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Review

Calcium, a pivotal player in photodynamic therapy?☆

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ABSTRACT

Photodynamic therapy combines three non-toxic components: light, oxygen and a photosensitizer to generate singlet oxygen and/or other ROS molecules in order to target destruction of cancer cells. The damage induced in the targeted cells can furthermore propagate to non-exposed bystander cells thereby exacerbating the damage. Ca^{2+} signaling is strongly intertwined with ROS signaling and both play crucial roles in cell death. In this review we aimed to review current knowledge on the role of Ca^{2+} and ROS signaling, their effect on cell-cell propagation via connexin-linked mechanisms and the outcome in terms of cell death. In general, photodynamic therapy results in an increased cytosolic Ca^{2+} concentration originating from Ca^{2+} entry or Ca^{2+} release from internal stores. While photodynamic therapy can certainly induce cell death, the outcome depends on the cell type and the photosensitizer used. Connexin channels propagating the Ca^{2+} signal, and presumably regenerating ROS at distance, may play a role in spreading the effect to neighboring non-exposed bystander cells. Given the various cell types and photosensitizers used, there is currently no unified signaling scheme to explain the role of Ca^{2+} and connexins in the responses following photodynamic therapy. This article is part of a Special Issue entitled: Calcium signaling in health, disease and therapy edited by Geert Bultynck and Jan Parys.

1. Introduction

Cancer has become one of the main causes of mortality and morbidity worldwide. In 2012 14.1 million new diagnosis and 8 million deaths worldwide could be related to cancer [1]. The aging population will see this number to double the next two decennia. Although an earlier diagnosis via screening programs and better treatment modalities have resulted in decreased mortality rates, cancer remains a major challenge in terms of morbidity and mortality [2]. Various, often very specialized treatment schedules are available depending on the cancer type, stage and location of the primary tumor. The best known treatment modalities are surgery, radio- and chemotherapy and in recent years also immunotherapy. Next to these therapies other approaches can be used or combined, including gene therapy and photodynamic therapy (PDT). Resistance to therapy is an emerging problem so unraveling the underlying mechanistic properties and consequences of all types of treatment is important to ascertain specific targeting of the tumor cells and efficiently eliminating them [3,4]. In this paper we aimed to provide an overview on the role of cytoplasmic Ca^{2+} in the execution of cell death and the spreading of the bystander effect to surrounding non-exposed cells following PDT. Connexin proteins play a special role in Ca^{2+} signaling, as they are involved in communicating $[Ca^{2+}]_i$ changes to neighboring cells and also contribute to other forms of Ca^{2+} dynamics such as oscillations (reviewed in Leybaert and Sanderson [5] and De Bock et al. [6]). We therefore also included a discussion on the role of connexins in the context of PDT, again with emphasis on Ca^{2+} signaling.

2. Photodynamic therapy

PDT is a treatment modality in which a photosensitizer (PS) is activated by visible or infrared light of the appropriate wavelength. This activation results in the conversion of the singlet state of the PS to the triplet state, often combined with fluorescence and heat [7]. The triplet state, which is the higher energy formation of the PS, generates cytotoxicity by formation of singlet oxygen (Type II reaction) and/or other reactive oxygen species (ROS) (Type I reaction) resulting from collisions with other molecules. Most PSs attain a combination of the two types following activation and hence, create a mixture of singlet oxygen and free radicals. PDT thus combines three non-toxic components: light, PS and oxygen to combine and form highly reactive ROS or singlet oxygen molecules. These molecules can subsequently inflict damage to the tumor cells or tumor vasculature, which may result in cell death and may mount an anti-tumor immune response, both leading to

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destruction of the tumor cell mass [7–9]. ROS formation is a process that is used in various cancer treatments to accomplish tumor cell death with less pronounced effects on healthy tissue. In tumor cells, a smaller amount of ROS is required to trigger cell death compared to normal cells since cancer cells have a high intrinsic oxidative activity and a dysfunctional antioxidant response [10].

Most PSs are cyclic tetrapyrrolic structures: porphyrins and analogues (e.g. chlorins, bacteriochlorins, phthalocyanines) [9]. The exact chemical structure of the PS determines its subcellular localization and efficacy in generating ROS and/or singlet oxygen [11]. The addition of PS alone without light exposure or light exposure without the presence of PS does not result in toxicity; the resulting photoactivation and ROS or singlet oxygen generation are thus necessary to provoke toxic reactions in the target cells. Ever since the first PSs were introduced in clinic, much research has been devoted to further improving them. This has resulted in the development of second generation PSs, mainly characterized by improved tissue penetration due to the usage of light with a longer wavelength in the infrared spectrum, and the third generation PSs, with compounds that are characterized by a better targeting towards tumor cells in order to avoid side effects on normal tissue. Targeting can be achieved in different ways; one of them is coupling of PSs to nanoparticles [12]. Another method to reduce general toxicity consists of better light focusing as well as reducing the illuminated area [8].

Today, the ideal PS has preferably (i) a high absorbance in the infrared spectrum to improve tissue penetration, (ii) a high singlet oxygen/ROS yield, (iii) photo stability, (iv) no intrinsic toxicity and (v) a high water solubility and a fast pharmacokinetic elimination to avoid the necessity for post-treatment protection from light exposure [7].

