Journal of Bioenergetics and Biomembranes, Vol. 31, No. 6, 1999

Mitochondrial Effects of Triarylmethane Dyes

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Received July 27, 1999; September 28, 1999

The mitochondrial effects of submicromolar concentrations of six triarylmethane dyes, with potential applications in antioncotic photodynamic therapy, were studied. All dyes promoted an inhibition of glutamate or succinate-supported respiration in uncoupled mitochondria, in a manner stimulated photodynamically. No inhibition of N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD) supported respiration was observed, indicating that these dyes do not affect mitochondrial complex IV. When mitochondria were energized with TMPD in the absence of an uncoupler, treatment with victoria blue R, B, or BO, promoted a dissipation of mitochondrial membrane potential and increase of respiratory rates, compatible with mitochondrial uncoupling. This effect was observed even in the dark, and was not prevented by EGTA, Mg^{2+} or cyclosporin A, suggesting that it is promoted by a direct effect of the dye on inner mitochondrial membrane permeability to protons. Indeed, victoria blue R, B, and BO promoted swelling of valinomycin-treated mitochondria incubated in a hyposmotic K^+-acetate-based medium, confirming that these dyes act as classic protonophores such as FCCP. On the other hand, ethyl violet, crystal violet, and malachite green promoted a dissipation of mitochondrial membrane potential, accompanied by mitochondrial swelling, which was prevented by EGTA, Mg^{2+}, and cyclosporin A, demonstrating that these drugs induce mitochondrial permeability transition. This mitochondrial permeabilization was followed by respiratory inhibition, attributable to cytochrome *c* release, and was caused by the oxidation of NAD(P)H promoted by these drugs.

KEY WORDS: Mitochondria; triarylmethane dyes; photodynamic therapy; respiration; mitochondrial permeability transition; cyclosporin A; calcium; proton transport.

INTRODUCTION

Recently, a large number of studies have shown that mitochondrial dysfunction precedes both apoptotic and necrotic cell death in a variety of conditions (Nicholls and Budd, 1998; Lemasters *et al.*, 1998; Marzo *et al.*, 1998; Fiskum *et al.*, 1999). Necrotic cell death is preceded by large-scale mitochondrial alterations, resulting in cellular ATP depletion, which leads to cell death. On the other hand, apoptosis may follow less intense or widespread mitochondrial alterations, since ATP is required in this process (Nicotera *et al.*, 1998; Lemasters, 1999). Indeed, mitochondria may actively regulate cell death through the release of pro-apoptogenic factors, which promote caspase activation resulting in apoptosis (Marzo *et al.*, 1998; Green and Reed, 1998). Two mitochondrial pro-apoptotic components, normally located in the mitochondrial intermembrane space, have been identified: a 57-KDa protease termed the apoptosis-inducing factor (AIF, Marzo *et al.*, 1998), and cytochrome *c*, a component of the mitochondrial respiratory chain (Green and Reed, 1998). Since AIF and cytochrome *c* are larger

[1] Key to abbreviations: AIF, apoptosis-inducing factor; CV^+, crystal violet; EV^+, ethyl violet; EGTA, ethylene glycol-*bis*-(β-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MG^+, malachite green; MPT, mitochondrial permeability transition; RLM, rat liver mitochondria; TAM^+, triarylmethane; TMPD, *N*,*N*,*N′*,*N′*-tetramethyl-*p*-phenylenediamine; VBB^+, victoria blue B; VBR^+, victoria blue R; $VPBBO^+$, victoria pure blue BO.

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0145-479X/99/1200-0581$16.00/0

than porin, the least selective outer mitochondrial membrane pore (Mannella, 1997), mitochondria must undergo a transformation, which renders the outer membrane more permeable in order to release either cytochrome *c* or AIF.

The best characterized mechanism which promotes outer mitochondrial membrane permeabilization is the mitochondrial permeability transition (MPT; for reviews, see Zoratti and Szabò, 1995; Bernardi *et al.*, 1998; Kowaltowski and Vercesi, 1999). MPT is a Ca^{2+}-promoted, nonselective permeabilization of the inner mitochondrial membrane, which results in coloidosmotic swelling of the organelle, followed by physical rupture of the outer membrane and release of cytochrome *c* and AIF (Scarlett and Murphy, 1997; Petit *et al.*, 1998). MPT can be prevented by the immune suppressor cyclosporin A or antioxidants, demonstrating that this process is triggered by mitochondrial oxidative stress (Zoratti and Szabò, 1995; Kowaltowski and Vercesi, 1999). MPT is also a reversible phenomenon (Castilho *et al.*, 1996), and may be compatible with the maintenance of cellular ATP levels necessary for the apoptotic process (Nicotera *et al.*, 1998).

The identification of a prominent role for mitochondria and MPT in the regulation of the apoptotic process suggests that this organelle may be an ideal target for drugs designed to promote cell death, such as those used in cancer therapy. The appropriate drug for this purpose would accumulate in mitochondria, preferentially in transformed cells, and lead to mitochondrial alterations which favor the induction of apoptosis rather than necrosis. In this sense, cationic compounds are favorable, since they can be actively accumulated in mitochondria because of the mitochondrial membrane potential and high inner membrane content of cardiolipin, an anionic lipid (Daum, 1985).

