



REVIEW ARTICLE

## Apoptosis and Cell Death Channels in Prostate Cancer

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Apoptosis, a type of programmed cell death, is a decisive mechanism in cell processes such as homeostasis, development, and many diseases including cancer. In mammals, the mechanisms that trigger and control the process of apoptosis are complex, because it has been observed that many molecules might be involved, acting in distinct ways and depending on the cellular type. The process of apoptosis is characterized by specific biochemical and morphologic changes. However, important specific messengers such as  $\text{Ca}^{2+}$  act in active proliferation as well as in apoptosis. At present, there is convincing evidence that a sustained increase in intracellular  $\text{Ca}^{2+}$  can activate cytotoxic mechanisms in various cells and tissues. Several ionic channels located in the cytoplasmic membrane might participate in the entry of calcium into the cytosol during apoptosis. Among these ionic channels, the purinoreceptors P2X and the channels of capacitative entry of calcium have been described. Pro- and anti-apoptotic molecules such as bax and bcl-2, respectively, have also been shown to participate in the process. We have recently found the activation of a  $\text{Ca}^{2+}$ -permeable, nonselective cation channel of 23 pS conductance in prostatic cancer (LNCaP) exclusively in cells previously induced to apoptosis. Our findings are discussed taking into account the different ion channels that might participate in programmed cell death in prostate cancer. © 2001 IMSS. Published by Elsevier Science Inc.

**Key Words:** Prostate cancer, Apoptosis, Calcium channels.

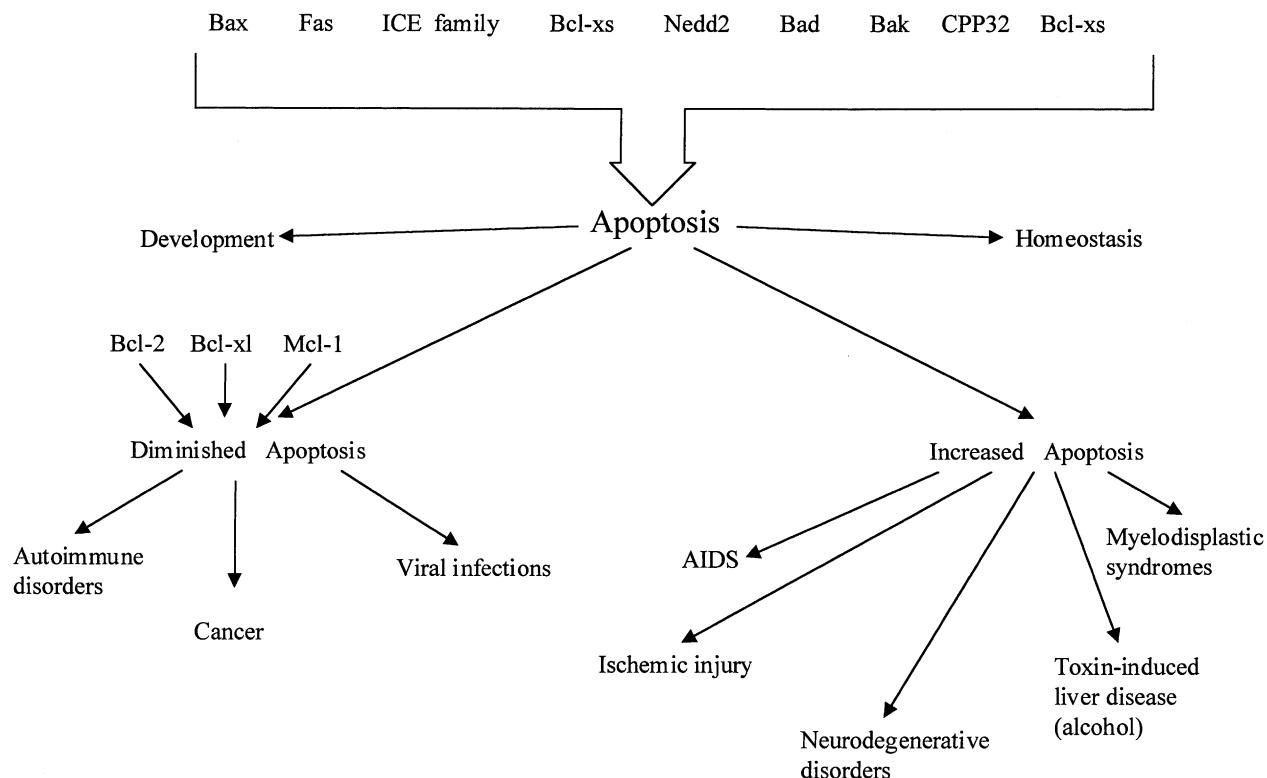
### Introduction

Apoptosis is a form of programmed cell death that is distinct from accidental cell death or necrosis. It occurs through the activation of a cell-intrinsic suicide program (1–3) and is carried out by means of internal as well as external signals similar to those leading to cell differentiation and proliferation (4,5) (Figure 1). A variety of different second messenger systems associated with the induction of apoptosis depends on cell type as well as several induction signals (4), some conserved among worms, insects, and vertebrates (1). The process of apoptotic cell death can be divided into various phases from the signals that initiate the process to the final events of cellular fragmentation (3,5,6) (Figure 2). In many cell types, DNA is degraded into fragments the size of oligonucleosomes, whereas in others larger DNA frag-

ments are produced (2). Apoptosis is also characterized by a loss of mitochondrial function (2). This important organelle controlling cell life and death presents at least three general mechanisms of activity associated with apoptosis: 1) alteration of oxidative phosphorylation, electron transport, and adenosine triphosphate (ATP) production; 2) liberation of molecules that initiate activation of the caspase family of proteases, and 3) in general, disruption of cellular reduction-oxidation (redox) potential (7). Activation of caspases such as caspase 9 (initiator caspase) appears to be regulated by the exit of cytochrome c from the mitochondria, in which Apaf-1 participates as an intermediate. Although the manner in which cytochrome c is released from the mitochondria is still under discussion, members of the bcl-2 family, such as bax, have been proposed to participate in the release of cytochrome c from the mitochondria (8–10).