Depending on the type of PS, different preferences exist in terms of subcellular localization. The fluorescence produced as a side effect of the photodynamic reaction can be used to monitor the subcellular localization of the PS [7]. Some PSs target the mitochondria or endoplasmic reticulum (ER), whereas others target lysosomes, the Golgi apparatus or the plasma membrane, or a combination of multiple organelles. The triggered cell death pathway and the speed of cell death onset depend on the subcellular localization of the PS [13]. Mitochondria are considered as the most effective target in cancer therapy because of their essential role in the cell's energy metabolism [9]. On top of that mitochondria are central players in ROS production and apoptosis induction [14]. Targeting mitochondria normally results in fast initiation of apoptosis. Porphyrins, which are widely used PSs, target the mitochondria via their high affinity for the benzodiazepine receptor present on the outer mitochondrial membrane [15]. Another important organelle is the ER because of its important Ca^{2+} storage function; additionally, perturbation of the ER can also result in cell death [7]. PSs targeting the lysosomes can induce cell death via the release of lysosomal enzymes or relocalization of the PS to other targets. TPPS4 localizes primarily in endosomes and lysosomes, although basal concentrations are also found in mitochondria; however exposure to light causes the lysosomal membrane to rupture, which leads to a redistribution of the photoactivated PS to the mitochondria and subsequent mitochondrial photodamage [16]. Apoptosis induced in this way appears to be slower compared to when mitochondria are directly targeted [15]. By combining different PSs or choosing a PS that targets multiple organelles, more efficient tumor cell death induction can be achieved [8]. Unlike ionizing radiation, PDT does not necessarily target the nucleus, which may represent a therapeutic advantage because it avoids the spreading of genomic instability [10]. Since ROS and singlet oxygen have a limited diffusion capability, the organelle in which the PS is present will preferentially receive most damage [7]. As a result, the subcellular localization [8] together with the concentration of the PS [17] and the light dose [18], determines the cell death execution pathway solicited in the targeted cells (see further below).

PDT is mainly used to target small, superficial lesions in which the PS can easily permeate and light is able to reach the PS, e.g. lung, esophagus and skin cancers. PDT can be combined with any other conventional cancer treatment and has little effect on the connective tissues, resulting in minimal scarring, which makes it an ideal treatment for lung tumors. PS for superficial skin tumors could be applied topically, resulting in less toxicity [3]. Despite the fact that PDT has been approved as cancer treatment for over 30 years, its use in clinic is still limited. This relates to the fact that it is only efficient in small, superficial lesions [19] and its side effects of pain and skin photosensitivity, which requires shielding from sun light or other bright light exposure during the time the photosensitizer is still present in the tissues. Coupling of the PS to antibodies that target specifically tumor cells or nanoparticles, which can be guided to the tumor area by magnetic fields, could improve the clinical outcome and minimize general side-effects [9]. In addition tumor areas can have hypoxic regions and since oxygen is necessary for the PDT reaction, this can compromise PDT effectiveness [17].

PDT can be used clinically in (i) a curative way, particularly in early stage tumors and in (ii) palliation of advanced cancers in order to prolong survival and improve quality of life. This treatment modality is mainly used to treat skin cancers, Barrett esophagus and unresectable cholangiocarcinoma [20]. It can be combined with surgery, chemotherapy or radiotherapy without interfering with the outcomes of these treatments. Because of the excellent cosmetic outcomes and negligible effects on the underlying tissue, PDT is best known for its use in the treatment of skin cancer [21]. The first generation of PSs has several drawbacks (as already discussed), thus research is progressing to discover new PS molecules that can overcome these negative aspects. Most work has been based on the adaptation of the first generation PSs including the use of other non-porphyrin-related structures and coupling of PSs to carriers such as nanoparticles. With the successes booked in these developments, it is expected that PDT will gain more attention in the clinical practice [22].

3. Effects in directly targeted cells: the role of the cytoplasmic Ca^{2+} concentration

Calcium ions function as an important messenger signal for both intracellular and intercellular communication. The free calcium ion concentration in the cytoplasm ($[Ca^{2+}]_i$) controls a large array of important physiological and pathological processes. In physiological resting conditions $[Ca^{2+}]_i$ is maintained at a very low values (~100 nM) whereas the extracellular concentration is ~1.8 mM. Various channels, combined with cytoplasmic buffers and primary or secondary transport mechanisms ensure that the $[Ca^{2+}]_i$ only increases in well-defined spatial and temporal domains in order to ensure specific signaling in the cell of this universal second messenger [5]. Increases in $[Ca^{2+}]_i$ are induced by the entry of Ca^{2+} via channels in the plasma membrane or by the release from internal Ca^{2+} stores in the cells. The ER is known as the largest and most important intracellular Ca^{2+} store containing 5–50 mM Ca^{2+} [23]. Sequestration of Ca^{2+} from the cytosol into the ER is mediated by the ER Ca^{2+}-ATPase (SERCA) pump [24]. Ca^{2+}-release from the ER is established by two receptor families: the ryanodine receptors (RyRs) and the inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs). While the endogenous activator of RyRs is Ca^{2+} itself, resulting in Ca^{2+}-induced Ca^{2+} release, IP_3 produced by activation of phospholipase C (PLC) activates IP_3Rs and subsequently triggers ER Ca^{2+} release [25]. Ca^{2+}-induced Ca^{2+} release acts to amplify microscopic initiation events into more globally propagating intracellular Ca^{2+} signals [26]. After ER release, Ca^{2+} is pumped back into the ER by the SERCA pump, and is taken up by the mitochondria, which help in shaping the temporal appearance of intracellular Ca^{2+} signals [27]. Moreover, plasma membrane Ca^{2+} ATPases (PMCAs) and Na^+/Ca^{2+} exchangers may contribute to the recovery process by pumping Ca^{2+} out of the cell. As a result, the Ca^{2+} that has left the cell needs to be taken up by the cell by store operated Ca^{2+} entry via STIM1/ORAI membrane channels that are activated by ER store depletion [28].

Finally, Ca^{2+} signals can also be propagated to surrounding cells as intercellular Ca^{2+} waves. Intercellular Ca^{2+} waves are propagated by the diffusion of IP_3 and Ca^{2+} in the cytoplasm and through gap junction channels connecting the cells; additionally the release of adenosine triphosphate (ATP) and other Ca^{2+} mobilizing paracrine messengers play an important role in this process [5,29]. ATP can be released via connexin hemichannels (see further below), Panx channels or P2X7 receptor channels [29–32]. Downstream of ATP, Ca^{2+} enters the cell via ionotropic P2X receptor channels or is released from the ER by the activation of the PLC-IP_3 axis via G-protein-coupled P2Y receptors [33]. When the uptake of Ca^{2+} by mitochondria results in Ca^{2+} overload in this organelle, this can lead to excess production of ROS, opening of the mitochondrial permeability transition pore, outer membrane permeabilization and eventually apoptotic cell death [34]. The intercellular propagation of $[Ca^{2+}]_i$ changes as Ca^{2+} waves have been reported in the context of pathological conditions such as ischemia, Alzheimer disease and epilepsy, and have been proposed to propagate cellular stress signals in glia cells [35]. On the other hand Ca^{2+}-related signaling is linked with NF-κB and calmodulin-dependent kinases II and IV, which support cell survival [36]. Altered Ca^{2+}-signaling pathways can provide means in which cancer cells can bypass anticancer defense mechanisms and contribute to dedifferentiation, induction of angiogenesis, increased telomerase expression and motility [37].