In this work, we study the mitochondrial effects of six cationic dyes with potential therapeutic applications: crystal violet (CV^+), ethyl violet (EV^+), malachite green (MG^+), victoria blue R (VBR^+), victoria blue B (VBB^+), and victoria pure blue BO ($VPBBO^+$) (see molecular structures in Fig. 1). Three of these drugs (CV^+, MG^+, and $VPBBO^+$; Moreno *et al.*, 1988; Bullough *et al.*, 1989; Modica-Napolitano *et al.*, 1990) have previously been shown to present mitochondrial effects at high concentrations, but no systematic study has been presented on the respiratory, membrane potential, and ion transport alterations promoted by treatment of mitochondria with these dyes. Furthermore, the concentrations of the drugs used here are submicromolar and more relevant to the conditions found during potential treatment with these compounds. In addition, all dye effects were studied both in the dark and upon photostimulation, since the dyes studied here may yield excited radical species when stimulated by light (Jones *et al.*, 1991). Thus, photostimulation of these drugs may further potentiate their ability to promote mitochondrial functional alterations, such as oxidative stress-triggered MPT (Kowaltowski and Vercesi, 1999). This suggests that these drugs may be appropriate for photodynamic therapy, in which chemotherapy is combined with photostimulation to provide a more intense and localized response (Stewart *et al.*, 1998).

MATERIALS AND METHODS

Materials

Chlorine salts of ethyl violet (EV^+), victoria blue R (VBR^+), victoria pure blue BO ($VPBBO^+$), victoria blue B (VBB^+) and malachite green (MG^+), purchased from Aldrich (Milwaukee, WI), and crystal violet (CV^+), from Sigma Chemical Co. (St. Louis, MO), were recrystallized from methanol and dried under vacuum. The purity of recrystallized triarylmethanes was assessed by thin-layer chromatography (silica gel, methanol–acetic acid 95:5, v:v) as described previously (Batista and Indig, 1998).

Preparation of Dye Solutions

The dye solutions were prepared daily, in water, with the exception of MG^+, which was prepared in 3 m*M* 3-(*N* morpholino)propanesulfonic acid buffer, pH 5.8, because of instability at higher pH. Solution concentrations were determined spectrophotometrically using the dye molar extinction coeficients (CV^+ = 9.5 × 10^4 M^{-1} cm^{-1}, at 590 nm; EV^+ = 9.2 × 10^4 M^{-1} cm^{-1}, at 595 nm; MG^+ = 9.5 × 10^4 M^{-1} cm^{-1}, at 617 nm; VBR^+ = 8.1 × 10^4 M^{-1} cm^{-1}, at 611 nm; VBB^+ = 6.8 × 10^4 M^{-1} cm^{-1}, at 618 nm; and $VPBBO^+$ = 8.3 × 10^4 M^{-1} cm^{-1}, at 614 nm). The concentrations of TAM^+ dyes used is not always equal, since the experiments were innitially conducted using preliminary molar extinction coeficients, and later corrected for the more precise values shown here. It is important to stress that since both MG^+ and VBB^+ are not stable in

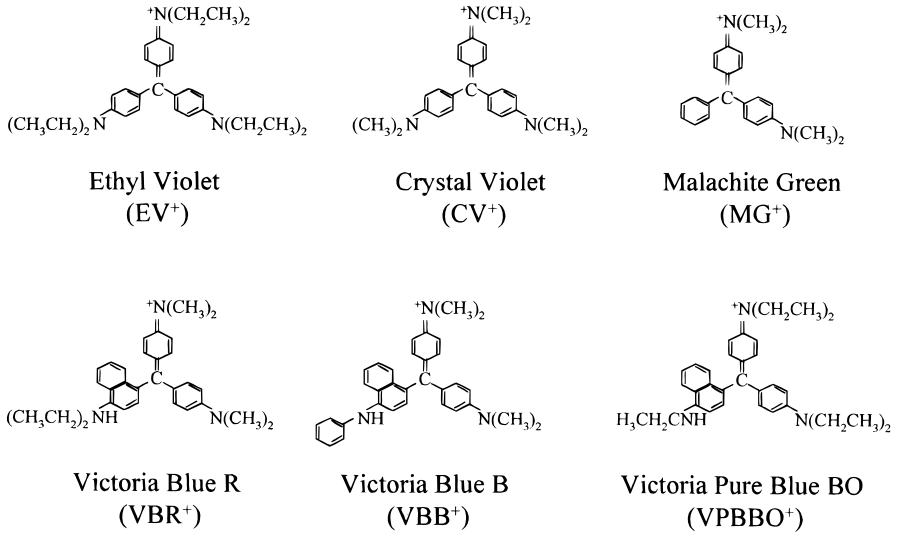

Fig. 1. Molecular structures of the six TAM^+ dyes studied.

aqueous solutions, part of the results obtained with these dyes may be attributable to byproducts not yet identified.

Isolation of Rat Liver Mitochondria

Mitochondria were isolated by conventional differential centrifugation (see Castilho *et al.*, 1996).

Standard Incubation Procedure

The experiments were carried out at 28°C, with continuous magnetic stirring, in a standard reaction containing 125 m*M* sucrose, 65 m*M* KCl, 2 m*M* inorganic phosphate, 10 m*M* HEPES buffer, pH 7.2, and 10 μ*M* Ca^{2+}. Other additions are indicated in the figure legends.

Data Analysis and Statistics

The results shown in Fig. 2–5 are representative of a series of at least three experiments. In Tables I and II, data represent the averages ± SD of at least three determinations. Statistical comparisons were conducted through a pairwise Student–Newmann–Keuls test, run by Sigmastat.