In *Caenorhabditis elegans*, the cysteine protease Ced-3 is important in the activation of programmed cell death (4,11,12), which can be blocked by the bcl-2 homolog ced-9. According to their function, at least 12 different homologs of Ced-3 have been reported and classified into two

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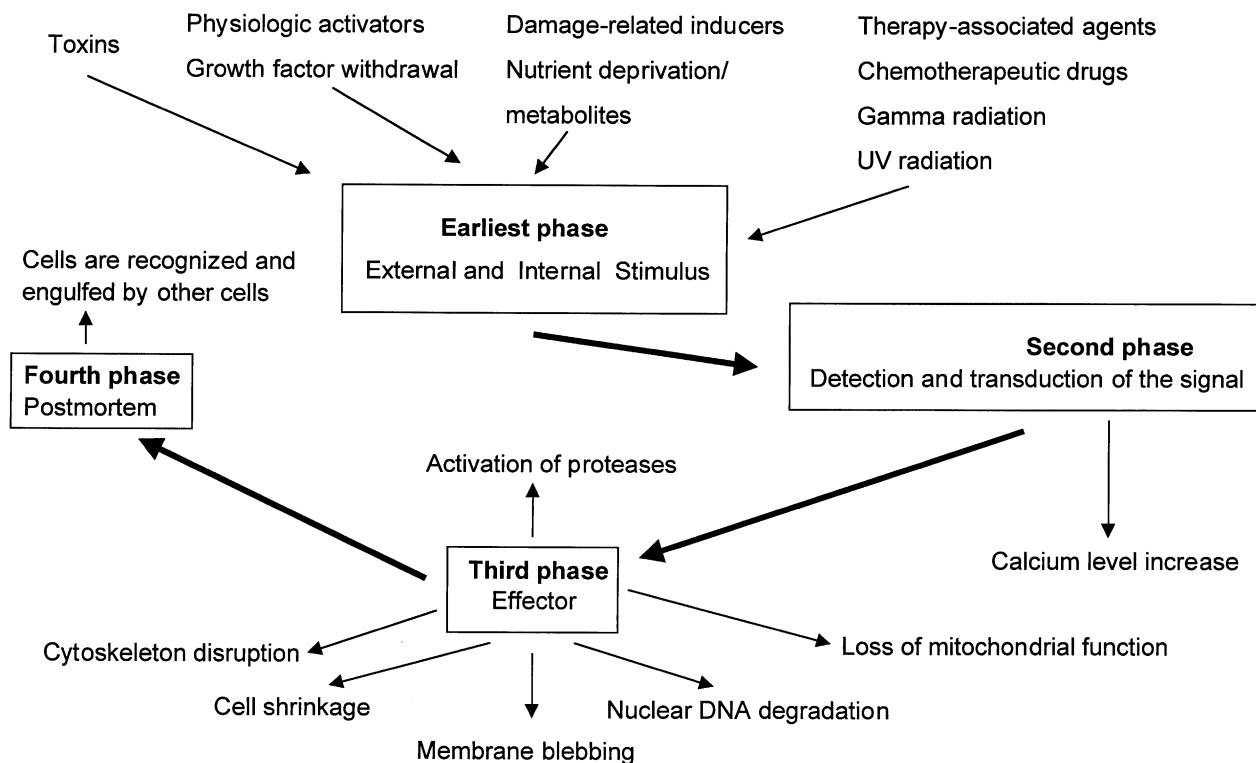
**Figure 1.** Molecules that regulate the process of apoptosis and their relationship to disease.

main groups (13): caspases related to the interleukin-1 $\beta$  converting enzyme (ICE) (caspase-1, -4 and -5) (14) and caspases whose functions are directly related to the apoptotic machinery (caspases 2, 3, 6, 7, 8, 9, and 10) (13–17). Some of these cysteine proteases or caspases are originally synthesized as inactive proenzymes (4,11,18,19).

Cell death can be inhibited by interfering with the protease function of these proteins and precursors, which are converted into the active enzyme through specific proteolytic processing or by autocatalysis triggered by union of cofactors and elimination of inhibitors (20). These proenzymes can be found in nucleated mammalian cells; to cause apoptosis, they must be cleaved at aspartate residues and assembled into heterotetramers. The active cysteine is located in the middle of a conserved QACRG motif common in most proteases (21). Although the final activation of these proteases is posttranslational, some pathways leading to their activation may involve steps that are transcriptionally controlled. In the apoptotic process, cells appear to initiate their own apoptotic death through activation of these endogenous proteases (20). Thornberry and Lazebnik in 1998 reported that caspases can be activated by two distinct mechanisms (20). Because all caspases have similar cleavage specificities, the simplest way to activate a procaspase has been to expose the molecules to a previously activated caspase. This caspase cascade is extensively used by cells in

activation of downstream effector caspases (caspase-3, -6, and -7). The second strategy is known as induced proximity, in which some cofactors reunite zymogen caspases and by intermolecular interaction in which the caspases are activated (20,22). Caspases have been defined as initiator and effector caspases. The activation of effector caspases is carried out using two pathways: via cytochrome c that activates initiator caspase 9, and via death receptors that activate initiator caspase 8; caspases 3, 6, and 7 are known as effector caspases (9,10,20).

