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## Review

# Ion Channels in Death and Differentiation of Prostate Cancer Cells.

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**Running title:** Ion channels in prostate cancer cells

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## ABSTRACT

Plasma membrane ion channels contribute to virtually all basic cellular processes, including such crucial ones for maintaining tissue homeostasis as proliferation, differentiation, and apoptosis. Enhanced proliferation, aberrant differentiation, and impaired ability to die are the prime reasons for abnormal tissue growth, which can eventually turn into uncontrolled expansion and invasion, characteristic of cancer. Prostate cancer (PCa) cells express a variety of plasma membrane ion channels. By providing the influx of essential signaling ions, perturbing intracellular ion concentrations, regulating cell volume, and maintaining membrane potential, PCa cells are critically involved in proliferation, differentiation, and apoptosis. PCa cells of varying metastatic ability can be distinguished by their ion channel characteristics. Increased malignancy and invasiveness of androgen-independent PCa cells is generally associated with the shift to a “more excitable” phenotype of their plasma membrane. This shift is manifested by the appearance of voltage-gated  $\text{Na}^+$  and  $\text{Ca}^{2+}$  channels which contribute to their enhanced apoptotic resistance together with downregulated store-operated  $\text{Ca}^{2+}$  influx, altered expression of different  $\text{K}^+$  channels and members of the Transient Receptor Potential (TRP) channel family, and strengthened capability for maintaining volume constancy. The present review examines channel types expressed by PCa cells and their involvement in metastatic behaviors.

**Key words:** prostate cancer; ion channels; proliferation differentiation and apoptosis; volume regulation; calcium signaling; store-operated channels, TRP channels.

**Abbreviations:** AR, androgen receptor; AVD, apoptotic volume decrease;  $[\text{Ca}^{2+}]_{\text{in}}$ , intracellular  $\text{Ca}^{2+}$  concentration; CRAC,  $\text{Ca}^{2+}$  release-activated channel; DAG, diacylglycerol;

ER, endoplasmic reticulum;  $I_{Cl,swell}$ , swelling-activated  $Cl^-$  current;  $I_{DAG}$ , current through DAG-gated cationic channels;  $I_K$ ,  $K^+$  current;  $I_{menthol}$ , menthol-activated current through cold/menthol-sensitive TRPM8;  $IP_3$ , inositol trisphosphate;  $I_{SOC}$ , store-operated membrane current; LVA, low voltage-activated; NE, neuroendocrine; PCa, prostate cancer; PLC, phospholipase C; RVD, regulatory volume decrease; SOC, store-operated channel; SOCE, store-operated calcium entry; TEA, tetraethylammonium; TTX, tetrodotoxin; VGCC, voltage-gated  $Ca^{2+}$  channel, VGSC, voltage-gated sodium channel; VRAC – volume-regulated anion channel.

## INTRODUCTION

Historically, the first important role ascribed to plasma membrane ion channels, over 60 years ago, was their participation in cellular electrogenesis and electrical excitability. However, numerous subsequent studies have firmly established the contribution of ion channels to virtually all basic cellular behaviors, including such crucial ones for maintaining tissue homeostasis as proliferation, differentiation, and apoptosis<sup>1,2</sup>. The major mechanisms via which ion channels contribute to these crucial processes include: providing the influx of essential signaling ions, regulating cell volume, and maintaining membrane potential. Malignant transformation of cells resulting from enhanced proliferation, aberrant differentiation, and impaired ability to die is the prime reason for abnormal tissue growth, which can eventually turn into uncontrolled expansion and invasion, characteristic of cancer. Such transformation is often accompanied by changes in ion channel expression and,

consequently, by abnormal progression of the cellular responses with which they are involved (figure 1).

Distinctions between prostate cancer (PCa) cells of varying metastatic ability can be made according to their ion channel characteristics. Because of unrestricted accessibility and convenience of experimentation, most studies on ion channel involvement in prostate carcinogenesis have been conducted on PCa epithelial cell lines of varying metastatic potential. Many cell lines are presently established from primary tissue sources and clonal derivatives of previously established lines<sup>3</sup> The data from native human PCa tissues is much sparser and are usually obtained in order to confirm major conclusions derived from cell line studies.

In this review, we describe the major types of ion channels in PCa epithelial cells, establish their role in apoptosis- and differentiation-related events, and track down how they evolve during transformation to apoptotic resistant cell phenotypes typical of advanced androgen-independent PCa.

