

ION CURRENTS AS INITIATORS OF LIGHT-INDUCED EFFECTS: STUDIES USING PHOTOSENSITIZERS

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The effects of reactive oxygen species, especially as generated by photosensitizers and light, may serve as a model for light-induced effects relying on endogenous chromophores. Early events in the series of reactions initiated by these processes are reviewed and representative data on block of ionic currents, membrane permeabilization and increase in intracellular calcium concentration are presented. A general model for the effects of oxidative stress ranging from no effect with mild challenge to necrosis following severe challenge is presented. The results suggest that a $^1\text{O}_2$ mechanism could be operative for some forms of low level laser therapy. Energetic considerations suggest that this mechanism is only likely for irradiation wavelengths shorter than 1270 nm.

Keywords: Cultured cells, photosensitization, photosensitizer, voltage clamp, patch clamp, GH₃ cells, leak current, calcium, cell killing, cell signaling

Introduction

For light to exert an effect on a biological preparation it must be absorbed by a chromophore, either endogenous or exogenous, promoting the chromophore to an excited state. The excited state must then transfer that energy of excitation in a manner that leads to a chemical or physical change in the system. From this point of view, photosensitization reactions involving exogenous chromophores may be quite similar to those involving endogenous chromophores. Although chromophore localization may affect the outcome, it is likely that similar signaling mechanisms may mediate both kinds of reactions. Here we examine recent developments related to early cell signaling events subsequent to photosensitized modification of cells as a model for light-induced effects in general, with or without a photosensitizer.

Photosensitization, Reactive Oxygen and Oxidative Stress

Photosensitized modification is generally viewed as an oxidative stress, and in particular, has often been viewed as a singlet oxygen ($^1\text{O}_2$) mediated effect. Singlet oxygen can be generated from a variety of sources, for example, from photosensitizers in the environment, such as hypericin in St. John's wort, a variety of drugs including nonsteroidal antiinflammatory agents and antidepressants, photodynamic pesticides, and certain dyes used in foods, drugs and cosmetics. These photosensitizers, as well as endogenous chromophores, generate $^1\text{O}_2$ and other reactive oxygen species (ROS) following absorption of light or ultraviolet radiation (UVR). The toxic effects of photosensitizers to

animals and humans have been well documented for many decades^(1,2), although the underlying mechanisms are only now beginning to be elucidated. We now, also, recognize that $^1\text{O}_2$ plays a role in common pathological conditions such as reperfusion following ischemia (I/R).

Singlet oxygen as a physiologically relevant oxidative stressor

Singlet oxygen is a relatively neglected member of a set of environmentally-relevant cellular stressors including heat shock⁽³⁾, Ca^{++} overload, ischemia⁽⁴⁾ and a variety of oxidative stressors. Oxidative stressors include superoxide radical anion (O_2^-), hydroxyl radical (OH), peroxynitrite (ONOO), and various other carbon-centered/lipid-centered radicals⁽⁵⁾. Currently, evidence is accumulating that $^1\text{O}_2$, previously thought to be of no physiological relevance, is likely to play a role in cellular oxidant stress^(6,7), and potentially in normal cell function⁽⁸⁾. For example, $^1\text{O}_2$ appears to be a trigger for protein synthesis and for gene activation^(9,10,11). Recently, Daub and co-workers have isolated a gene from a plant pathogenic fungus that confers resistance to $^1\text{O}_2$ when incorporated into $^1\text{O}_2$ susceptible organisms⁽⁸⁾.

Homologs of this $^1\text{O}_2$ resistance gene have been found in a large number and diversity of organisms including bacteria, yeast, plants⁽⁸⁾, and animals including humans⁽¹²⁾. A variety of reactions can generate $^1\text{O}_2$ *in vivo*⁽¹³⁾ including the reaction of ONOO \cdot with H_2O_2 ⁽¹⁴⁾, with O_2 ⁽¹⁵⁾ or with model organic peroxides⁽¹⁶⁾. In fact, some effects attributed to ONOO \cdot *in vivo* might result from $^1\text{O}_2$ production, a possi-

bility supported by the observation that mutations induced by ONOO⁻ differ significantly from those induced by OH[•], but show similarities to those reported for ¹O₂ (17).