The function of Ca<sup>2+</sup> as an intracellular regulator in cell physiology is well established based on many reports concluding that an increase in intracellular Ca<sup>2+</sup> activates several deleterious mechanisms proven to be toxic in some cell types (23,24) (Figure 3). For many years, Ca<sup>2+</sup> has been considered an activator of mechanisms involved in the catabolism of proteins, phospholipids, and nucleic acids. A sustained intracellular Ca<sup>2+</sup> concentration above the physiologic level has been related to an uncontrolled breakdown of macromolecules, critical in the maintenance of cell structure and function. A number of cytoskeletal proteins including spectrin, fodrin, caldesmon, aducin, tubulin, MAP-2, tau factor, vimentin, and cytokeratin has been shown to be susceptible to proteolytic attack (24). Two cytoskeletal proteins, vinculin, and the actin-binding protein, reported to be directly involved in the stabilization of microfilaments asso-



**Figure 2.** Cellular events occurring during the different phases of apoptosis.

ciated with the plasma membrane, have been shown as preferred substrates for  $\text{Ca}^{2+}$ -dependent proteases (24). The breakdown of the intermediate filament-rich nuclear envelope secondary to a transient increment in the intracellular-free calcium concentration can be prevented when the  $\text{Ca}^{2+}$  increase is buffered with  $\text{Ca}^{2+}$  chelators. Activation of the multifunctional  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase has shown to be essential during breakdown of the nuclear envelope (24). The  $\text{Ca}^{2+}$ -dependent regulatory cofactor calmodulin may link these  $\text{Ca}^{2+}$  alterations to the effector machinery, as calmodulin antagonists can interfere with apoptosis in some of these systems (24).

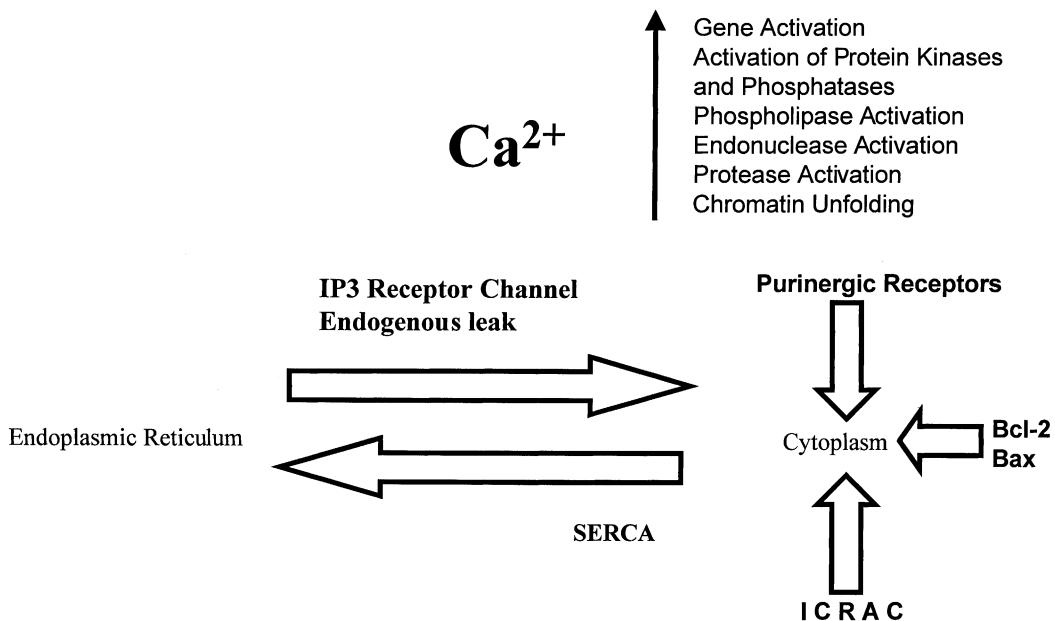
There are various mammalian genes similar in structure to bcl-2 that act in a similar manner to protect cells, as well as others that antagonize the protection offered by bcl-2 (4,11,18) (Figure 1). In different tissues tested, cell survival depends on the presence of survival signals given by the different cells forming the tissue as well as the extracellular matrix. Because death occasionally occurs in the absence of protein synthesis, proteins that mediate apoptosis are thought to be constitutively expressed in several cell types (5,25). It can be considered that most cells are programmed to kill themselves if survival signals are not received from the environment at regular intervals (5,25). Moreover, viruses have developed strategies to inhibit the process of apoptosis and to receive optimal replication through the ex-

pression of viral genes following the induction of cellular suppressors (4).

### Apoptosis and Prostate Cancer

Repair of damaged DNA is the only natural way to ensure survival in unicellular organisms. In metazoans, however, the optimal way to control occurring cell DNA damage is much more complex because a number of mutations, such as those expressed in genes controlling cell proliferation and apoptosis, often lead to neoplasms. Repair, growth arrest, and cell suicide (apoptosis) at present can be considered a response to DNA damage, although their fate in many cases depends on cell type, location, environment, and extent of damage. Therefore, alterations either in the machinery that senses DNA damage or in the mechanisms that implement a response to DNA damage are considered important in predisposition to cancer (26). There are two main checkpoints in the control of cell cycle progression: G1/S prior to the replication process of DNA, and G2/M preceding the process of mitosis (10). Along these transitions, damage to DNA is sensed and molecules such as p53 activated as a response, followed by cell cycle arrest and activation of apoptosis (27).