Since the working mechanism of PDT is based on the formation of ROS and singlet oxygen, leading to cell death, and Ca^{2+} signaling is intertwined with ROS and cell death, it is evident that Ca^{2+} signaling has been the subject of interest in many research papers covering the effect of PDT. ROS can induce Ca^{2+} overload in the cell by inducing Ca^{2+} release from the ER, inhibition of Ca^{2+} pumps on the plasma membrane and ER, stimulation of Ca^{2+} induced Ca^{2+} release and opening of the mitochondrial transition pore. Ca^{2+} can, on its turn also influence ROS signaling in two opposite ways: it can induce secondary ROS generation in the mitochondria by stimulating the oxidative phosphorylation and possibly also via opening of the mitochondrial permeability transition pores, but it can also mitigate ROS signaling by activating anti-oxidant enzymes such as catalase and superoxide dismutase [38,39].

The studies that examined $[Ca^{2+}]_i$ all reported an increase following PDT. The only PS for which no increase was observed was TPPS1 in murine colon adenocarcinoma cells; however the absence of $[Ca^{2+}]_i$ changes might be due to measurement immediately after PS illumination and lack of data at later time points [16]. Song et al. reported that $[Ca^{2+}]_i$ changes peak at 2 h post-PDT, indicating that the timing of Ca^{2+} measurement might indeed be crucial for the result [40]. The source of this increased $[Ca^{2+}]_i$ is however still in debate. Some papers report an important role for the release from internal stores [41–45], whereas others claim that Ca^{2+} influx [46–48] has a predominant role or a combination of both pathways is at play [49,50]. It is not completely clear if the localization of the PS is the main determinant for the way the $[Ca^{2+}]_i$ changes are achieved, nevertheless it is obvious that this localization does play an important role in it, especially when organelles contributing to Ca^{2+} storage are targeted. The fact that a multitude of PSs, cell lines and experimental conditions have been used in the papers published on this theme, makes comparisons and conclusive statements difficult.

Localization of the PS in the ER as a primary target site, results in the damage of this organelle. Because of its high Ca^{2+} content, ER damage will release significant amounts of Ca^{2+} into the cytoplasm [40,45,51]. Next to ER Ca^{2+} release, damage to the SERCA2 pump in the ER membrane will diminish Ca^{2+} re-uptake from the cytoplasm into the ER further elevating $[Ca^{2+}]_i$ [41,45,51,52]. SERCA can be damaged by oxidation of the thiol-groups on the pump or by direct ROS attack on ATP binding sites [53]. Some papers report Ca^{2+} entry in a second phase of $[Ca^{2+}]_i$ elevation [40]. Granville et al. indeed reported a rapid damage of SERCA2 and release of Ca^{2+} from the ER (and also mitochondria) following verteporfin-PDT on HeLa cells but the resulting $[Ca^{2+}]_i$ increase did not seem to be involved in cytochrome *c* release from the ER and subsequent apoptosis [45]. Ding et al. on the other hand claimed that SERCA dysfunction and the ensuing $[Ca^{2+}]_i$ increase is responsible for cytochrome *c* release and activation of caspase 3 following HMME-PDT in the same cell type [41].

Targeting of the PS MPPa to the mitochondria of prostate carcinoma cells can lead to opening of the mitochondrial permeability transition pores which results in Ca^{2+} release from the mitochondria into the cytosol and subsequent $[Ca^{2+}]_i$ increase. Of note, this $[Ca^{2+}]_i$ increase occurred at a later time point than the ROS peak. It is hypothesized that ROS oxidize the matrix GSH leading to permeability transition pore opening. An increase in NO concentration was also reported in this paper, however, because the concentration was not as high as the ROS concentration, they concluded that it was less important and did not investigate this into depth [54].

For some PSs, the cellular targets are yet unknown or are multiple in nature and time dependent. Photofrin, a hematoporphyrin derivative localizes in lipophilic membrane structures including the plasma membrane and mitochondria; this PS has been investigated by several groups. In C6 glioma cells, it was found that Photofrin-PDT caused Ca^{2+} entry as a result of glutamate activation of Ca^{2+}-permeable AMPA receptors containing the GluR2Q subunit. The exact mechanism responsible for this elevated glutamate concentration activating the AMPA receptors has not been elucidated yet [47,48]. Another group proposed that the accumulation of Photofrin and the resulting ROS molecules in the membrane of squamous cell carcinoma cells and murine colon adenocarcinoma cells activated the PLC-IP_3 signaling axis resulting in Ca^{2+} release from the internal stores. The authors suggested PLA2 involvement but mechanistic linkage lacks [55]. PLC-IP_3 linked Ca^{2+} release has also been reported by Cui et al. for the PS PLMGdB in isolated rat pancreatic acini [56]. While these data suggest that Photofrin may trigger both Ca^{2+} entry and Ca^{2+} release, it is more likely that the type of response largely depends on the cell type under study. As a general remark, it needs to be realized that pure Ca^{2+} entry or pure Ca^{2+} release are probably rather rare events: Ca^{2+} entry may certainly be associated with Ca^{2+}-induced Ca^{2+} release (CICR), and Ca^{2+} release may activate store-operated Ca^{2+} entry to compensate for the lost Ca^{2+} pumped out by plasma membrane Ca^{2+} ATPases.