Importance of oxidative stress and cell death

Oxidative stress and associated cell death are integral components of dysfunctional processes in many systems including the heart and vasculature. In addition to its role in I/R injury, oxidative stress is thought to play a role in myocardial stunning (18). Stress-induced cell death also contributes to heart failure due to acute myocardial infarction, cardiac hypertrophy and aging (19) and vascular wall injury, and similar stress may initiate ischemic preconditioning (3). Considerations such as these have led us to study photosensitization-induced effects on cardiac cells in an attempt to understand the early signaling mechanisms related to these consequences.

Early Events in Photosensitization-Induced Oxidative Stress

Block of Ionic Currents

One of the earliest effects of photosensitization is alteration of ionic fluxes across cell membranes (20,21). In a variety of cell types we have shown that most ionic currents

are blocked by photosensitization (Fig. 1). For example, in freshly isolated cardiac cells sodium (I_{Na}), calcium (I_{Ca}) and potassium (I_K) currents are all blocked. Typical of most currents studied, I_K is blocked in a single exponential process by photosensitization (22). The rate of block of I_K is considerably slower at depolarized membrane potentials compared to the normal resting potential (23).

The rate of block of ionic currents by photosensitization suggests that they are not involved in signaling processes that lead to irreversible cell damage. In frog atrial cells Na⁺, K⁺ and Ca⁺⁺ currents are all blocked with considerably less illumination than is needed to cause the cells to go into contracture (24). However, the contracture seen in frog atrial cells may be a different process than that leading to cell death in other cell types. Therefore, we performed similar studies using cultured GH₃ cells, assessing cell death with 3 different assays. We demonstrated that, in the presence of 0.5 μM RB, cell death occurred with 1.6 to 3.9 J/cm² of light. Under the same conditions the known ionic currents in GH₃ cells, namely I_{Ca}, delayed rectifier K⁺ current and Ca⁺⁺-activated K⁺ current, were all blocked with less than 1.5 J/cm² of light.