Activation of p53 is carried out through a phosphoryla-



**Figure 3.** Membrane molecules that participate in the influx of  $\text{Ca}^{2+}$  and their roles in the modulation of apoptosis.

tion process that regulates its DNA-binding affinity. Phosphorylation appears to regulate conformational changes occurring in the carboxy-terminal regulatory domain. Interaction with other molecules appears to be carried out with domains located proximate to the amino end. It has been observed that a change in serine 20 to alanine abrogates p53 stabilization as a response to ionizing radiation and ultraviolet light (UV) (27). p53 has been considered a tumor suppressor because the lack of its function has been found in a high percentage in human primary tumors. Moreover, p53 undergoes other types of mutations as a consequence of binding to several cellular and viral oncoproteins (28,29).

Different cell types from human tumors have been described as having a decreased ability to undergo apoptosis in response to a physiologic stimulus (5). Specifically, overexpression of Bcl-2 prevents cells from initiating the process of apoptosis in response to a number of different stimuli, and its expression is commonly associated with a poor prognosis in prostate and colon cancer (5,30). The process of apoptosis has been implicated in the regression of prostate and mammary tumors induced by removal of androgens and estrogens. Therefore, activation of this process could provide a form of protection against carcinogenesis (6). In hormone-dependent tumors, induction of apoptosis by hormonal ablation has been associated with tumor regression (6). Because most of our current chemotherapeutic agents kill cells by mechanisms other than apoptosis, enhancement of apoptosis in malignancy could be therapeutically valuable (31).

With castration, the presence of a  $\text{Ca}^{2+}$ -dependent endonuclease in rat prostate nucleus extracts appears to be coordinated with increase in fragmentation of nuclear DNA

(32). Androgen ablation is considered a rarely curative therapy in prostate cancer because metastatic cancer within individual patients is heterogeneous, including both androgen-dependent and -independent prostatic cancer cells. Therefore, androgen ablation does not eliminate preexisting androgen-independent cancer cells. Moreover, although an enormous effort is being made, there are no chemotherapeutic agents discovered to date that could be effective in controlling proliferation of androgen-independent prostatic cancer cells. The relationship between rate of proliferation and death is the main factor that determines the proliferation of any cancer cell. Only when the rate of cell death is greater than cell proliferation are cancer cells eliminated. Based on this argument, an important therapy for androgen-independent cancer cells could be obtained either by lowering the rate of proliferation and/or by raising the rate of cell death to a point where the latter exceeds the rate of cell proliferation.

Several antiproliferative chemotherapeutic agents that are cytostatic and/or cytotoxic to sensitive target cells have been reported (33). However, these agents frequently lead to cancer cell death only when cell proliferation is in progress. Cancer cells that are not proliferating at the time of treatment are resistant to these cytotoxic agents. This is based on the fact that cells have sufficient time to repair cellular damage that might have occurred prior to the next cell proliferation cycle. Nevertheless, it is known that the majority (>90%) of prostatic cancer cells in an individual patient is not actively proliferating and is thus resistant to standard cytotoxic chemotherapy. Therefore, some type of cytotoxic therapy that induces death of androgen-independent pros-

tatic cancer cells without the requirement of having cells in active proliferation is urgently needed (33). However, it is known that increased expression of bcl-2 and inactivation of p53 is important in progression of the disease (34).

Androgen ablation produces an energy-dependent process of programmed death in nonproliferating, androgen-dependent prostatic cancer cells; this process involves fragmentation of DNA into nucleosomal multimers catalyzed by nuclear  $\text{Ca}^{2+}$ . In contrast, androgen-independent prostatic cancer cells are not induced to undergo such a programmed cell death by androgen ablation. One possibility for explaining the inability of androgen ablation to induce programmed death of androgen-independent prostatic cancer cells is that the procedure does not permit a steady increase in their intracellular  $\text{Ca}^{2+}$  concentration (33). This phenomenon might support the idea that androgen-independent prostatic cancer cells can be induced to undergo programmed death if an increase in cytoplasmic calcium is sustained by other means without the use of hormones. Therefore, androgen-independent, highly metastatic Dunning R-3327 AT-3 rat prostatic cancer cells have been chronically exposed *in vitro* to the calcium ionophore ionomycin to sustain elevation in their intracellular  $\text{Ca}^{2+}$  concentration. Ionomycin is a polyether ionophore that chelates calcium (and cadmium) in the form of dibasic acids (35). Its site of action is initially found at the level of internal  $\text{Ca}^{2+}$  stores (36), inducing an elevation of intracellular  $\text{Ca}^{2+}$  three- to sixfold above baseline. If induction is sustained for more than 12 h, cell death occurs. These observations identify intracellular  $\text{Ca}^{2+}$  as a potential target for therapy against androgen-independent prostatic cancer cells (33).

It has been known for some time that calcium accumulates within the endoplasmic reticulum of cells through the function of sarcoplasmic reticulum and endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase, the catalytic function of the calcium pump (37). The resulting equilibrium regulates important functions (Figure 3). Treatment of androgen-independent prostatic cancer cells of both rat and human origin with thapsigargin (TG), a sesquiterpene  $\gamma$ -lactone that selectively inhibits sarcoplasmic reticulum and endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase, results in an important increase of intracellular  $\text{Ca}^{2+}$  within minutes of exposure (37). A secondary influx of extracellular  $\text{Ca}^{2+}$  results in morphologic (cell-rounding) and biochemical changes within 6 to 12 h. This influx of  $\text{Ca}^{2+}$  also increases tissue transglutaminase expression and calmodulin concentration associated with decreased expression of the G1 cyclins (37). Androgen-independent prostatic cancer cells stop progression through the cell cycle when treated with TG. It appears that  $G_0$ -arrested cells undergo double-strand DNA fragmentation in parallel to a loss of plasma membrane integrity and cell fragmentation. These authors demonstrate that TG induces programmed cell death in androgen-independent prostatic cancer cells and that this process is critically dependent upon a