Oxygen Uptake Measurements

Oxygen concentration was monitored using a Clark-type electrode in a sealed glass cuvette equipped with magnetic stirring.

Photostimulation

Unless specified, all respiration studies were conducted in the dark; while membrane potential and swelling assays were submitted to the light of the spectrophotometer or spectrofluorimeter itself, during the assays. Photostimulation of the mitochondrial suspension during oxygen consumption measurements or TPP^+-measured membrane potential was provided by shining a 500 W halogen light bulb, at a distance of 43 cm, using a cut-off glass filter (500 nm wavelength mean value ± 50 nm half bandwidth).

Mitochondrial Dye Uptake

Mitochondria (1 mg/ml) were incubated in 3 ml standard reaction medium containing 200 μ*M* TMPD, 2 m*M* ascorbate, 0.1 m*M* EGTA, and 2 m*M* Mg^{2+}, in the presence or absence of 1 μ*M* FCCP. After 2 min, 3 μmol of CV^+, EV^+, MG^+, VBR^+, VBB^+, or $VPBBO^+$ were added. After an additional 2-min incubation, samples were centrifuged and the dye bound to the mitochondrial pellet was extracted

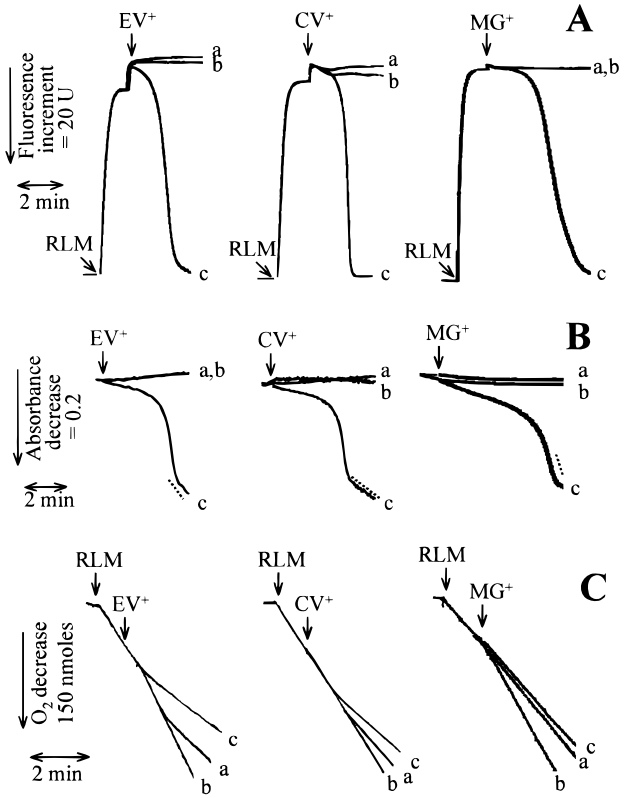

Fig. 2. Effects of EV^+, CV^+, and MG^+ on mitochondrial membrane potential (A), swelling (B), and respiration (C). RLM (0.5 mg/ml) were added to standard reaction media containing 5 μM safranin O (Panel A), 200 μM TMPD (all traces), and 2 mM ascorbate (all traces), in the presence of 100 μM EGTA plus 2 mM Mg^{2+} (lines a), 1 μM cyclosporin A (lines b), or no further additions (lines c). EV^+ (0.60 μM), CV^+ (0.60 μM), and MG^+ (0.55 μM) were added where indicated. The dotted lines represent experiments incubated in the dark until the beginning of the traces.

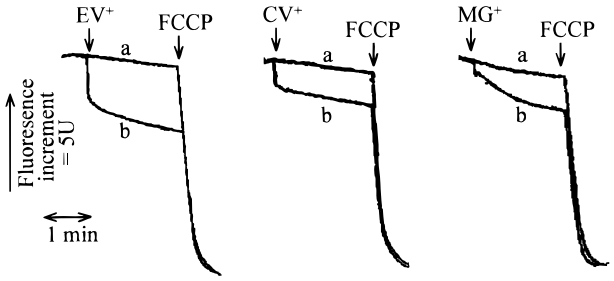

Fig. 3. Effects of EV^+, CV^+, and MG^+ on mitochondrial NAD(P)H redox state. RLM (0.5 mg/ml) were added to standard reaction media containing 200 μM TMPD, 2 mM ascorbate, 100 μM EGTA, and 2 mM Mg^{2+} (all traces).). EV^+ (0.60 μM), CV^+ (0.60 μM) and MG^+ (0.55 μM) (lines b) and 0.5 μM FCCP (all traces) were added where indicated.