The PS 2-BA-2-DMHB mainly triggers Ca^{2+} entry from the extracellular space but a small contribution of store release was observed in zero extracellular Ca^{2+} conditions. Since $[Ca^{2+}]_i$ decreases again but remains at a new steady state with a higher concentration, a mechanism is proposed in which the membrane is the primary target of this PS which results in damage of the plasma membrane and resulting Ca^{2+} influx. Recovery of the membrane then leads to a decrease of the $[Ca^{2+}]_i$ but damage of Ca^{2+} buffering systems and intracellular stores leads to a sustained elevation of the $[Ca^{2+}]_i$. These experiments have been carried out in a human breast cancer cell line and a human gastric adenocarcinoma cell line [49,50].

The role of Ca^{2+} in PDT treatment was also examined in vivo. Therefore tumor masses in a mouse model were treated with the PS phthalocyanine chloride. In these experiments $p53^{-/-}$ and $p53^{+/+}$ cells were used. The $p53^{-/-}$ had an altered Ca^{2+} signaling which resulted in a decreased amount of apoptosis [57].

Overall, we conclude that exposure of cells to PDT results in an increase of $[Ca^{2+}]_i$ triggered by Ca^{2+} entry or Ca^{2+} release from the internal stores depending on the intracellular target of the PS, the cell type and the experimental conditions (see Fig. 1a) (Table 1).

3.1. The role of Ca^{2+} in the execution of cell death

PDT has been shown to result in different modes of cell death [17]. Some groups reported that lower dose, e.g. lower light fluency or lower dose of the PS, results in apoptosis, whereas higher doses execute necrosis [18,58]. Other papers describe an influence of the cell type or targeted organelle by the PS [15,59]. So multiple cell death pathways