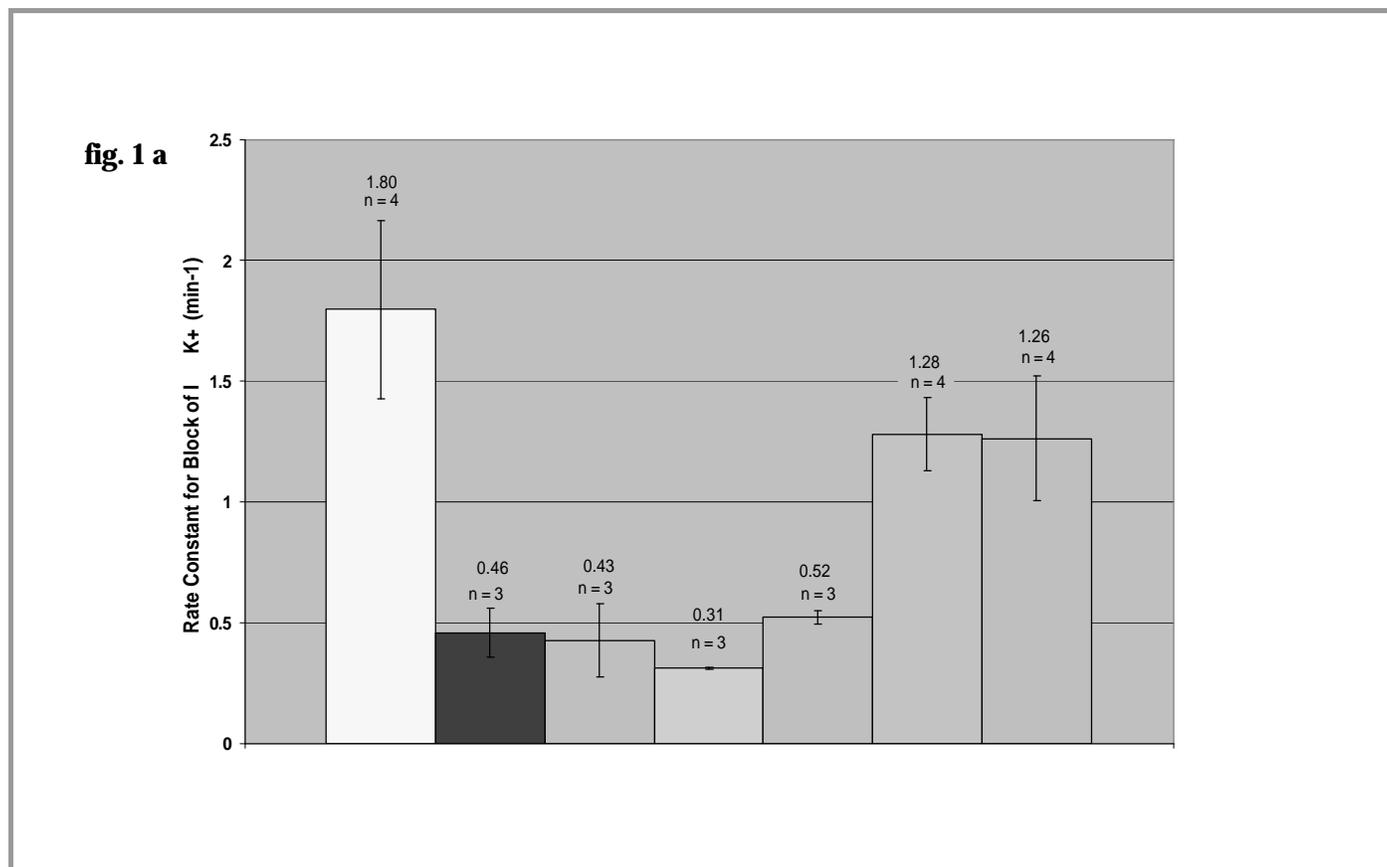


fig. 1 b

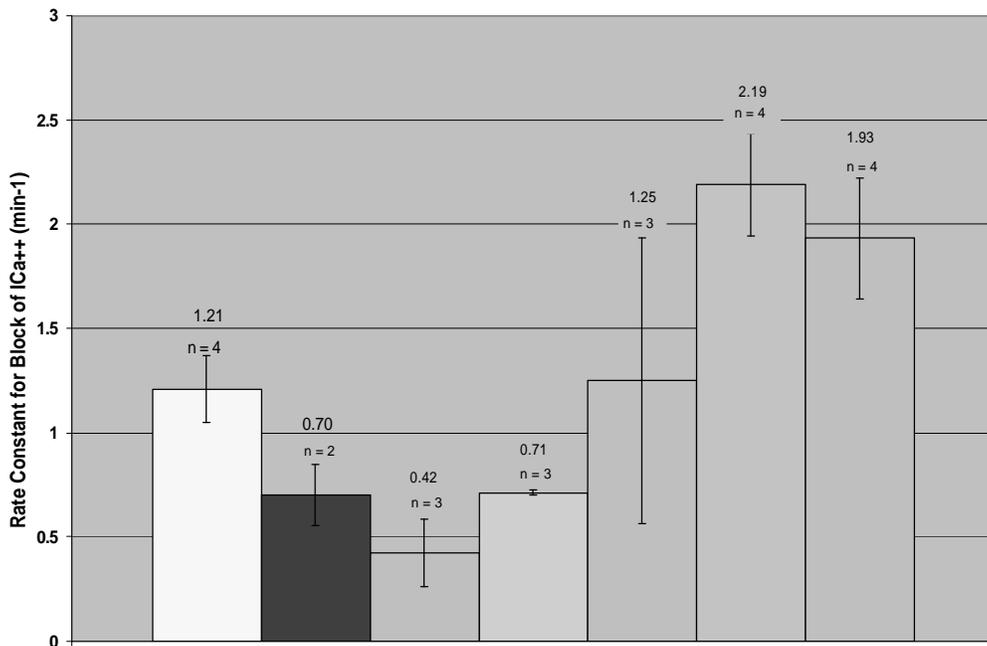
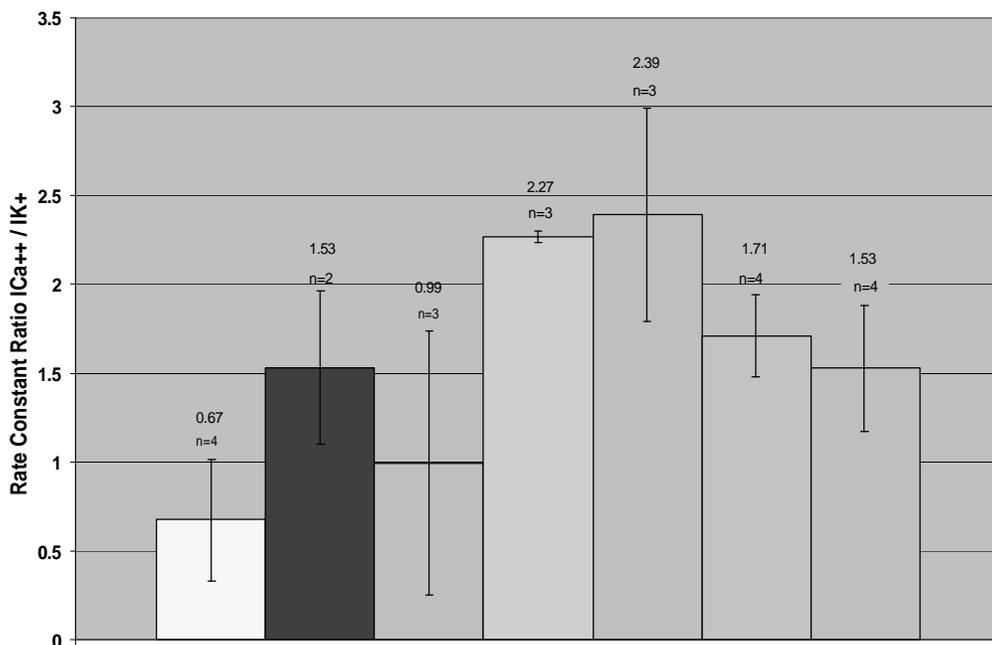


