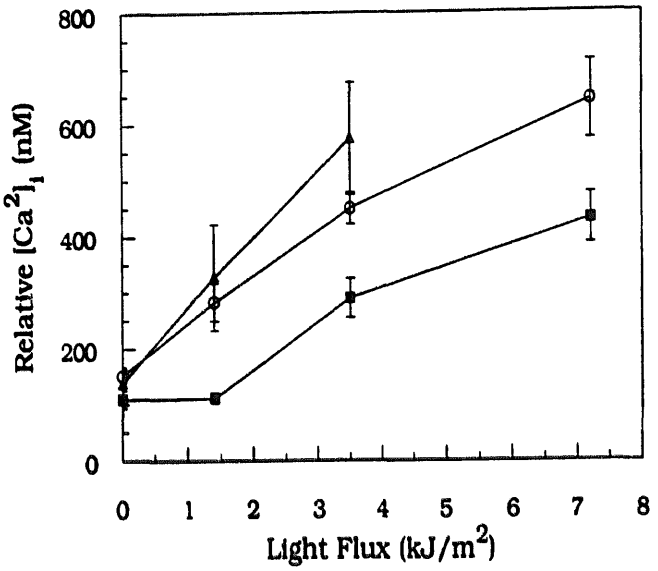

Fig. 4. Changes in the concentration of intracellular calcium $[Ca^{2+}]_i$ by photodynamic action for several concentrations of ZnPCS. Mouse myeloma cells were incubated with fura-2/AM and $ZnPcS_2$ for 1 h, then washed and resuspended in HPS. Calcium concentrations were determined spectrofluorimetrically following each light treatment. Each point represent three trials, error bars represent the standard error. ▲, 50 nM $ZnPcS_2$; ○, 10 nM $ZnPcS_2$; ■, 2 nM $ZnPcS_2$.

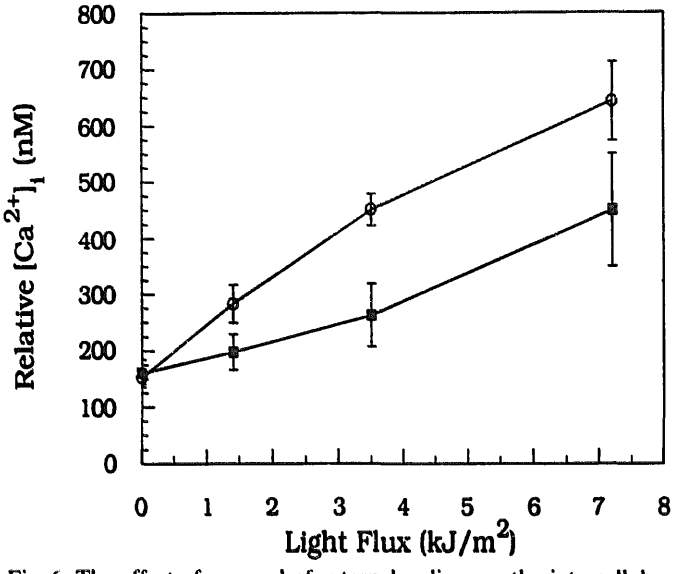

Fig. 6. The effect of removal of external sodium on the intracellular calcium concentration changes induced by 10 nM $ZnPcS_2$ and light. $[Ca^{2+}]_i$ was determined in cells irradiated in Na^+-free buffer (155 mM choline) which showed half the rate of depolarization. For details see Fig. 4. ○, Normal HPS; ■, Na^+-free HPS.

decrease in the accumulation of cytosolic Ca^{2+}. These results, together with those shown in Fig. 4, indicate that membrane depolarization and/or increased intracellular sodium concentrations are correlated with an accumulation of intracellular free calcium.