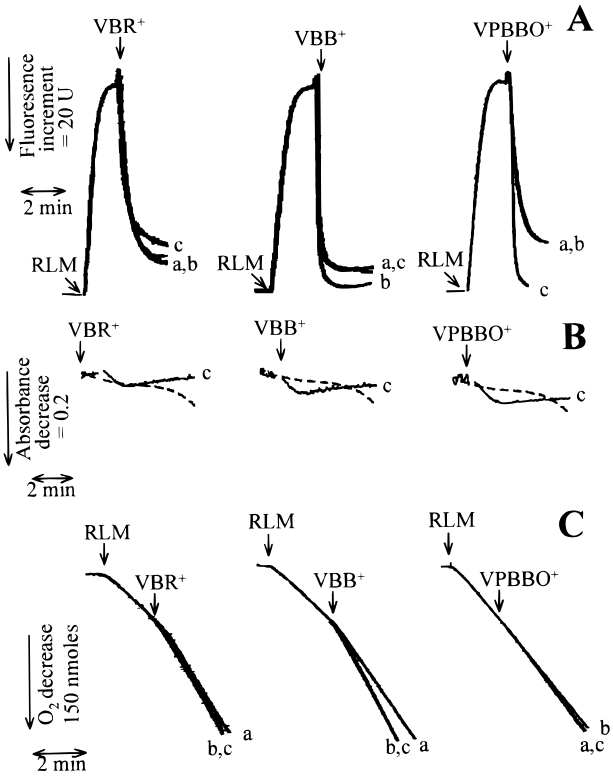

Fig. 4. Effects of VBR^+, VBB^+, and $VPBBO^+$ on mitochondrial membrane potential (A), swelling (B), and respiration (C). RLM (0.5 mg/ml) were added to standard reaction media containing 5 μM safranin O (Panel A), 200 μM TMPD (all traces), and 2 mM ascorbate (all traces), in the presence of 100 μM EGTA plus 2 mM Mg^{2+} (lines a), 1 μM cyclosporin A (lines b), or no further additions (lines c). VBR^+ (0.36 μM), VBB^+ (0.52 μM) or $VPBBO^+$ (0.30 μM) were added where indicated. The dashed lines represent experiments in which no VBR^+, VBB^+, or $VPBBO^+$ were added.

in 1 ml ethanol. Samples were centrifuged again and the absorbance of the dye extracted in ethanol was measured at the respective dye peak, according to the molar extinction coefficients in ethanol (CV^+ = 10.4 × 10^4 $M^{-1}cm^{-1}$, at 588 nm; EV^+ = 10.1 × 10^4 $M^{-1}cm^{-1}$, at 592 nm; MG^+ = 11.0 × 10^4 $M^{-1}cm^{-1}$, at 622 nm; VBR^+ = 7.4 × 10^4 $M^{-1}cm^{-1}$, at 574 nm; VBB^+ = 7.3 × 10^4 $M^{-1}cm^{-1}$, at 597 nm; and $VPBBO^+$ = 8.1 × 10^4 $M^{-1}cm^{-1}$, at 589 nm). The bound dye content is expressed as a percentage of the initial dye content.

Measurements of Mitochondrial Transmembrane Electrical Potential

Mitochondrial membrane potential was estimated through fluorescence changes of safranin O (5 μM),

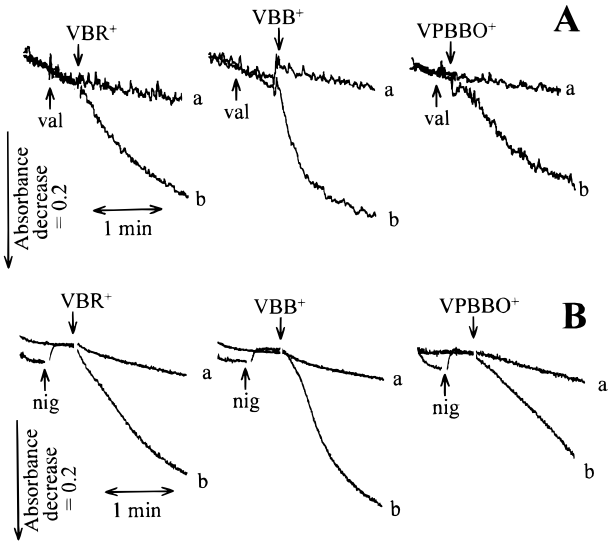

Fig. 5. Effect of VBR^+, VBB^+, and $VPBBO^+$ on inner mitochondrial membrane permeability to protons. RLM (0.5 mg/ml) were added to reaction media containing 10 m*M* HEPES, pH 7.2, 0.2 μ*M* antimycin A, 200 μ*M* propranolol, and 55 m*M* K^+-acetate (Panel A) or 55 m*M* KSCN (Panel B). Valinomycin (val, 0.5 μ*M*), nigericin (nig, 0.2 μ*M*), VBR^+ (0.36 μ*M*), VBB^+ (0.52 μ*M*) or $VPBBO^+$ (0.30 μ*M*) were added where indicated. Mitochondrial swelling was monitored as described in Materials and Methods.

recorded on a model F-4010 Hitachi spectrofluorimeter (Hitachi, Ltd., Tokyo, Japan) operating at excitation and emission wavelengths of 495 and 586 nm, with a slit width of 5 nm. Alternatively, to study the effect of the dyes in the dark, membrane potential was measured using a TPP^+-sensitive electrode, as described in Castilho *et al.*, 1996.

Mitochondrial Swelling

Mitochondrial swelling was monitored by following the turbidity of the mitochondrial suspension at 520 nm, using a Hitachi U-3000 spectrophotometer.

Pyridine Nucleotide Redox State

NAD(P)H fluorescence was followed at Ex 352 nm and Em 464 nm, on a Hitachi F-4000 spectrofluorimeter.