fig. 1 c



- = 0.125 μM RB;
- = 1 μM AO;
- ▒ = 1 μM MB;
- ▓ = 15 μM EY, voltage clamp pulse to 0 mV;
- ▒ = 15 μM EY, voltage clamp pulse to +40 mV;
- ▓ = 0.625 μM PB, voltage clamp pulse to 0 mV;
- ▒ = 0.625 μM PB, voltage clamp pulse to +40 mV.

Figure 1. Block of ionic currents. Isolated frog atrial cells, bathed in 150 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 10⁻⁴ M tetrodotoxin and photosensitizer. The patch pipette contained 150 mM KCl, 20 mM tetraethylammonium chloride and 10⁻⁵ M cyclic AMP. After pipette attachment voltage clamp currents from a -70 mV holding potential were recorded during pulses to 0 mV and +40 mV. Once currents had stabilized control records were recorded and illumination was started (approximately 6.5 mW/cm²). Currents were recorded at appropriate intervals during continuous illumination. Potassium and calcium currents were extracted from the voltage clamp currents⁽²⁵⁾ and rate constants for current block were calculated from logarithmic plots of current versus time of illumination⁽²²⁾. Values are means ± S.E.M. A) Exponential rate constants for block of I_k; B) Exponential rate constants for block of I_{ca}; and C) Ratio of rate constants for I_{ca} to that for I_k.