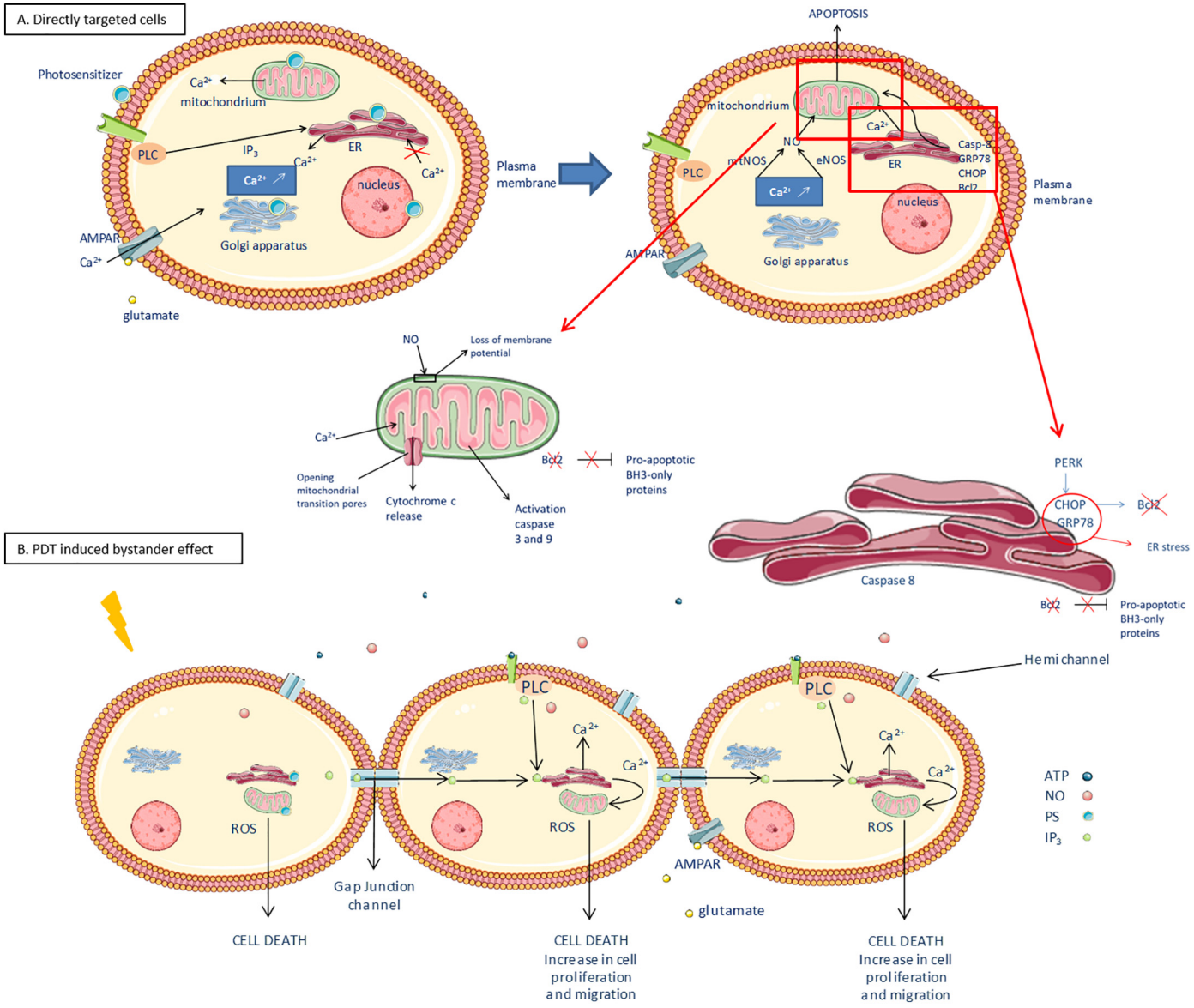

Fig. 1. The role of Ca^{2+} in directly targeted cells and bystander cells.
A. The influence of PDT on $[Ca^{2+}]_i$ and the role of Ca^{2+} in the execution of apoptosis. Depending on the subcellular localization of the PS, PDT results in an inhibition of Ca^{2+} uptake from the cytosol in the ER caused by damage of SERCA2, an IP_3 mediated Ca^{2+} release from the ER, Ca^{2+} release from the mitochondria or an increase in the entry of extracellular Ca^{2+}. One proposed mechanism of Ca^{2+} entry consists of glutamate-activation of Ca^{2+} permeable AMPA receptors.
The $[Ca^{2+}]_i$ increase may then result in Ca^{2+} overload in the mitochondria which results in apoptosis. This induction of apoptosis can be via the loss of membrane potential, release of cytochrome *c*, activation of caspases or loss of inhibition of pro-apoptotic BH3-only proteins. Ca^{2+} can also directly be transferred from the ER to the mitochondria. And Ca^{2+} independent pathways encompassing Caspase-8, GRP78, CHOP and Bcl2 from the ER are also possible.
B. The role of Ca^{2+} and Cx channels in PDT induced bystander effect focusing on the cell-cell propagation of Ca^{2+} changes. ROS and/or NO are mentioned as the responsible molecules for the effects in the bystander cells (for a review on the role of NO in cell death [119]). However, due to their limited life-time and resulting diffusion distance, especially for ROS, other messengers contribute to their regeneration and bystander cells. A mechanism is proposed in which both NO and most importantly Ca^{2+} signaling via IP_3 and ATP are involved in the propagation and the regeneration of ROS and NO production in the bystander cells which then cause bystander effects. The effects depend on the cell type and experimental conditions and can range from cell death to increase in cell proliferation and migration. Next to this active propagation of bystander signals, a passive process whereby toxic substances from the cells are released from the cell because of membrane rupture is hypothesized by some groups (not shown on the figure).

can result from PDT depending on different parameters. Apoptosis, also known as programmed cell death, can be activated by two pathways, the intrinsic and the extrinsic pathway. The latter results from activation of death receptors on the plasma membrane, while the former results from intracellular events directly or indirectly targeting mitochondria. Both pathways converge to the execution phase where caspase 3 is activated. Apoptotic cells are characterized by several morphological features: cell shrinkage, chromatin condensation and fragmentation, plasma membrane blebbing and shedding of apoptotic bodies [60,61]. These apoptotic bodies are recognized, engulfed and degraded by phagocytes or neighboring cells in vitro [62]. The execution of apoptosis necessitates energy in the form of ATP [63]. The second form of cell death reported following PDT is necrosis. Morphologically necrotic cells exhibit different characteristics compared to apoptotic cells, these include: increased cell volume, rounding and swelling of the cell and organelles, irreversible rupture of the cell membrane and loss of the content of the cell in the extracellular space [64]. Since necrosis does not require energy in the form of ATP, this cell

Table 1
Relation between target of the PS, source of Ca^{2+} and role of Ca^{2+} in cell death.

Photosensitizer	Target	Source of Ca^{2+}	Role of Ca^{2+} in cell death
Rose bengal [40,43]		Internal stores	Activation of NOS resulting in loss of MMP and activation of caspases 9 and 3
Photophrin [42,46–48,55,78]	Membranes Mitochondria	Extracellular [46,78] Internal stores [42,55]	PLC activation leading to Ca^{2+} increase and apoptosis [55]
HAL [51]	ER + mitochondria	Internal stores	Increased Ca^{2+} following ER stress cleaves caspase 8
MPPa [54]	Mitochondria		
PPa [66]			BAPTA-AM inhibited activation of caspase 3 and apoptosis [66]
Verteporfin [45]	ER	ER + mitochondria	BAPTA-AM does not inhibit cytochrome *c* release
2-BA-2-DMHB [49,50]		Mainly extracellular	Ca^{2+} activates mtNOS, the resulting increase in NO contributes to cytochrome *c* release
AE	ER + mitochondria		ER stress
Radachlorin [44]		ER	
HMME [41]		ER	Cytochrome c release + activation of caspase 3
TBR [68]	Golgi		Ca^{2+}-dependent but caspase 3-independent apoptosis via Golgi
Porphyrins [11]			
- Meta	ER	Intracellular (Ca^{2+} entry was excluded)	Activation of calpains and stress caspases 12 and 4
- Para	Lysosomes		Mitochondrial apoptosis
Ruthenium red [67]	Mainly ER, also mitochondria		Calcium chelator BAPTA does not inhibit apoptosis
PS I [70]	ER and mitochondria		Apoptosis induction via mitochondrial damage and ER stress
AlPcSn (n = 2, 3 or 4) [120]			Ca^{2+} and cAMP mediated signaling involved in induction of necrosis
DH-II-24 [58]	Mainly ER, also mitochondria and lysosomes		Low dose: transient increase in Ca^{2+} leading to apoptosis, high dose: prolonged increase in Ca^{2+} leading to necrosis
Hypericin [52]	ER	ER	Triggers autophagy in apoptosis-deficient $Bax^{-/-}Bak^{-/-}$DKO MEF cells

death mode will occur when the mitochondrial insult is extreme and the energy production is inhibited [63]. In the absence of phagocytes in vitro membrane integrity can also be lost in apoptotic cells, which is known as secondary necrosis [65].

Given that $[Ca^{2+}]_i$ was increased in most cases, regardless of the PS target, and Ca^{2+} does play a role in the induction of cell death in general, it is obvious that a number of papers investigated the connection between Ca^{2+} and the execution of cell death following PDT. Tajiri et al. were one of the first to propose a relationship between these two [55].

Most of the work covering Ca^{2+} and cell death focus on caspase 3 dependent apoptosis, and NO has been described to play a role in this process. Chan et al. reported $[Ca^{2+}]_i$ increases in HUVEC cells following Rose Bengal PDT; these increases are caused by singlet oxygen which then activate eNOS and consequently increase NO production. NO is an upstream regulator leading to loss of mitochondrial membrane potential and activation of caspase-9 and -3, which execute apoptosis [43]. The contribution of NO in this process has been reported by other groups as well. Lu et al. discovered that the increased $[Ca^{2+}]_i$ observed following 2-BA-2-DMHB treatment in a breast cancer cell line, activates mitochondrial NOS (mtNOS) which then causes an increase in NO and contributes to the induction of apoptosis together with ROS [50].