RESULTS

Table I shows the effect of triarylmethane (TAM^+) dyes EV^+, CV^+, MG^+, VBR^+, VBB^+, and $VPBBO^+$ on mitochondrial respiratory rates supported by glutamate (a mitochondrial Complex I electron donor), succinate (a Complex II electron donor) or TMPD/ascorbate, which donates electrons to Complex IV. Mitochondria were pretreated with FCCP, to generate maximal respiratory rates, and EGTA plus Mg^{2+}, to inhibit possible Ca^{2+}-promoted mitochondrial membrane alterations (see Kowaltowski and Vercesi, 1999). Under these conditions, when incubated in the dark, respiration supported by glutamate was significantly inhibited by EV^+, CV^+, VBR^+, and VBB^+. When photoexcited (+ irr.), all six dyes promoted significant inhibitions of glutamate-supported respiration and the effect of EV^+ and CV^+ was increased, demonstrating that the respiratory inhibition observed is stimulated by excited spe-

Table I. Effects of TAM^+ Dyes on Mitochondrial Respiration[a]

	EV^+	CV^+	MG^+	VBR^+	VBB^+	$VPBBO^+$
Glutamate	83.0% ± 4.2%[b]	81.0% ± 2.6%[b]	100.0% ± 1.0%	82.5% ± 5.1%[b]	83.0% ± 4.2%[b]	85.0% ± 14.1%
Glutamate + irr.	72.8% ± 3.9%[b,c]	73.3% ± 5.7%[b,c]	90.0% ± 7.1%[b]	76.0% ± 9.1%[b]	73.0% ± 8.5%[b]	78.3% ± 12.3%[b]
Succinate	92.0% ± 7.8%	80.0% ± 5.3%[b]	96.0% ± 0.0%[b]	94.3% ± 2.3%	78.5% ± 3.5%[b]	86.0% ± 6.5%[b]
Succinate + irr.	83.0% ± 7.3%[b]	66.0% ± 5.6%[b,c]	88.0% ± 1.0%[b,c]	82.7% ± 8.4%[b,c]	65.0% ± 1.4%[b,c]	75.8% ± 8.0%[b,c]
TMPD	106.3% ± 6.4%	97.3% ± 5.7%	104.3% ± 7.5%	106.5% ± 6.4%	100.0% ± 0.0%	102.8% ± 1.9%
TMPD + irr.	107.8% ± 6.8%	97.8% ± 5.9%	104.3% ± 7.5%	112.5% ± 9.3%	101.0% ± 1.4%	108.0% ± 9.7%

[a] Rat liver mitochondria (RLM, 0.5 mg/ml) were added to 1.3 ml of standard reaction media containing 100 μ*M* EGTA, 2 m*M* Mg^{2+}, and 0.5 μM FCCP. Glutamate (10 m*M*), 2 m*M* succinate or 200 μ*M* TMPD plus 2 m*M* ascorbate were used as respiratory substrates, as shown. Respiratory rates (expressed as percentages of initial respiratory rates in the absence of dyes) were calculated 1–2 min after treatment with EV^+ (0.60 μ*M*), CV^+ (0.60 μ*M*), MG^+ (0.55 μ*M*), VBR^+ (0.36 μ*M*), VBB^+ (0.52 μ*M*) or $VPBBO^+$ (0.30 μ*M*) in the dark and after photostimulation (irr.). Typical respiratory rates in the absence of dyes were 35–45 nmol/min/mg (glutamate), 100–130 nmol/min/mg (succinate), and 60–90 nmol/min/mg (TMPD).

[b] $p < 0.05$ as compared to control (untreated) respiratory rates.

[c] $p < 0.05$ as compared to respiratory rates of suspensions treated in the dark.

Table II. Percentage of TAM^+ Dye Bound to the Mitochondrial Fraction

	EV^+	EV^+ + FCCP	CV^+	CV^+ + FCCP	MG^+	MG^+ + FCCP	VBR^+	VBR^+ + FCCP	VBB^+	VBB^+ + FCCP	$VPBBO^+$	$VPBBO^+$ + FCCP
Mean	93.9%	80.1%[b]	96.0%	77.9%[b]	20.6%	6.8%[b]	67.4%	67.7%	103.0%	56.6%[b]	56.8%	49.9%
S.D.	6.2%	7.4%	3.5%	11.1%	2.3%	1.2%	4.6%	6.9%	18.5%	6.1%	2.5%	6.4%

[a] Affinity of EV^+, CV^+, MG^+, VBR^+, VBB^+, and $VPBBO^+$ to coupled or deenergized (+ FCCP) mitochondria was determined as described in Materials and Methods.

[b] $p < 0.05$, as compared to samples not treated with FCCP.

cies generated through photostimulation of TAM^+ dyes. Similarly, succinate-supported mitochondrial respiration was significantly inhibited by CV^+, MG^+, VBB^+, and $VPBBO^+$ in the dark, and all dyes when stimulated by light. Interestingly, TMPD-supported respiration was not inhibited by any dye, even when photoexcited. This indicates that mitochondrial Complex IV is resistant to respiratory inhibition promoted by TAM^+ dyes.

Next, we determined the efficiency with which TAM^+ dyes are taken up by isolated mitochondria and the role played by the mitochondrial membrane potential on dye uptake. To do so, the fraction of each dye taken up by the mitochondria after a brief incubation period was determined (Table II). Under these conditions, differences in mitochondrial membrane potential secondary to respiratory inhibition promoted by these dyes were avoided via energization of the mitochondria with TMPD/ascorbate. We found that the mitochondrial binding of EV^+, CV^+, and VBB^+ was very high (above 90%) and was significantly decreased in the absence of a membrane potential (+ FCCP). MG^+ binding to mitochondria could also be prevented in the absence of mitochondrial energization, but the binding to energized mitochondria was much lower than that of the previous dyes. VBR^+ and $VPBBO^+$ bound at intermediate levels to mitochondria and were not significantly affected by the lack of a mitochondrial membrane potential. For these last compounds, membrane partitioning is conceivably the dominant contribution toward dye binding, while EV^+, CV^+, MG^+, and VBB^+ were, in part, actively accumulated in mitochondria down their membrane potential.