sustained elevation of intracellular  $\text{Ca}^{2+}$  (37). Taken in conjunction, these studies have identified the ER  $\text{Ca}^{2+}$ -ATPase as a new therapeutic target for activating programmed cell death of nonproliferating, androgen-independent prostatic cancer cells (37). Considering that a sustained increase in the concentration of intracellular calcium might be considered as a consequence of the influx of calcium through the plasma membrane (33,37), it was important to evaluate the participation of  $\text{Ca}^{2+}$  fluxes through the plasma membrane in these cancer cells. In this sense, our research group has demonstrated the activation of a  $\text{Ca}^{2+}$ -permeable, nonselective cation channel of 23 pS conductance in prostatic cancer cells (LNCaP) (androgen-independent) exclusively during apoptosis onset, using two different inducers of apoptosis, i.e., ionomycin and serum removal (38). In perforated patch recordings of single LNCaP cells, a membrane potential of  $-40$  mV and an intracellular calcium concentration ( $\text{Ca}^{2+}\text{i}$ ) of 90 nM were found. Application of 10  $\mu\text{M}$  ionomycin produced a biphasic increase in  $\text{Ca}^{2+}\text{i}$ . The initial rise in  $\text{Ca}^{2+}$  was due to release of  $\text{Ca}^{2+}$  from internal stores and was associated with membrane hyperpolarization to  $-77$  mV. The latter was probably due to the activation of high conductance,  $\text{Ca}^{2+}$ , and voltage-dependent  $\text{K}^+$  channels (maxi-K). Moreover, the second rise in  $\text{Ca}^{2+}\text{i}$  was always preceded by and strictly associated with membrane depolarization.

Simultaneous recordings of  $\text{Ca}^{2+}\text{i}$  and ion channel activity in the cell-attached configuration of patch clamp revealed a  $\text{Ca}^{2+}$ -permeable channel of 23 pS conductance. This channel did not require membrane depolarization for its activation. The 23 pS channel did not open at rest or on the first increment in  $\text{Ca}^{2+}\text{i}$ . However, activity of this channel was evident during the second increment in  $\text{Ca}^{2+}\text{i}$ . The absence of serum also activated the 23 pS channel, albeit with a lower frequency than ionomycin. Thus, the 23 pS channel activated by two unrelated inducers of apoptosis might be considered an important molecule in the  $\text{Ca}^{2+}$  influx mechanism in programmed cell death of androgen-independent LNCaP cells (38).

It has also been demonstrated that oxidative stress activates a  $\text{Ca}^{2+}$ -permeable, nonselective cation channel in endothelial cells, suggesting a role for oxidative stress in apoptosis (39). Because this channel shares important characteristics with the new 23 pS channel as well as with others related to the apoptotic mechanism emphasizing the capacitative entry of calcium, it is important to evaluate the novelty of the 23pS channel based on the properties of several well-known channels present in healthy as well as in neoplastic cells most probably associated with the process of apoptosis. However, it should be mentioned that two research groups have recently proposed that the release of store-retained calcium in androgen-dependent LNCaP cells might be sufficient to trigger the apoptotic process without the apparent need to activate store-operated channels (SOC channels) or a sustained entry of calcium (40,41).

**Table 1.** Purinergic receptors

Purinoceptor	Subtype	Isolation	Activity	Distribution	Molecular and biochemical characteristics	Electrophysiological characteristics	References
P2T			G protein-coupled	Numerous cultured cell lines			44
P2U				Coupling with apoptosis			
P2Y	P2Y1						
P2Z							
P2X	P2X1-P2X7		ATP ligand-gated cation channel G protein-coupled	Mast cells, macrophages, fibroblasts Neural and neuromuscular tissues	Carry Na <sup>+</sup> , K <sup>+</sup> , Ca <sup>2+</sup> currents		44
P2R				Mouse tissues spleen, testis, kidney, liver, lung, heart, brain			44
P2X1	Rat <i>vas deferens</i>			<i>Vas deferens</i> , bladder, lung, liver, low levels in dorsal root ganglion	19 pS between –140 and –80 mV, depolarization, entry of Ca <sup>2+</sup>		45
P2X1	Human bladder			Leukocytes, pancreas, spleen, testis, prostate, ovary, small intestine, colon, fetal liver	coupling with apoptosis		48
P2X2	PC12 cells			Bladder, brain, spinal cord, intestine, <i>vas deferens</i> , pituitary gland, adrenal gland	Rat P2X1 89% P2X2-P2X4 40–50%		49
P2X3	Dorsal root ganglion sensory neuron			Dorsal root ganglion	1416 bp, 472 aa, 52557 K, identity primary structure RP-2 40%		52
hP2X	Cells surrounding human tumor of bladder			Urinary bladder (human tumor), spleen, lung, thymus, liver, adrenal gland, human promyelocytic cell line, HL60	3800 bp, 397 aa, 47 K, identity primary structure with other P2X receptors 40/47% 2643 bp, 399 aa, identity primary structure, P2X1 41%		47
P2X4	Rat brain			Central nervous system neurons, blood vessels, leukocytes	1164 bp, 388 aa, 43 K, identity primary structure, rP2X1 50%, P2X2 44%, P2X3 44%		54
P2X5	Rat heart			Rat heart, brain, spinal cord, adrenal gland	455 aa, identity primary structure, P2X1 41%, P2X2 45%, P2X3 45%		57
rP2X7(P2Z)	Rat brain		ATP-dependent lysis of macrophages permeable to large molecules and small cations	Macrophages, microglia, brain, spinal cord, lung, spleen	595 aa, carboxyl-terminus is longer and important for the lytic actions for ATP		58
hP2X7	A human monocyte library			Pancreas, liver, heart, thymus, brain, skeletal muscle, lung, placenta, testis, leukocytes, prostate, spleen	Identity primary structure, rP2X7 80%	ATP EC <sub>50</sub> value was approximately 10-fold greater than that of rP2X7	59