Ding et al. showed that $[Ca^{2+}]_i$ elevation takes place before cytochrome C release and caspase 3 activation, and speculated that this increase plays a role in the apoptotic process [41], an observation also made by Inanami et al. The latter reported that $[Ca^{2+}]_i$ and cAMP are independent regulators of apoptosis upstream of caspase 3. Ca^{2+} facilitated the release of cytochrome *c* from the mitochondria whereas cAMP inhibits the apoptotic signals that occurred after cytochrome *c* release [66]. However, some groups reported the occurrence of $[Ca^{2+}]_i$ elevation but without any linkage to the induction of apoptosis. Here, it is assumed that Bcl2 on the ER and mitochondria, is sequestered following PDT treatment, which then results in a lost brake on proapoptotic BH3-only proteins [67]. Ogata et al. on the other hand reported a Ca^{2+} dependent apoptosis pathway that is independent of caspase 3. This group investigated PDT with the PS TBR in HeLa cells. TBR exclusively targets the Golgi apparatus [68]. The outcome of the experiments can thus also depend on the method used to investigate cell death. Most groups investigated the induction of apoptosis by the visualization of activated caspase 3; however different biochemical pathways leading to apoptosis may be activated. Release of cytochrome C is another widespread method to investigate apoptosis. Granville et al. reported no influence of the Ca^{2+} chelator BAPTA-AM on cytochrome C release following verteporfin-PDT in HeLa cells but this does not exclude the fact that Ca^{2+} can play an important role in apoptosis induction via other pathways [45]. Zn-BC-AM is a PS that localizes at both the ER and mitochondria; it triggers the mitochondria-mediated apoptotic pathway directly and indirectly via the ER. The Ca^{2+} release from the ER was responsible for an increase in cell sensitivity to the mitochondrial apoptotic pathway [69]. Li et al. investigated PSI, a PS that targets both organelles in HeLa cells and came to a similar conclusion. They concluded that the Ca^{2+} overload resulted in ER stress which they measured both by CCAAT-enhancer-binding protein homologous protein (CHOP), an ER-stress inducible transcription factor and GRP78, an ER chaperone linked to ER stress [70]. CHOP would then downregulate Bcl2 [71]. Verfaillie et al. discovered a role for PERK in the induction of apoptosis following hypericin-PDT. PERK is responsible for sustaining the pro-apoptotic CHOP, a protein already discussed above, but on top of that it also contributed to the propagation of ROS signals between the ER and the mitochondria [72]. Activation of caspases 9 and 3 was also reported [70]. The PS HAL localizes at the ER; illumination of this PS results in a Ca^{2+}-dependent activation of caspase 8 which in turn activates caspase 3 [51]. MPPa targets the mitochondria and results in the opening of mitochondrial transition pores results next to release of Ca^{2+} also in release of cytochrome *c* which leads to apoptosis via activation of caspases. Unfortunately, this group did not investigate the connection between Ca^{2+} and the release of cytochrome C and activation of caspases [54] in this particular context (for a general review of Ca^{2+}, mitochondrial permeability transition and cytochrome C release see [73]). Kuchay et al. used xenografts and PC3 cells to show that a decrease in IP3R3 and an increase in the F-box protein 2 (FBXL2), the receptor subunit of one of the human SCF ubiquitin ligase complexes, resulted in a limitation of the mitochondrial Ca^{2+} overload, which eventually inhibited cell death induction in PTEN deficient mice. By increasing the IP3R3 or using GGTi-241, an inhibitor of FBXL2, the induction of apoptosis was restored suggesting this strategy shows some therapeutic potential t [74].

Some studies show that even with a deficient apoptotic pathway,

the overall effect of PDT treatment is still the same when overall cell survival is measured. This is beneficial for the treatment of tumors which are resistant to other therapies because of escape of apoptosis [15,75,76]. One of the other cell death modalities that has been described in Bax/Bak-deficient HeLa cells that are unable to undergo apoptosis, is autophagy. Buytaert et al. investigated hypericin-PDT in these cells and reported a perturbation of the Ca^{2+} homeostasis resulting from the inability to refill the ER because of a damaged SERCA, which was followed by autophagy. Excessive autophagy could then lead to cell death [52]. Yoo et al. described a role of Ca^{2+} in differentiating between apoptosis following low dose PDT and necrosis following high dose PDT: the former was characterized by transient $[Ca^{2+}]_i$ increase while the latter displayed a massive and prolonged $[Ca^{2+}]_i$ elevation [58].

From this discussion, we conclude that the increase in $[Ca^{2+}]_i$ following PDT can lead to apoptosis via different pathways and that the outcome of the studies depends on the subcellular localization of the PS, the cells used and the method to quantify apoptosis or other cell death modalities. This conclusion is supported by the general observation that the role of Ca^{2+} in cell death and apoptosis in particular, involves effects at several key points and not just at a single step of the cascade [77] (see Fig. 1a)

3.2. The role of connexins in PDT

Connexins (Cxs) are tetraspan proteins consisting of two extracellular loops (EL-1 and EL-2), a cytoplasmic loop (CL) and a cytoplasmic N-terminus (AT) and the C-terminus (CT). In humans 21 different Cx isotypes are known and they are named according to their molecular weight. Cxs show a tissue/cellular specificity with Cx43 being the most abundant and wide-spread isoform in mammals [79]. Cxs are transmembranous proteins contributing to cell signaling via (i) the formation of hemichannels, i.e. the assembly of 6 Cx proteins into half a gap junction channel that connect the cytoplasm of the cell with the extracellular medium and as such contribute to paracrine signaling, and (ii) the formation of gap junctions consisting of two hemichannels and connecting the cytoplasm of two adjacent cells thereby allowing direct cell-cell communication. Both types of connexins channels allow the passage of molecules and ions up to 1–1.5 kDa, including ATP, IP_3 and Ca^{2+}; the latter three substances are crucial in communicating Ca^{2+} signals between cells as intercellular Ca^{2+} waves [5,37]. One hemichannel can be composed of Cx proteins of the same (homomeric) or different (heteromeric) isotypes, on top of that gap junctions can comprise two different (heterotypic) or two equal hemichannels (homotypic). Although not all combinations are possible, this does result in an enormous variety of channels and resulting permeabilities for different components [79]. Under physiological circumstances, gap junctions allow the transfer of intercellular signals and metabolites; in contrast, hemichannels are normally closed but may open in response to cell stress conditions including ischemia, pro-inflammatory conditions [80], oxidative stress [81], increased $[Ca^{2+}]_i$ [82] or decreased extracellular Ca^{2+} concentration [83]. Cxs have a short half-life in the order of 4 to 6 h and are therefore capable of rapidly responding to alterations in the environment [79,84]. Modulation of connexin channel function can occur via different ways: Ca^{2+} is one of the known ions that, via its interaction with calmodulin, inhibit gap junction channels [85]. The effect of $[Ca^{2+}]_i$ on hemichannels is more complex: it acts to open hemichannels at moderately elevated concentrations (up to ~500 nM) and closes hemichannels at higher concentrations (full closure at 1 μM) (reviewed in Leybaert et al. [86]). High $[Ca^{2+}]_i$ closure results from actomyosine activation that disrupts loop/tail interaction of the connexin protein, bringing the channel in a state that is not available anymore for opening [87,88]. Interestingly extracellular Ca^{2+} has received a lot of interest in the cancer field, either linking to signaling via the Ca^{2+}-sensing receptor [89–92] or via other less well defined mechanisms [93–95]. Opening of hemichannels in response to low extracellular Ca^{2+} can influence the signaling by release of ATP into the extracellular medium, it thus has to be taken into account when interpreting those results [83]. Posttranslational modifications also strongly influence the function of Cx channels and the best characterized modification is phosphorylation. A manifold of biomolecules like growth factors and neurotransmitters also influence these channels [84]. Cx expression is strongly altered in cancerous tissues: because they have tumor-suppressing effects, they are commonly downregulated in the primary tumor. However, Cxs also play a role in making cell contacts during metastasis, in which case they become upregulated [96]. Their contribution to damage and spreading of damage following PDT can thus differ depending on the tumor cell and the stage.