In Fig. 2A, we examined the effects of EV^+, CV^+, and MG^+ on mitochondrial membrane potential, estimated through absorbance decreases of the fluorescent membrane potential probe safranin O. Again, TMPD/ascorbate was used as an electron donor to avoid membrane potential loss secondary only to effects of the dyes on mitochondrial respiration. Under these conditions, no change in mitochondrial membrane potential was observed after treatment with EV^+, CV^+, or MG^+ in the presence of EGTA and Mg^{2+} (Panel A, lines a). However, if EGTA and Mg^{2+} were omitted from the reaction media and the experiments were conducted in the presence of 10 μM Ca^{2+}, control mitochondria were able to retain a membrane potential for at least 10 min (results not shown), while a dissipation of mitochondrial membrane potential was observed with mitochondria treated with EV^+, CV^+, or MG^+ (lines c). Since a Ca^{2+}-dependent inner mitochondrial membrane permeabilization is characteristic of MPT (Zoratti and Szabò, 1995), the effect of the MPT inhibitor cyclosporin A on this membrane potential drop was investigated. The membrane potential dissipation induced by EV^+, CV^+, or MG^+ was completely prevented by 1 μM cyclosporin A (lines b), confirming that the effect observed was associated to MPT.

MPT is a nonselective inner mitochondrial membrane permeabilization, which is usually accompanied by coloidosmotic mitochondrial swelling, secondary to membrane permeabilization to the osmotic support (Zoratti and Szabò, 1995). In accordance with the results obtained while monitoring mitochondrial membrane potential (Fig. 2A), a decrease in turbidity of mitochondrial suspensions, as a result of mitochondrial swelling, was observed upon treatment with EV^+, CV^+, or MG^+ (Fig. 2B, lines c). This mitochondrial swelling was prevented by cyclosporin A (lines b), EGTA plus Mg^{2+} (lines a), EGTA or Mg^{2+} alone (results not shown). In addition, if the dye was added in the dark, and absorbance measurements were initiated only when the photoexcited mitochondria (lines c) had already undergone extensive swelling, no stimulation of the mitochondrial permeabilization could be observed (dotted lines). In addition, when mitochondrial membrane potential drops induced by EV^+, CV^+, or MG^+ were followed using a TPP^+-sensitive electrode (see Materials and Methods), photoexcitation stimulated the drop only slightly (results not shown).

This suggests that these dyes promote MPT in the dark, and that under our experimental conditions, the photochemical enhancement of this process is modest. Measurements of mitochondrial respiration conducted in the dark showed that these dyes promoted mitochondrial respiratory inhibition (Fig. 2C, lines a), attributable to the release of mitochondrial cytochrome *c* secondary to MPT (Green and Reed, 1998). This respiratory inhibition was only marginally stimulated by photoexcitation (results not shown), and could be completely prevented by cyclosporin A (lines b). In the presence of EGTA and Mg^{2+}, respiratory inhibition was also prevented (lines a), but respiratory rates were lower than those observed in the presence of cyclosporin A, probably due to the inhibition of mitochondrial dehydrogenases in the absence of Ca^{2+} (Moreno-Sanchez *et al.*, 1990).

We have previously shown that, with the exception of treatment with dithiol reagents, MPT is promoted by an accumulation of reactive oxygen species in mitochondria, which leads to membrane protein thiol oxidation (see Kowaltowski and Vercesi, 1999). TAM^+ dyes are known to bind to a variety of biopolymer polyelectrolytes in aqueous media, including proteins and nucleic acids. Under these circunstances, the electronic excitation of TAM^+ dyes leads the formation of triplet species and free radicals (Baptista and Indig, 1998; Bartlett and Indig, 1999), and these reactive reaction intermediates are the species responsible for the phototoxic effects of photosensitizers in biological systems. However, any photosensitizer can also show substantial dark toxicity and since EV^+, CV^+, and MG^+ were capable of promoting MPT in the dark, we have explored whether these dyes can lead to mitochondrial oxidative stress by depleting the reductive resources of the organelle.

Mitochondrial NAD(P)H is one of the key mitochondrial reductive resources, since it maintains the reduced state of glutathione, the substrate for glutathione peroxidase (Kowaltowski and Vercesi, 1999). Two lines of evidences were obtained for the direct reaction of TAM^+ dyes with NAD(P)H. First, the reactions of NADH with CV^+, EV^+, VBR^+, and $VPBBO^+$ were investigated in 45 m*M* Tris buffer, pH 7.3. In all cases, the reaction of NADH with the TAM^+ dye was easily observed (results not shown). Second, CV^+, EV^+, and MG^+ were found to promote the oxidation of mitochondrial NAD(P)H, as measured by the decrease in NAD(P)H fluorescence upon treatment with the TAM^+ dyes (Fig. 3, lines b). Since these fluorescence experiments were conducted in the presence of EGTA and Mg^{2+}, the oxidation of NAD(P)H could not be attributed to mitochondrial uncoupling. Further evidence that the decrease in fluorescence observed was caused by NAD(P)H oxidation, was provided by the observation that control mitochondrial suspensions (lines a) presented a larger fluorescence decrease than those treated with EV^+, CV^+ or MG^+, when uncoupled by FCCP.