## Ligand-Gated Ion Channels

It has been proposed that ATP might function as an extracellular messenger in addition to its essential role as a molecular energy source (42,43). ATP has been shown to be stored within secretory vesicles, and solely in response to well-defined stimuli appears to interact with purinergic receptors (P2 receptors) (43). Purinergic receptors called P2 purinoreceptors include receptors of the ligand-gated ion channel type as well as the G protein-linked superfamily of receptors. Historically, these receptors have been termed P2X and P2Y for the first group (42) and P2T, P2U, and P2Z for the G protein-coupled receptors (44) (Table 1). To study the biophysical characterization of the P2X purinoreceptor, the electrophysiologic approach has been valuable for studying a number of ATP-gated membrane currents in several cell types (42). When optimal recording conditions are employed in the presence of ATP, smooth muscle and cardiac muscle cells show an inward current at a resting membrane potential that corresponds to -50 to -70 mV. These currents have been demonstrated to be inwardly rectifying and supported by channels that are permeable

not only to K<sup>+</sup> and Na<sup>+</sup> but also to Ca<sup>2+</sup>. No purinoreceptor has shown a significant homology with any other known ligand-gated ion channel or receptor superfamily (42). Therefore, the mechanism by which extracellular ATP interacts with these P2X purinoreceptors is still unknown. It is important to mention that the consensus sequence region (G)(X4)GK(X7)(I/V) for ATP binding is absent in these receptors (42).

Based on the potent growth inhibition shown by ATP on a variety of human and murine tumor cells, administration of AMP or ATP to tumor-bearing murine hosts has been shown to be associated with dramatic cytostatic and cytotoxic effects. Such anticancer activity is likely to be related to the activation of P2Z purinoreceptors that mediate cell death through apoptosis (44,45–59) (Table 1). The rat P2X7 receptor (rP2X7) functions as a channel permeable to small cations and as a cytolytic pore. It is important to point out that in this channel, the carboxyl-terminus corresponds to 239 aa, compared to 27–120 aa in other purinergic receptors (Table 2). Channel activation does not occur if rP2X7 lacks the last 177 residues of its carboxyl terminus (59). Brief (1–2 sec) applications of ATP open the channel transiently,

**Table 2.** Ca<sup>2+</sup>-permeable channels involved in apoptosis

Channel type	Activation way	Current type	Conductance	Blockade	Expression	References
P2X	ATP ligand-gated cation channel low pH and divalent ions Zn <sup>2+</sup> increased the affinity for the agonist	Carry Na <sup>+</sup> , K <sup>+</sup> , Ca <sup>2+</sup> currents, depolarization	12–20 pS	Trivalent cations	Rat, mouse, human neural and neuromuscular tissues hP2X1 and hP2X7 expressed in prostate	42, 44, 45, 47, 50, 52, 54, 57, 58, 78
ICRAC CCE	Diffusible messenger and interaction between endoplasmic reticulum membrane and cytoplasmic membrane	Unselective; Ca <sup>2+</sup> , Na <sup>+</sup> , Ba <sup>2+</sup> , Hyperpolarization	24fS	Trivalent and bivalent	Drosophila, mammals, Xenopus laevis, Caenorhabditis elegans Trp photoreceptors, Htrp3 in brain, ovary, colon, small intestine, lung, prostate, placenta, and testis, TRPC1 (human) fetal brain, adult heart, brain, testis, and ovaries, prostate, mammalian genes trp-3, Trp-4, trp-5 and trp-6 in brain. Co-expressed in the same type cell, although varied levels	62, 64, 65, 66, 67, 68, 69, 71
Bcl-2	Pore-forming activity with dependence on low pH and acidic lipid membranes	Cation-selective channels protein translocation-selective	18 ± 2 pS most frequent 41 ± 2 pS and 90 ± 10 pS	pH acts as modulator	Cytoplasmic membrane, mitochondrial membrane, nuclear envelope, and parts of the endoplasmic reticulum	72
Bax	Pore-forming activity with dependence on low pH		Pred 250 ± 25 pS, 80 ± 25 pS and 180 ± 25 pS	pH acts as a modulator	Cytoplasmic membrane, mitochondrial membrane	75
23 pS	Ionomycin serum removal	Ca <sup>2+</sup> -permeable, non-selective cation channel	23 pS		Cytoplasmic membrane of LNCaP cells induced to apoptosis	38

similar to other P2X receptors. Some important differences have been shown between rP2X7 and the human purinoreceptor hP2X7, because high concentrations of agonists are required to activate the latter group. The half-maximal concentration ( $EC_{50}$ ) of 2'- 3'-(O)-(4-benzoyl benzoyl) ATP (BzATP) (60) to activate hP2X7 receptors is 25-fold greater than that observed for the rP2X7 receptor; the ATP  $EC_{50}$  value is approximately 10-fold greater (59) (Table 2).