Next to Cx proteins, another family of related transmembrane proteins exists, called pannexins (Panxs). These proteins have a similar topology as Cxs but are less likely to form functional gap junctions [97]. As a result they are present as “hemichannels” that have been proposed to be better referred to as “channels” [98].

The role of Cxs in cell responses to PDT has only been investigated by a few groups and available data are thus limited. Wu et al. reported a larger effect of PDT with Photofrin in HeLa cells when they expressed Cx32 both in vitro and in vivo; the increased effect was related to enhanced ROS and Ca^{2+} signaling [99]. Similar results were found with Cx26 expressing HeLa cells [78]. A larger uptake of Photofrin itself was excluded as the reason of this increased effect since quantification of the uptake of Photofrin was comparable for cells with or without Cx-channels present. The authors speculated on increased Ca^{2+} entry and Ca^{2+} release of Ca^{2+} stores but data supporting this was absent [99]. Making use of Cx32 expressing HeLa cells exposed to HPPH-PDT, Liu et al. reported that gap junctions can enhance the effect in the treated zone by the spreading of ROS, membrane depolarization and caspase 3 apoptosis between the treated cells [100]. No reports are currently available investigating the role of hemichannels in PDT.

Taken overall, the limited studies available all reported a role for connexins in enhancing the effect of PDT.

4. Bystander effects

Radiation induced bystander effects, i.e. cell death or other effects occurring in cells not directly irradiated are a well-known and thoroughly investigated subject. Bystander effects are not limited to radiotherapy but have been reported in other circumstances such as cell death following localized application of cytochrome *c* and spreading of the effects in HIV infected cells [101–103]. The bystander effect may provoke enhanced cell killing in the tumor itself, but may equally result in toxic effects in healthy surrounding tissues following cancer treatment. The spreading of these signals occurs via direct cell-cell communication and/or paracrine signaling. Knowledge of this phenomenon and its underlying mechanisms can thus help to take advantage of it to amplify the effect of the cancer treatment on the tumor and minimize unwanted side-effects on the surrounding healthy tissue by inhibiting this effect [104].

Focal photodynamic injury has been used as a model to investigate bystander signaling [105–107]; because it is light-based, optical or microscopy-based approaches intrinsically allow precise focusing of the light to targeted cells. Other methods to investigate bystander signaling in this context are: the use of conical flexi-Perm-ConA silicone rings that separate exposed from non-exposed cells [108], co-cultures with non-exposed cells [109] and transwell cell culture approaches [110]. Computational modeling studies have also been applied to predict the number of dead cells based on Monte Carlo simulations [111–113]. The mechanisms reported to contribute to the PDT-induced bystander effect are summarized in Fig. 1B.

Dahle et al. observed more pronounced bystander effects upon using PSs that target the plasma membrane; it was observed that the ensuing process of necrotic cell death was involved in releasing toxic substances

in the medium, thereby causing bystander effects [111]. A similar process was also demonstrated by Thomas et al. Retinoblastoma cell lines with different cell density were implanted in nude mice. Following 5,10,15-Tri(para-O-(2-(2-O-α-D-manosyloxy)-ethoxy)-ethoxy-phenyl)-20-phenyl porphyrin-PDT it was shown that necrotic cells release toxic substances in the vicinity of bystander cells thereby causing apoptosis in these cells. The exact nature of these toxic substances has not been investigated in depth [114]. In contrast to this passive diffusion scenario, others have suggested the contribution of more 'active' signaling scenario's whereby DP-PDT in EMT-6 cells and WTK1 cells only resulted in a bystander effect when sublethal doses of PDT were applied [107,110]. The differences in these observations can result from different experimental set-ups, the latter two made use of in vitro investigations with a transwell system or focal irradiation whereas Dahle et al. made use of Monte Carlo simulations to estimate the amount of cell death caused by bystander signaling, Thomas et al. on the other hand performed their experiments in vivo. The latter made use of retinoblastoma cell lines with different cell density which were implanted in nude mice [114].