Figure 4A shows the effects of VBR^+, VBB^+, and $VPBBO^+$ on mitochondrial membrane potential. Interestingly, these three dyes promoted mitochondrial uncoupling even in the presence of Mg^{2+} and EGTA (lines a). The extent of the uncoupling promoted by VBR^+ or VBB^+ in the presence of EGTA plus Mg^{2+} was almost complete when compared to the uncoupling promoted by FCCP (not shown), and was not significantly modified when mitochondria were incubated in the absence of EGTA and Mg^{2+} (lines c) or in the presence of cyclosporin A (lines b). This suggests that this uncoupling cannot be attributed to MPT. The uncoupling promoted by $VPBBO^+$ was slightly smaller in the presence of EGTA plus Mg^{2+} (line a) or cyclosporin A (line b), when compared to an experiment conducted in the presence of 10 μM Ca^{2+} (line c), suggesting that only a small part of this uncoupling could be secondary to MPT.

The hypotheses that VBR^+, VBB^+, and $VPBBO^+$ promoted primarily mitochondrial uncoupling instead of MPT was strengthened by the observation that no significant mitochondrial swelling was observed in suspensions treated with these dyes (Fig 4B). Indeed, VBR^+, VBB^+, and $VPBBO^+$ even prevented the slight mitochondrial permeabilization promoted by Ca^{2+} alone (dashed lines), probably by preventing mitochondrial Ca^{2+} uptake because of the lack of a membrane potential. Furthermore, treatment with VBR^+, VBB^+, or $VPBBO^+$ increased the respiratory rates of mitochondria (Fig. 4C), demonstrating that these dyes promote mitochondrial uncoupling, but not cytochrome *c* release.

The results presented in Fig. 4 suggest that VBR^+, VBB^+, and $VPBBO^+$ can act, at the concentrations used, as classical mitochondrial uncouplers such as FCCP. To confirm that these dyes presented protonophoric properties, we conducted the experiments shown in Fig. 5A, in which mitochondria were incubated in a hyposmotic K^+-acetate based media. Normally, mitochondria do not swell in this media, since the inner mitochondrial membrane is permeable only to acetic acid and impermeable to K^+, acetate, and H^+. However, if K^+ and H^+ ionophores are added (such

as, valinomycin and FCCP) to equilibrate K^+ and H^+ gradients formed during acetic acid redistribution, mitochondrial swelling occurs (Garlid and Nakashima, 1983). Indeed, we observed that mitochondria incubated in the presence of valinomycin presented large-amplitude swelling when treated with VBR^+, VBB^+, or $VPBBO^+$ (lines b). In the absence of valinomycin, no swelling was observed (lines a). These experiments suggest that VBR^+, VBB^+, and $VPBBO^+$ can act as protonophores. However, because of the cationic properties of these dyes, it is possible (although very unprobable) that they may promote mitochondrial swelling by direct binding to acetate. To exclude this possibility, we conducted a second assay to assess possible protonophoric properties of these dyes (Fig. 5B). In this assay, mitochondria were suspended in hyposmotic KSCN-based media. Mitochondria are permeable to SCN^-, but not to K^+. Thus, if incubated with the H^+/K^+ exchanger nigericin, mitochondria swell if in the presence of a pronotophore, to maintain a free proton flow. Confirming their protonophoric effects, VBR^+, VBB^+, and $VPBBO^+$ promoted swelling in nigericin treated (lines b), but not untreated (lines a) mitochondria.

DISCUSSION

Previous studies have shown that CV^+, MG^+, and $VPBBO^+$ may promote alterations to mitochondrial functions, such as respiratory inhibition or uncoupling (Moreno *et al.*, 1988; Bullough *et al.*, 1989; Modica-Napolitano *et al.*, 1990). However, the details of the mechanisms of action of these dyes toward mitochondria are not well understood. Here, we have conducted a more detailed examination of the effects of six TAM^+ dyes on mitochondrial respiration, membrane potential, and membrane integrity, both in the dark and under photostimulation. We also characterize dye partitioning between osmotic support and mitochondria. The characterization of mechanisms of mitochondrial toxicity promoted by TAM^+ is especially pertinent when considering the recent findings implicating mitochondria in the regulation of apoptosis and induction of necrosis (Nicholls and Budd, 1998; Lemasters *et al.*, 1998; Marzo *et al.*, 1998). Since cationic TAM^+ dyes actively accumulate in mitochondria assisted by the mitochondrial membrane potential, these drugs may prove useful as triggers for cell death in cancer therapy. Thus, we hope our studies may help the selection of dyes most suitable for antioncotic treatment. In addition, electronic excitation of TAM^+ dyes can lead to the formation of reactive oxygen species (Jones *et al.*, 1991; Baptista and Indig, 1998; Bartlett and Indig, 1999), known to further enhance the cytotoxic effects of these compounds (Docampo *et al.*, 1983; Fiedorowicz *et al.*, 1997). This property may make TAM^+ dyes suitable for photodynamic therapy.

We found that all dyes studied promoted glutamate and succinate-supported respiratory inhibition, in a manner stimulated by irradiation (Table I). The extent of the respiratory inhibition observed in the presence of glutamate and succinate was mostly very similar, suggesting that the main mitochondrial respiratory complex inhibited was Complex III. However, EV^+ and VBR^+ promoted a significantly higher respiratory inhibition in the presence of glutamate than in the presence of succinate, indicating that they promote the inhibition of both Complexes I and III. This respiratory inhibition promoted by TAM^+ dyes can probably be attributed to the oxidation of essential protein thiols in Complexes I and III, which are more sensitive to oxidation-promoted respiratory inhibition. On the other hand, mitochondrial Complex IV is not commonly affected by oxidants and was not inhibited by TAM^+ dyes, as verified by the lack of effect of these dyes on TMPD-supported mitochondrial respiration.