ATP apparently induces cell death in thymocytes, hepatocytes, and several lymphocytic cell lines by increasing intracellular calcium concentration. Although P2Z and P2Y purinoreceptors appear to be involved in this calcium entry, P2X receptors have been those related to the apoptotic process (Table 1). These results might indicate that the P2X receptor and related molecules present active functions in programmed cell death as well as in synaptic transmission (46).

### Ionic Channels Involved in the Capacitative Entry of Calcium

Inositol 1,4,5-trisphosphate production provokes a biphasic  $Ca^{2+}$  signal in many cells: the first phase is effected through the mobilization of  $Ca^{2+}$  from internal stores that drive the initial burst, and the second phase by a store-operated  $Ca^{2+}$  entry (formerly capacitative  $Ca^{2+}$  entry) (61,62). Store-operated  $Ca^{2+}$  channels (SOC), which require decrements of  $Ca^{2+}$  in the endoplasmic reticulum (ER), are a subfamily of receptor-activated calcium channels (RACCs) (63). A signal for activation of calcium entry at the level of the plasma membrane is provided through depletion of intracellular  $Ca^{2+}$  pools. Depletion of intracellular  $Ca^{2+}$  pools is capable of activating a nonvoltage-sensitive plasma membrane  $Ca^{2+}$  conductance (62,64). The transmitting signal from intracellular stores to the membrane could be afforded by the following three main mechanisms: vesicle secretion, diffusible messages, and conformational coupling (61,65).

Closely resembling a capacitor from an electric circuit, calcium stores do not allow the entry of more cation when they are charged. However, they begin to promote entry as soon as stored calcium is discharged. This capacitative entry mechanism (CCE) is present in many cell types (66) and describes the influx of  $Ca^{2+}$  into cells. Therefore, this process that replenishes  $Ca^{2+}$  stores appears to be emptied through the action of inositol 1,4,5-trisphosphate (InsP3), in that CCE is an essential component for the cellular response to hormones and growth factors (67). To distinguish this from other calcium entry channels, the term calcium-release-activated calcium currents (ICRAC) has been used to refer to the current flowing through these capacitative calcium entry channels (Table 2).

The protein named as transient receptor potential or Trp has been found to be involved with functions of CCE in *Drosophila* photoreceptors. Trp is considered a member of a family of store-operated channels (SOCs) conserved from *Caenorhabditis elegans* to mammalian organisms (66,68).

Trp could be similar to voltage-gated  $Ca^{2+}$  channel in the regions named S3–S6, including the S5–S6 linker that forms the ion-selective pore. It has been observed that the positively charged residues in S4 that confer voltage sensitivity to voltage-gated channels are absent in Trp (66,67). Several homologs of Trp are known to be present in man as well as in mouse and *Xenopus*. This fact provides evidence that Trp might be the functional analog of the mammalian ICRAC channel (Table 2). Although these two channels do not present identical permeability properties, the Trp channel presents a higher conductance than the ICRAC channel, while also being much less specific.

As soon as stores have been partially or completely depleted, entry is initiated by a sudden hyperpolarization of the membrane. These series of experiments reveal that entry is importantly biphasic and switches on and off very quickly (66). The existence of six Trp-related genes has been reported in the mouse genome in which the expression in L cells of small portions of these genes in antisense orientation suppressed CCE. cDNAs encoding the human Trp homologs Htrp1 and 3 expressed in COS cells increased CCE. The activity of Htrp1 and 3 increases  $Ca^{2+}$  entry into COS cells by 75 and 230%, respectively. Northern analysis detected an Htrp3 mRNA of 4 Kb predominantly in brain and at much lower levels in several other tissues (67). The model of transmembrane topology for a monomeric Trp protein is proposed to be a six-spanning transmembrane protein with a 350-amino acid cytosolic N-terminus and a 200-amino acid cytosolic C-terminus. In analogy to voltage-gated  $Ca^{2+}$  channels, six consensus glycosylation sites are encoded in Htrp, none predicted to be extracellular (67).

There is evidence of tissue- and cell-specific co-expression of multiple Trp forms, implying that the subunit composition of a particular CCE channel may vary depending on cell type. Several Trp homologs of mammalian cells are involved in CCE, demonstrating that expression of antisense Trp sequences abolishes capacitative  $Ca^{2+}$  entry. This leads to speculation that the six Trp homologs are subunits of CCE channels (67). García and Schilling in 1997 (69) showed a distribution pattern for Trp mammalian genes (trp-1, -3, -4, -5, and -6). These are expressed in brain and are barely detectable in liver. These results also showed that several genes could be co-expressed in the same cell type at different levels of expression. However, interpretation of these results is difficult to assess because mRNA levels do not always equal protein levels and amounts of protein encoded by these genes may be required at varied levels by each cellular type. Mammalian Trp genes are ubiquitously expressed albeit at varying levels (69). This situation additionally suggests that these genes encode for subunits of distinct channels in the same cell, suggesting that the subunit composition of a particular CCE channel, for instance ICRAC, might be different according to the type of cell.

Taking into account the very low expression of mammalian Trp genes in liver compared to other tissues, there is the

possibility that there are other genes coding for CCE channel subunits that might not have similarity to other Trp family members (69).

One of the most common characteristics of ion currents associated with CCE is the activation they demonstrate upon depletion of intracellular  $\text{Ca}^{2+}$  stores induced by inhibitors of endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase, such as thapsigargin, removal of extracellular  $\text{Ca}^{2+}$ , or  $\text{Ca}^{2+}$  ionophores (70). Nevertheless, several differences have been found in ion permeability and unitary conductance that support the possibility that ion channels associated with CCE are encoded by closely related genes. The native currents that can be activated by  $\text{Ca}^{2+}$ -store depletion in various non-excitable cells are characterized by inward rectification (70). Wes et al. (61) described the molecular characterization of the human homolog of Trp. TRPC1 (human transient receptor potential channel-related protein 1) was 40% identical to *Drosophila* Trp; this molecule lacked the S4 transmembrane region required as a voltage sensor in many voltage-gated ion channels. TRPC1 is expressed at different levels in several tissues. Although expression levels of TRPC1 are observed in prostate, they are lower than those in testis and ovaries. Additionally, the authors identified a second human gene that encodes for a Trp homolog protein, named TRPC3 (61).