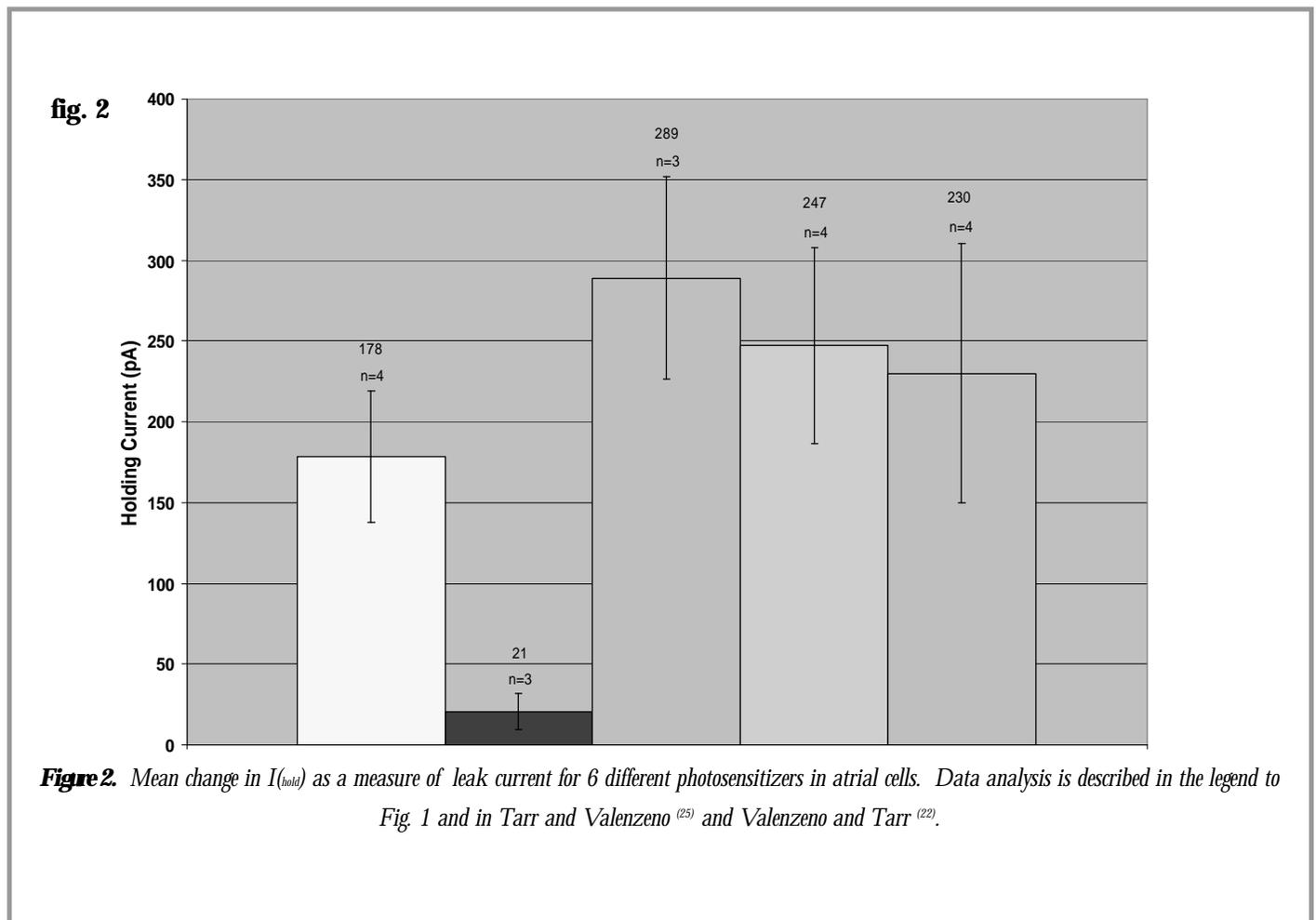
Thus, photosensitization can block a variety of ionic currents but the rates of block are not identical for all photosensitizers and for all ionic currents. In fact, some specificity can be observed among different photosensitizers. Figure 1 shows the rate constants for block of I_K and I_{Ca} by 5 different photosensitizers. Figure 1A shows that Rose Bengal (RB) is particularly effective in blocking I_K . Acridine orange (AO), methylene blue (MB) and eosin yellow (EY) were much less effective. On the other hand, Fig. 1b indicates that Phloxine B (PB) appears to be the most effective photosensitizer for block of I_{Ca} . RB and MB are less effective and about equivalent. These differences are emphasized in Fig. 1C, where the ratio of rate constants for block of I_K to that for block of I_{Ca} are presented. The non-equivalence of the data in Fig. 1c indicates photosensitizer selectivity for current block. EY shows the greatest selectivity between I_{Ca} and I_K ; MB shows the least (ratio $\gg 1$).