## **POTASSIUM CHANNELS**

Potassium channels are involved in the maintenance of resting potential, thereby they represent an integral part of all cells. Since  $K^+$  channels provide an efflux of  $K^+$ , which is the dominant cation of the intracellular medium, they are also important regulators of cell volume.  $K^+$  channels represent one of the most diverse groups of channels, consisting of five major classes: (i) voltage-gated ( $K_v$  class), (ii)  $Ca^{2+}$ -activated ( $K_{Ca}$  class), (iii) inwardly rectifying ( $K_{ir}$  class), (iv) ATP-sensitive ( $K_{ATP}$  class), and (v) background two-pore domain-containing ( $K_{2P}$  class)<sup>4</sup>. Some of them have been identified in various types of carcinomas where there are involved in the proliferation and apoptosis of tumor cells<sup>4</sup>. This is consistent

with the paradigm according to which the enhanced  $K^+$  efflux is associated with apoptosis promotion and, conversely, that apoptosis is attenuated if  $K^+$  efflux is decreased<sup>4-6</sup>. The mechanisms for pro-apoptotic effects of enhanced  $K^+$  efflux include: (i) decay of the membrane potential and associated calcium ( $Ca^{2+}$ ) overload, (ii) apoptotic cell shrinkage (apoptotic volume decrease, AVD) and activation of intracellular pro-apoptotic effectors<sup>4-6</sup>. In particular, decreases in intracellular  $K^+$  appear to promote critical events during the early phases of cell death, including proteolytic cleavage of pro-caspase-3 and enhanced endonuclease activity<sup>5</sup>.

Among numerous  $K^+$ -channel types, a member of the  $K_v$  class eag1 (*ether-a-go-go*) (or  $K_v10.1$ ), has been found to be involved in tumorigenesis<sup>7-9</sup>. eag1  $K^+$  channel expression is exaggerated in several human cancers where it is involved in cell proliferation<sup>9</sup>. Inhibition of endogenous eag1 commonly reduces cell proliferation, while heterologous overexpression enhances proliferation rate<sup>7</sup>.

PCa epithelial cells are generally characterized by quite prominent voltage-gated  $K^+$  current ( $I_K$ ). However, the existing data on its molecular nature are quite diverse and sometimes conflicting. This probably reflects the multi-channel origin of the current as well as the multiplicity of factors that influence the patterns of their expression. Historically, in prostatic androgen-dependent LNCaP cells, the inhibition of  $I_K$  exerted antiproliferative effects, although it did not induce apoptosis<sup>10-12</sup>. Comparison of prostatic androgen-dependent LNCaP and androgen-independent PC-3 cell lines has shown that increased malignancy is associated with lower density of voltage-gated  $K^+$  current, which potentially makes their membrane “more excitable”<sup>13</sup>. However, due to their combined biophysical properties and despite the fact that primary prostate carcinoma tissue has been shown to be highly enriched with eag1 mRNA and protein<sup>9</sup>, the endogenous  $I_K$  in PCa epithelial cells could not specifically be linked to the activity of a specific  $K^+$ -channel type.

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The existing data suggests that  $K_v1.3$  is the dominant  $K_v$ -class channel expressed in normal and cancerous rat and human prostate tissues, as well as in prostatic cell lines with different metastatic potentials, with lesser contributions from  $K_v1.4$  and  $K_v1.6$ <sup>14-16</sup>. The difference between strongly metastatic rat MAT-LyLu and weakly metastatic AT-2 cell lines was again mostly found with regard to  $I_K$  density, rather than biophysical properties<sup>16</sup>. This is consistent with the altered expression of the same channel types as opposed to the appearance of new ones. Facilitation of  $K^+$  efflux by  $K^+$ -channel openers, (minoxidil, 1-ethyl-2-benzimidazolinone (EBIO) or diazoxide) was able to increase growth of PC-3 cells by 30-50%, while  $K^+$ -channel inhibitors (dequalinium, amiodarone and glibenclamide) caused a dose-dependent, growth inhibition of both androgen-sensitive (LNCaP, MDA-PCA-2B) and androgen-insensitive (PC-3, DU-145) human PCa cell lines<sup>15</sup>. The same blockers induced PC-3 apoptosis within 4 hours treatment<sup>15</sup>.

Thus we can conclude that PCa epithelial cells that preserve androgen sensitivity, and display relatively weak metastatic potential, are generally characterized by higher  $I_K$  and  $K^+$ -channel expression. On the one hand, this promotes their proliferation, but on the other hand it makes them more prone to programmed cell death. On the contrary, lower  $I_K$  and  $K^+$ -channel expression of highly metastatic, androgen-insensitive cells, although reducing their proliferative activity, contributes to their apoptotic resistance.