Although thapsigargin activates Trp channels selective for  $\text{Ca}^{2+} > \text{Na}^+ > \text{Ba}^{2+}$ , Trp1 channels are nonselective with respect to  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ , and  $\text{Ba}^{2+}$  and are not activated by thapsigargin or  $\text{Ca}^{2+}$ -store depletion. Experiments employing channel chimeras in which C-terminal domains of Trp and Trp1 have been exchanged led to the conclusion that the carboxy-terminal of Trp seems important for thapsigargin sensitivity. When Trp1 is expressed in SF9 cells under basal conditions, it is constitutively active and its selectivity corresponds to  $\text{Na}^+ > \text{Ca}^{2+} > \text{Ba}^{2+}$ . Moreover, Trp1 is not activated by store depletion and is known to initiate membrane currents. TRPC1 appears to correspond to characteristics shown by Trp1, with the exception of the type of membrane currents both present (71).

### Bcl-2 and Bax-Forming Pores on the Cellular Membrane

Bcl-2 is the most characteristic member of the family of apoptosis-regulating proteins, regulating the homeostasis of blockers and promoters of cell death (72–74) (Table 2). Because many homologs of bcl-2 have been described, several of which form homo- or heterodimers, this might indicate that these molecules become organized and function through protein-protein interactions (73).

Purified recombinant bcl-2 exhibits pore-forming activity with dependence on low pH and acidic lipid membranes. Nevertheless, a mutant of bcl-2 not presenting the two-core hydrophobic  $\alpha$ -helices (helices 5 and 6) essential for its insertion into the membrane only showed nonspecific effects

(72). Employing planar lipid bilayers, bcl-2 was shown to form discrete ion-conducting channels selective to cations. This property was totally lost when the bcl-2 mutant was tested (72).

The most frequent conductance shown to be related to bcl-2 is  $18 \pm 2$  pS in 0.5 M KCl at pH 7.4. Nevertheless, higher channel conductances ( $41 \pm 2$  pS and  $90 \pm 10$  pS) have been shown with progressively lower occurrence, indicating that generation of larger multimers of bcl-2 might occur (72). Bcl-2 and the majority of its homologs show a series of hydrophobic residues near their carboxy-terminal, which closely associates them with several intracellular membranes (72).

Channels formed by bcl-2 are principally voltage-independent because current conducted over a wide range exhibits a linear current-voltage relation. Recordings at low pH demonstrated that acidity promotes bcl-2-channel insertion into planar bilayers. For instance, at pH 5.4, membrane conductance moved from high to low values, a phenomenon supporting the progressive insertion of more channels into the membrane, indicating that pH might be considered a modulator (72). Bcl-2 has been shown to be abundant at contact sites that approximate inner and outer mitochondrial membranes. Single-channel conductances of 20, 40, and 90 pS have been found at pH 5.4 appearing as bursts (72).

Bcl-x as well as bax has shown to present pore-forming activity in synthetic lipid membranes (75,76) (Table 2). Bax, a pro-apoptotic member of the family, was added to neuronal cultures at a concentration of 10  $\mu\text{M}$ , which showed to be lytic within 3–6 h. Nevertheless, addition of bax and bcl-2 together delayed neuronal lysis by 12 h (75,76). To determine whether bax might also be considered a pore-forming protein, the hypothesis that bax could allow liberation of the carboxyfluorescein contained in liposomes was tested. It was shown that bax induces dye efflux from these liposomes in a concentration-dependent manner at neutral pH. The release of dye was also demonstrated to be pH-dependent (75,76). Because bcl-2 was as efficient as bax at pH 4 and the channel-forming ability of only bcl-2 decreased at pH 5, it was concluded that the pore-forming properties of bax and bcl-2 are most probably different.

Bax predominantly opens at  $250 \pm 25$  pS with sporadic changes at two main sublevels ( $80 \pm 25$  pS and  $180 \pm 25$  pS) (75,76). Due to the localization of bax in mitochondrial membranes, permeability changes and therefore disruption of transmembrane potential might be considered as important events in the genesis of apoptosis (75,76).

The process of apoptosis in several cell types occurs frequently on removal of cytokines and several growth factors. It has been proposed that these molecules could trigger a response in the nucleus with the activation of apoptotic-related genes such as c-myc, bcl-2, and p53 that might be directly involved through several pathways in initiation of apoptosis (21,77). In contrast, during the final stage of apoptosis a series of common processes such as fragmentation of

DNA and changes in the structure of cells is frequently observed. Between the process of initiation and the final events of the apoptotic pathway, a picture begins to emerge of how the basic properties of several plasma-membrane  $\text{Ca}^{2+}$  channels might be shared to elucidate common characteristics related to the delivery of a specific intracellular  $\text{Ca}^{2+}$  signal appearing to participate in the initiation and/or support of apoptosis. As described previously, such  $\text{Ca}^{2+}$  fluxes play a central role in the regulation of apoptosis, directly associated with normal and malignant cell proliferation. Therefore, understanding the molecular mechanisms of apoptotic cell death should lead to fundamental advances in the therapy of many diseases, including prostate cancer.

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