As already alluded to, ROS, NO and Ca^{2+} are likely messengers of bystander effects following PDT. ROS are, however, short-lived molecules that are unlikely to travel from cell-to-cell in order to inflict damage at distance. It is more likely that other messengers may help in cell-cell propagation thereby perhaps facilitating de novo generation of ROS and RNS in bystander cells. One of the candidate messengers in this process is Ca^{2+}, more specifically IP_3-driven Ca^{2+} waves propagating between cells. Feine et al. reported $[Ca^{2+}]_i$ transients in bystander cells 5–10 min post-PDT [106]. Cali et al. distinguished two NO peaks in the bystander cells: a fast direct one and a second Ca^{2+} wave-linked slower one [105]. In fact, NO itself has been implicated as a signal that is also important for Ca^{2+} wave propagation in its own right [115].The results of Cali et al. were confirmed in tumors grown within a dorsal skinfold chamber implanted in BALB/c mice, thus confirming the importance of both NO and Ca^{2+} in vivo in this context [105]. A role for NO produced by targeted cells in spreading of the bystander effect has been reported by Bazak et al. as well, although in this context an increase in cell proliferation and migration of the bystander cells was reported instead of an increase in cell death. The contribution of a Ca^{2+}-driven NO release has not been investigated in this paper [108]. The difference could be because of a difference in NO concentration. Low concentrations of NO have been shown to stimulate survival, proliferation and migration of cancer cells whereas high levels of NO cause a cytotoxic response [116].

Feine et al. also implicated the importance of ROS in bystander cell death but assumed that another intermediate messenger is transmitted via GJs given its short lifetime. Alternatively, it remains possible that ROS with longer lifetimes may diffuse over distances in the order of cell diameters [106]. Activation of NADPH-oxidase in the targeted cells has been shown to play a role in bystander responses following PDT. They proposed that H_2O_2, a longer lived species with a half-life of 1 ms compared to 10^{-9} s for OH [117], is the actual intercellular messenger. Bystander cells in turn also produce ROS following PDT but during a shorter timeframe and at lower concentrations. They point out that H_2O_2 can diffuse freely in the extracellular medium of the cell cultures used in their experiments, which might not be the case in vivo [107]. Chakraborty et al. proposed a role for lipid peroxidation in mediating the bystander effect. Deuteroporphyrin localizes at the plasma membrane, resulting in a local production of ROS and/or singlet oxygen. These molecules can cause lipid peroxidation producing lipid hydroperoxides that would have sufficient lifetime to diffuse to bystander cells to initiate oxidation processes [110].

In summary, ROS and/or NO are frequently proposed as responsible for the PDT-induced bystander effect. However, due to their limited diffusion distance, a propagation mechanism based on Ca^{2+}-signaling via IP_3 and ATP may likely be involved in inducing NO and/or ROS regeneration in bystander cells.

Cxs have been reported to play a prominent role in the spreading of damage to neighboring cells following different types of insult including ionizing radiation (reviewed in Decrock et al. [118]), localized loading of cells with cytochrome *c* [101] or the spreading of apoptosis in HIV infected cells [102]. The role of Cxs in the spreading of bystander effects provoked by PDT has been examined by some groups, with evidence for involvement of Cx43 [105,106] and Cx26 and Cx32 [78,99,100]. The results depend on the cell-type and PS used and on the method used to evaluate involvement of Cx channels.

Feine et al. showed that bystander signaling following WST-11-PDT was cell contact dependent, based on experiments with a scratch in the cell culture at which the bystander effect stopped. They furthermore showed that bystander spread was inhibited by the gap junction blockers Carbenoxolone (Cbx) and glycyrrhizic acid (GZA), and that spread was also diminished in transformed bEnd.3 cells with dominant-negative Cx43 expression [106]. Cali et al. investigated the role of Cx channels in C26GM cells in the bystander effect following AlClPc-PDT. In a first step the coupling of the cells via gap junctions was confirmed via patch clamp and voltage clamp experiments. In a next step these channels were inhibited by Cbx and Flufenamic Acid (FFA), this significantly reduced the Ca^{2+} wave and changes in $[Ca^{2+}]_i$ and NO in the bystander cells, thus showing that Cx channels are important players in the spreading of this bystander effect and cell death induction in these cells [105].

To the best of our knowledge the role of Cxs in the bystander effect following PDT has only been studied in vitro, although this gives us an indication about the role of these proteins in bystander signaling, in vivo studies are still necessary. Expression of Cxs can be altered by different factors including shear stress which is present in endothelial cells in vivo but not in vitro [106]. The contribution of HCs to this process has not been investigated into depth within the studies mentioned above. The blockers used in the studies are pharmacological blockers with known aspecific side-effects. In some studies they are combined with genetic models in which Cx expression is forced or knocked down in the specific cell line. The expression of Cxs is often altered in cancer cells, depending on the stage of the tumor and the cell type, Cxs can be down or upregulated [96]. It is thus important to investigate the role of Cx-channels in the cell type of interest, malignant or normal, in order to obtain biological relevant results.

Taken overall, the limited number of studies available suggests a role for Connexin channels in the spreading of the PDT-induced bystander effect.

5. Concluding remarks

PDT is not one general therapy but depending on the PS used, different cellular responses occur and give rise to the activation of various cell death pathways. This makes it difficult to pin down the role of Ca^{2+} and Cxs to a single effect. On top of that not all PS molecules have been characterized in depth yet, for some of them not all cellular targets are known or targets may vary with incubation time. Interpretation of the results in terms of the cellular location of PSs is thus not possible for all PSs currently available. However we can conclude that PDT results in an increase in $[Ca^{2+}]_i$ and that depending on the cell type, the PS used and the end-point used to quantify cell death this increase in $[Ca^{2+}]_i$ does play a role in the execution of cell death following PDT.

Upon comparing the proposed signaling cascades mediating bystander effect propagation following PDT with those involved in radiation induced bystander effects (reviewed in [118]), several similarities can be noticed. Comparing results from both types of research can thus be beneficial in discovering new possibilities and new research hypotheses. In both cases, ROS invariably emerges as a central player; as already referred to, ROS may prove to be decisive for bystander effects but there is probably an extensive underlying signaling machinery communicating an intercellular message resulting in local activation of ROS production. It looks obvious that the ER-mitochondrial axis plays a

crucial role in local ROS activation, while the intercellular message may well involve intercellular Ca^{2+} wave propagation based on connexins as well as paracrine signaling. It needs to be added that for the moment, there are little in vivo data available on PDT-linked Ca^{2+} involvement and bystander signaling. Further research in this domain would help to improve the efficiency of PDT and its use in the clinic, and may help in reducing the side-effects on the surrounding healthy tissue.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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