Induction of Leak Current

In a variety of cell types we have shown that while most

ionic currents are blocked by photosensitization, a new current that we refer to as leak (I_{LEAK}) develops (Fig. 2). For example, in freshly isolated cardiac cells the block of ionic currents, discussed above, precedes development of I_{LEAK} , which is induced with complicated kinetics, when studied at a single voltage⁽²²⁾. However, I_{LEAK} is initially a K^+ current, but it becomes a mixed K^+ and Na^+ current as its magnitude increases⁽²⁶⁾. A similar current in opossum kidney cells appears to be carried by both cations and anions⁽²⁷⁾.

Figure 2 demonstrates the induction of I_{LEAK} in frog atrial cells assessed as the current required to clamp the membrane potential at a -70 mV holding potential (I_{hold}) in the same set of cells used for Fig. 1. The figure shows that, like the block of voltage-gated ionic currents, induction of I_{LEAK} is also dependent on the photosensitizer used. AO was very ineffective in inducing I_{LEAK} compared to the other photosensitizers tested. MB, which was relatively ineffective at blocking voltage-gated currents, is the best photosensitizer of the group in inducing leak current.



Leak Current, $^1\text{O}_2$ and Membrane Depolarization

We have compared ⁽²⁸⁾ block of cardiac delayed rectifier K^+ current (I_K) to activation of the permeabilization-related leak current (I_LEAK) using a $^1\text{O}_2$ generator (RB) or a non- $^1\text{O}_2$ generator, menadione (MQ). The photosensitizer MQ is well known to produce redox reactions in the dark, but at lower concentrations and shorter incubation times it also produces a light-dependent photomodification of cells and tissues that can be separated from the dark effects ^(29,30). Both photosensitizers blocked I_K and activated I_LEAK . However, when we adjusted photosensitizer concentrations and illumination intensity so that both produced the same rate of I_K block, we found that RB was about 12-fold more effective at activating I_LEAK . In the same study we demonstrated via time-resolved infrared flash photolysis that RB produced the expected yield of $^1\text{O}_2$, whereas MQ produced no detectable $^1\text{O}_2$. These studies point toward $^1\text{O}_2$ as a very effective permeabilizer of the cardiac cell membrane.

An expected consequence of membrane permeabilization is membrane depolarization, and this depolarization occurs while cells are still viable and have the potential of being rescued from ROS-induced cell damage. It is well established, for example, that cardiac cells depolarize following exposure to ROS generated by a variety of means including H_2O_2 ⁽³¹⁾, dihydroxyfumaric acid ^(32,33), xanthine plus xanthine oxidase ⁽³²⁾, iron chelates ⁽³⁴⁾ and cumene hydroperoxide ⁽³⁵⁾ as well as RB and light ⁽³⁶⁾. Permeabilization and depolarization also occur in non-cardiac cells exposed to oxidant stress. For example, alloxan and its auto-oxidation product H_2O_2 irreversibly depolarize insulin-secreting cells ^(37,38), and the oxidant *tert*-butylhydroperoxide depolarizes pulmonary artery endothelial cells in a manner similar to that produced by intracellular oxidized glutathione ^(39,40). Oxidant stress initiated by cell exposure to long wavelength UVR depolarizes a variety of cell types in a manner similar to that produced by the oxidant *tert*-butylhydroperoxide ⁽⁴¹⁾.

Calcium influx

In addition to the damaging effects membrane depolarization has on the electrophysiological properties of excitable cells, the underlying sustained increase in membrane permeability to Na^+ , K^+ and Ca^{++} poses a threat to cell survival. First, it increases the energy demands for maintenance of electrolyte and water balance. Second, the increased Ca^{++} influx increases the likelihood of cell damage related to Ca^{++} overload. The increase in intracellular calcium concentration following exposure to photosensitizer-generated reactive oxygen can be detected using fluorescent indicators for calcium, as shown in Fig. 3. In other studies we demonstrated that Ca^{++} influx linked to photosensitization-Laser Therapy Vol. 13

induced membrane permeabilization produces intracellular Ca^{++} overload and hypercontracture of cardiac cells ⁽²¹⁾. In light of the stress that increased sarcolemmal permeability can impose on a cell, it is reasonable to hypothesize, as other investigators have recently done ^(38,42,43), that this permeabilization may be both an early and important event in initiating cell death.