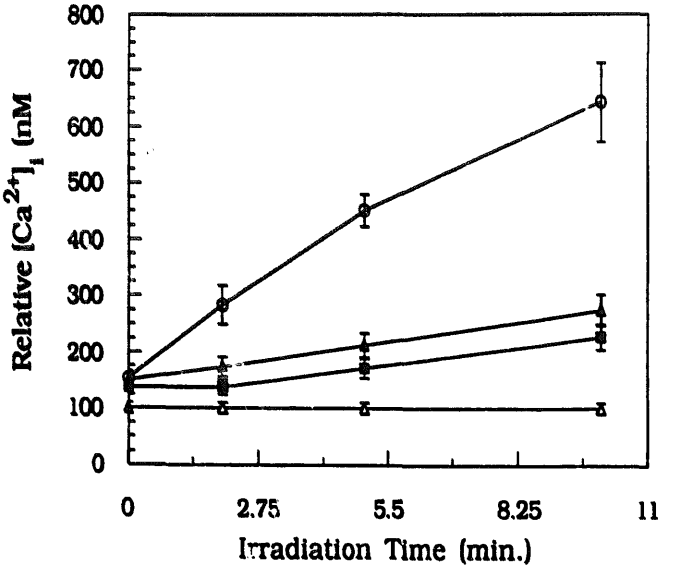

Fig. 5. The effect of 10 nM $ZnPcS_2$ on the relative $[Ca^{2+}]_i$. Fura-2 was used to determine $[Ca^{2+}]_i$ of mouse myeloma cells under various treatment conditions. In the case of light treatment, cell suspensions were exposed to the same light dose as in previous experiments, but in this case it has been reported as treatment time to be consistent with dark controls. ○, Cells treated with 10 nM $ZnPcS_2$ resuspended in normal HPS plus light; ■, 10 nM $ZnPcS_2$ in normal HPS without light (dark control); ▲, light only (no sensitizer); △, 10 nM $ZnPcS_2$ in Ca^2-free HPS with light. Each point represents the average of three trials, error bars represent the standard error.

Discussion

Depolarization of the plasma membrane of mouse myeloma cells by $ZnPcS_2$ could either result from direct membrane damage or as a secondary effect caused by changes in metabolic processes or mitochondrial function. No changes in cellular ATP levels were observed during this treatment. From this it can be concluded that alteration of metabolic processes is not responsible for the membrane potential changes. It is highly likely that in this case the membrane potential changes result from direct damage by photodynamic action to the plasma membrane.

There are at least two explanations that are consistent with these results. One explanation is that photodynamic action induces a decrease in potassium conductance which would cause the resting membrane potential (V_m) to be more strongly affected by the Nernst potential for sodium (E_{Na}) and hence the cell is depolarized. Studies on excitable cells [16–18] suggest there may be some precedence for such behavior. The second explanation is that there is a loss of the normal potassium selectivity caused by photodynamic action so that the overall cation permeability (including Na^+ and Ca^{2+}) increases Results from non-excitable cells [5,9,10,29] and observations of increased leakage conductance [16,18] are examples of cases where such a process has been shown to occur by photodynamic action.

It does not appear likely that the first explanation is important in this case. The only instances where K^+ conductance was shown to decrease was in excitable cells such as axons and atrial cells where specific voltage-activated channels were blocked by photodynamic action. This blockage was shown to correspond to changes in the action potential but was not necessarily responsible for the changes in the resting membrane potential, since significant leakage conductance was also measured. It has recently been reported that this leakage conductance induced by photosensitization in frog cardiac cells can be attributed to Na^+ influx [32] since it was reduced when tetramethylammonium or choline were substituted for sodium. Instead, the results reported here appear to be better explained as a loss of the normal K^+ selectivity owing to a general increase in cation permeability.

Our results indicate that sodium strongly contributes to the membrane depolarization process. From sodium-substitution experiments it is concluded that at least part of the depolarization involves sodium permeation. Much slower rates of depolarization were observed when choline was substituted for sodium. The depolarization observed in the sodium-free samples might indicate a limited permeability of the organic cation through the membrane.