The small differences in effects of different dyes on mitochondrial respiration, evidenced in Table I, seem to be more related to the concentration of the dye used than distinct affinities of these dyes for the mitochondria (Table II). EV^+, CV^+ MG^+, and VBB^+ binding to the mitochondrial membrane was significantly lower when the dyes were added to mitochondria pretreated with FCCP, to prevent the formation of a mitochondrial membrane potential. This demonstrates that mitochondria actively accumulate these TAM^+ dyes when energized. The lack of a membrane potential may not have significantly altered the uptake of VBR^+ and $VPBBO^+$ since the dyes promote mitochondrial uncoupling under these conditions (see Fig. 4A and C).

Interestingly, when studying the effects of the dyes on mitochondrial membrane potential supported by TMPD, we noted that TAM^+ dyes present two distinct classes of behaviors. EV^+, CV^+, and MG^+ promote a drop in mitochondrial membrane potential, accompanied by mitochondrial swelling, which was inhibited by EGTA, Mg^{2+} and cyclosporin A (Fig. 1A and B), effects characteristic of MPT. These dyes also promoted a decrease of mitochondrial respiratory rates (Fig. 1C), attributable to cytochrome *c* release following mitochondrial swelling (Green and Reed, 1998).

Indeed, the respiratory rates could be partially restored by the addition of high concentrations of exogenous cytochrome *c* (results not shown). In addition, MPT promoted by these dyes could be observed even in the dark and was only marginally stimulated by photoirradiation. This suggests that these dyes promote MPT independently of photodynamically generated radical species. In fact, EV^+, CV^+, and MG^+ are capable of directly oxidizing mitochondrial NAD(P)H (Fig. 3), leading to mitochondrial oxidative stress which favors MPT (Kowaltowski and Vercesi, 1999). These experiments have characterized EV^+, CV^+, and MG^+ as new MPT inducers, which act by promoting the oxidation of mitochondrial pyridine nucleotides, leading mitochondrial oxidative stress (Kowaltowski and Vercesi, 1999).

Differently from EV^+, CV^+, and MG^+, victoria blue dyes VBR^+, VBB^+ and $VPBBO^+$ promoted a decrease in mitochondrial membrane potential even in the presence of EGTA and Mg^{2+} or cyclosporin A (Fig. 4). In fact, EGTA and Mg^{2+} or cyclosporin A had no effect on mitochondrial uncoupling promoted by VBR^+ and VBB^+. Only a small part of $VPBBO^+$-induced mitochondrial uncoupling could be prevented by EGTA and Mg^{2+} or cyclosporin A under our conditions. When very low concentrations ($0.03–0.125 \mu M$) of $VPBBO^+$ were used, the preventive effect of EGTA, Mg^{2+}, and cyclosporin A was larger (results not shown), suggesting that under some conditions this dye may also cause primarily MPT (see Morgan *et al.*, 1998). Confirming that mitochondrial uncoupling induced by VBR^+, VBB^+, and $VPBBO^+$ could not be attributed to MPT, no mitochondrial swelling or respiratory inhibition secondary to cytochrome *c* release was observed. We have demonstrated that the drop in membrane potential and increased respiratory rates in the presence of these dyes could be attributed to their protonophoric activities, as confirmed by two distinct tests for proton conductance activity across the inner mitochondrial membrane (Fig. 5).

The characterization of protonophofic effects of TAM^+ dyes at submicromolar concentrations is novel and surprising, since these compounds are cationic. It is possible that VBR^+, VBB^+ and $VPBBO^+$ act similarly to amphiphilic amines such as bupivacaine, forming transient linear complexes across the inner mitochondrial membrane, which provide binding sites for protons (Sun and Garlid, 1992). However, the presence of bupivacaine multimers are required for this process and the concentrations of victoria blue dyes used in our experiments was low. Further studies are necessary to understand the mechanism through which these TAM^+ dyes promote proton transport across the inner mitochondrial membrane.

In summary, we found that, to different extents, all TAM^+ dyes may promote mitochondrial respiratory inhibition, enhanced by light. In the absence of respiratory inhibition, during TMPD-supported respiration, the dye effects present two distinct patterns. While VBR^+, VBB^+ and $VPBBO^+$ act as classical mitochondrial uncouplers, which would lead predominantly to necrotic cell death, EV^+, CV^+, and MG^+ promote MPT, which could result in apoptosis, an ideal form of cell death in antioncotic therapy. These observations suggest that TAM^+ dyes are of potential use for the development of novel mitochondrial-targeting strategies for chemo- and photochemotherapy, and, we hope, should help further research in antioncotic and photodynamic drug design.

ACKNOWLEDGMENTS

We thank Claudia O. Silva for the preparation of rat liver mitochondria. This work was supported by grants from FAPESP, FAEP-UNICAMP, PRONEX, CNPq-PADCT, and the University of Wisconsin at Madison. A.J.K. and J.T. are students supported by FAPESP fellowships. G.L.I. is supported in part by the Burroghs Wellcome Fund–American Foundation of Pharmaceutical Education (AACP Grant Program for New Investigators).

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