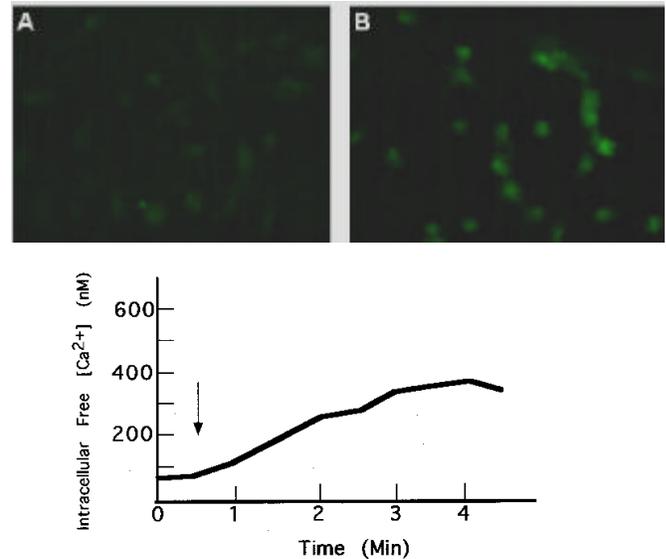


Figure 3. Calcium green fluorescence from H9c2 cultured rat cardiac cells before (top left) and after 3 min (top right) of RB-sensitized photomodification. The plot is the increase in Ca^{++} concentration in one cell. Illumination via the halogen arc lamp illuminator of the fluorescence microscope began at the time indicated. Determinations were made following 4 h of incubation of cells, 2 days post-plating, in the presence of $10 \mu\text{M}$ calcium green 2 acetoxymethyl ester at 37°C in a humidified $5\% \text{CO}_2$ incubator.

Ionic Currents and Cell Killing

The available evidence suggests that I_LEAK plays a significant role in photosensitization-induced cell killing, probably related to its ability to mediate calcium influx. In isolated frog atrial cells we have demonstrated a correlation between the time of onset of contracture with the development of I_LEAK in response to RB and light. In associated studies isolated membrane patches also showed an analogous permeability increase ⁽⁴⁴⁾, and this permeability pathway allowed passage of both sodium and calcium ⁽⁴⁵⁾. The latter is similar to results in whole cells showing that I_LEAK is a relatively nonspecific cation pathway ⁽²⁶⁾. However, since the contracture seen in frog atrial cells is not identical to cell death, we assessed cell killing using cultured GH₃ cells and 3 different assays of cell death. We demonstrated that in the presence of $0.5 \mu\text{M}$ RB cell death occurred with 1.6 to $3.9 \text{ J}/\text{cm}^2$ of light. As noted above, block of Na^+ , Ca^{++} and K^+ currents was complete after an exposure to only

1.5 J/cm². In contrast, I_{LEAK} began to increase with a few tenths of a J/cm² of light, but was still increasing at 2 J/cm², the longest illumination studied. Thus, I_{LEAK} is the only ionic current that changes with illumination in the range of fluences that produces killing of GH₃ cells⁽⁴⁶⁾. This is the first demonstration of the relative time courses of photomodification of membrane ionic currents compared to cell killing.

Oxidative Stress, Ionic Currents and Cellular Responses

There is now a substantial body of evidence that membrane permeabilization, Ca⁺⁺ influx, and increased intracellular free ionized Ca⁺⁺ are early and ubiquitous phenomena associated with cellular oxidative stress. There is also considerable evidence that these events are linked. The ways that cells respond to oxidant stress may be instructive in understanding their responses to other stresses, since there are many similarities among the responses to stresses such as alcohol, heat and oxidants. Certainly, the photosensitizer and light effects that have been reviewed above are similar. Recent discussions of the effects of oxidant stress have concluded that depending on the level of stress, cells may respond in different ways. As the level of stress increases responses occur in the following progression.