Amiloride, an agent which blocks many types of sodium channels, also attenuated the depolarization. This might indicate that some type of sodium channel opens as a result of photodynamic action of $ZnPcS_2$ on mouse myeloma cells either as a result of direct damage to the channel protein or to the lipid environment of the membrane which could alter the regulation of the channel protein [30]. Conducting Na^+ channels have been observed in some lymphocytes [24] and would not be blocked by tetrodotoxin as are the Na^+ channels of excitable cells. A general finding in excitable cells is that conducting sodium channels are blocked before any large changes in the resting membrane potential are observed [16,17]. Another possibility is that a Na^+/Ca^{2+} exchange channel is involved in this process. Such a channel could be blocked by 1 mM amiloride [31].

Damage to the plasma membrane could also occur in such a way that cation permeabilities are altered without affecting any specific channel proteins. Such an effect was recently reported in erythrocytes treated with photosensitizers [33] and is also a possibility in this situation. This would imply that the barrier which normally prevents sodium influx breaks down, perhaps by formation of small pores, which would allow sodium to enter the cell down its electrochemical gradient thus enhancing passive Na^+/K^+ exchange. If such is the case then perhaps the effect observed for amiloride is coincidental in that it may block the pore rather than by acting at a specific protein channel.

A large decrease in intracellular K^+ was observed under these treatment conditions. Potassium permeability changes have been previously reported to result from oxidative damage. It has been suggested that potassium leakage resulting from the photodynamic action of chloroaluminum phthalocyanine tetrasulfonate in erythrocytes may be associated with the formation of aqueous pores permeable to polar compounds [6]. Such pores were suggested to result from the oxidation of intrinsic membrane proteins. It has also been reported that superoxide anion generated in low concentrations by phenazinemethosulfate in human erythrocytes in vitro increased the passive K^+ and Na^+ permeability without inducing any major effects on any specific K^+ carrier such as the Na^+/K^+ pump or cotransport system such as the Na^+/K^+ cotransporter [9]. Previously we showed that ouabain, which blocks the Na^+/K^+ pump was also ineffective in preventing the depolarization [11]. Although damage to K^+ channels is a possibility, as mentioned above, the results from these K^+ channel blocker experiments are inconclusive at this time. Further measurement of single channel conductances are necessary before these results can be completely understood.

This study also indicates that the intracellular free Ca^{2+} levels increased as a result of photodynamic action induced by zinc phthalocyanine-disulfonate and red light in mouse myeloma cells in vitro. This effect was found to be correlated with the membrane potential changes but did not affect the membrane potential directly since removal of external Ca^{2+} did not prevent the depolarization. Direct damage to calcium channels does not appear to be involved, since removal of external sodium should not have affected the calcium influx if this was the case. It is possible that the Ca^{2+} influx is Na^+ dependent, perhaps involving activation of a Na^+/Ca^{2+} exchange mechanism due to increased levels of intracellular sodium. A general increase in cation flux, as suggested above, would also account for the observed results.

The relationship between depolarization of the plasma membrane and the decrease in cell survival is not clearly understood. Alteration of cellular ion homeostasis is presumed to modify the regulation and function of many cellular substituents including proteins and organelles. It is possible that osmotic swelling could ultimately occur which would subsequently lead to cell lysis. Changes in calcium levels may also contribute to the induction of cell death processes in a number of ways including alteration in enzyme regulation and increased binding to cellular ligands resulting in loss of normal cell function [34–36].

In conclusion, photodynamic action by $ZnPcS_2$ induces an imbalance of ionic homeostasis in mouse myeloma cells which can be observed as the depolarization of the plasma membrane. This was found to

occur before there is a significant loss of membrane integrity as determined by trypan blue exclusion. It also occurs before intracellular ATP levels and presumably related metabolic processes are significantly altered. An increase in sodium permeability, either resulting from pore formation or sodium channel activation appears to play an important role in the membrane depolarization process.

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