1. Very low-level stress - within normal limits, physiological signaling, survival
2. Low-level stress - stress signaling, antioxidant induction, survival
3. Moderate-level stress - stress signaling, apoptotic program, apoptotic death
4. High-level stress - gross damage, necrotic death

The foregoing is adapted from the proposal of Girotti⁽⁴⁷⁾ relating to oxidant initiated lipid peroxidation, but is typical of other stress response schemes. For example, Berridge, et al.⁽⁴⁸⁾ have described similar effects of calcium signaling and overload as life and death determinants for cells. While the latter steps leading to cell death in these schemes may be related to cell permeabilization, the earlier steps that do not lead to cell death may depend on lower levels of permeabilization, or they may depend on the changes in the well-defined ionic currents of cells, most likely block or reduction of those currents.

Ionic currents as cell signals

Non-receptor-mediated signals, specifically reactive oxygen intermediates, may be linked to intracellular second messenger systems⁽⁴⁹⁾ and thereby effect a wide variety of cel-

lular responses. A potential link is via reactive oxygen effects on ion currents. Reactive oxygen intermediates are now well recognized as mediators capable of regulating ion channel activity and gene expression⁽⁵⁰⁾. For example, critical cysteine residues in potassium channels have been shown to be susceptible to reactive oxygen attack resulting in modifications of the inactivation behavior of the channel. Channels lacking these cysteine residues were not sensitive to attack⁽⁵¹⁾. Our work has shown that singlet oxygen generated by photosensitizers modifies a wide variety of ion channels resulting in changes in kinetics and channel block, and here we show that there is selectivity among photosensitizers in terms of their effects on ionic currents. Cloned potassium channels also show susceptibility to photosensitizer generated ¹O₂, but not all channels are equally sensitive. Kv1.3, Kv1.4, Kv1.5, Kv3.4 and IRK3 are very sensitive, while Kv1.2, Kv2.1, Kv2.2, Kv4.1, IRK1, ROMK1 and hIsK are much less sensitive or not sensitive at all⁽⁵²⁾. In addition to block of existing channels, photosensitizer generated ¹O₂ induces a new "leak" pathway in a variety of cell types as shown by us and others⁽⁵³⁾.

Implications for Low Level Laser Therapy

Identification of cellular mechanisms mediating the effects of low level laser therapy is likely to provide a significant boost to this therapeutic modality. Singlet oxygen generation as a result of energy transfer from endogenous chromophores with consequent modification of cellular structures should be considered as a possible mechanism. Here we have emphasized the cell signaling effects that ¹O₂ can produce via actions on membrane ionic currents. Since different cell types possess different ionic channels, and even the same cell types have different ionic channels at different stages of development, a variety of cell responses could be produced by the same laser treatment. Thus, the proposed mechanism is quite robust.

On the other hand it also places limitations on the laser irradiation protocols that could operate in this manner. To produce ¹O₂, sufficient energy must be transferred to molecular oxygen to promote it from the ground state triplet level to the excited singlet state. This energy requirement amounts to 22.5 kcal/mol, equivalent to a photon wavelength of 1270 nm, or the phosphorescence emission wavelength of singlet oxygen⁽⁵⁴⁾. To transfer energy of this magnitude to oxygen, the chromophore excited state must have at least this much energy. Thus, a singlet oxygen mechanism resulting from photoactivation of an endogenous (or exogenous) chromophore can only be operative for irradiation wavelengths below 1270 nm.

Conclusions

Responses of cells to photosensitizers and light and the established pattern of cellular responses to stress suggest that early signaling events resulting from exposure to low-level light might be found in $^1\text{O}_2$ -mediated effects operating via changes in ionic currents - inhibition or block of those currents - in affected cells.

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WALT President, Dr. Nick Nicolopoulos (4th from right) poses for a picture with dignitaries at the 3rd Congress held in Athens, Greece. Adults in the photo include (L to R) Astronaut _____, Dr. Isaac Kaplan, Dr. Nicolopoulos and Dr. Toshio Ohshiro, May, 2000.



A cross-section of dignitaries at the Opening Ceremony of the Japanese Society for Laser Medicine and Surgery (from L to R are an official of the Congress, Professors Atsumi, Rao and Enwemeka), Fall, 2000



Some of the delegates from South America at the 3rd Congress of WALT held in Athens, Greece (from L to R are Professors Fernando Soriano, Tony Pinhiero, and Aldo Brugnera), May, 2000



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