The importance of augmented  $K^+$  efflux in apoptosis was directly confirmed in experiments with KChAP, a  $K^+$ -channel regulatory protein that increases  $K^+$ -channel expression in a “chaperone-like” fashion in heterologous expression systems<sup>23</sup>. Overexpression of KChAP in LNCaP cells, decreased the average cell size due to enhanced AVD, promoted spontaneous cells apoptosis<sup>17</sup>. Moreover, repetitive overexpression of KChAP during 19-day in LNCaP and DU-145 tumor xenografts in nude mice significantly suppressed tumor growth due to the apoptosis of infected tumor cells. The mechanism of pro-

apoptotic KChAP action could be due to direct interaction with  $K^+$  channels, thereby increasing their expression. Overexpression of KChAP in LNCaP cells also produced  $G_0/G_1$  cell cycle arrest *via* the activation of p53 (the tumor suppressor protein) acting as a transcription factor. However, the involvement of p53 in pro-apoptotic KChAP activity was ruled out based on the fact that KChAP was able to induce similar apoptosis in DU-145 cells expressing mutated p53, rendering it non-functional as a transcription factor<sup>17</sup>.

Pharmacological data also suggest the presence of  $K_{Ca}$ -class channel representatives in PCa cells. Indeed, in LNCaP and PC-3 cells, the activation of  $K_{Ca}$  channels enhanced cell proliferation. The mechanism underlying the regulation of cell proliferation by  $IK_{Ca}$  channels remains to be elucidated, these results highlight the importance of  $Ca^{2+}$ -dependent  $K^+$  efflux in general on  $IK_{Ca}$  channels, especially in the proliferation of human PCa cells.

Interestingly, a recent electrophysiological study has also identified large-conductance ( $BK_{Ca}$ )  $K_{Ca}$  channels in LNCaP cells, though with quite unusual voltage- and  $[Ca^{2+}]_{in}$ -dependence. This may be due to a unique subunit composition of the channel<sup>18</sup>.  $BK_{Ca}$  channel expression was regulated by serum-derived factors, as serum deprivation strongly reduced whole-cell current density. Current decrease in serum-deprived medium was unaffected by either an antagonist (bicalutamide, Casodex®) or an agonist (R1881) of androgen receptor (AR), suggesting that these factors were apparently not androgens<sup>18</sup>. It is known that serum starvation induces neuroendocrine (NE) differentiation of LNCaP cells<sup>13,14</sup>. Therefore, reduction of  $BK_{Ca}$  channels may play an important role in this process, especially in light of the simultaneous increase in the expression of low voltage-activated (LVA) calcium channels in NE cells<sup>13</sup>.

In conclusion,  $K^+$  channels seem to play an important role in the control of prostate cancer cells growth by regulating membrane potential and passive calcium influxes. However, further studies are needed to identify the precise role of each type of  $K^+$  channels in

carcinogenesis for their potential utilization as diagnostic/prognostic markers and/or therapeutic targets.

## **VOLTAGE-GATED SODIUM AND CALCIUM CHANNELS**

The notion that increased malignancy of PCa cells is associated with the shift to a “more excitable” phenotype of their plasma membrane is supported not only by the decrease in  $K^+$  conductances, as described above, but also by the appearance of inward currents characteristic of excitable cells, such as voltage-gated  $Na^+$  and  $Ca^{2+}$  currents. Indeed, in several PCa epithelial cells the expression of voltage-gated  $Na^+$  channels (VGSCs) on functional, protein and mRNA levels has been firmly established<sup>19-22</sup>. Moreover, VGSCs activity can enhance the metastatic behavior of cells, including their proliferation. VGSC opener veratrine has been shown to increase growth of not only androgen-insensitive PC-3 and DU-145 cells (for which functional channel activity was documented), but also of androgen-sensitive LNCaP and MDA-PCA-2B cell lines, which apparently do not display such activity<sup>20</sup>. At the same time, VGSC blockers (flunarizine, and riluzole) induced dose-dependent growth-inhibition of all four cell lines<sup>20</sup>.

RT-PCR analysis identifies tetrodotoxin-(TTX)-sensitive  $Na_v1.7$  as the most upregulated (approximately 20-fold)  $Na^+$ -channel  $\alpha$  subunit in PCa<sup>22</sup>. Furthermore, TTX has been shown to directly reduce the invasiveness of the cells<sup>23</sup>, thus suggesting  $Na^+$  channels as a viable target for anti-PCa research. All this strongly supports the notion that expression of VGSCs and the metastatic behaviors of prostate carcinoma cells are functionally related. However, the mechanism(s) responsible for VGSCs upregulation, as well as their pro-metastatic action, are still poorly understood. It is suggested that VGSCs expression may endow the membranes of PCa epithelial cells with electrophysiological properties that enhance their motility<sup>24</sup> and/or

secretory activities<sup>25</sup>, as well as perturb intracellular ionic homeostasis. Indeed, it has been directly demonstrated that, whereas VGSC blockers (TTX and phenytoin) reduce, VGSC openers (aconitine, ATX II) enhance the migration of metastatic human PC-3 or rat MAT-LyLu cells without influencing the motility of weakly metastatic human LNCaP and rat AT-2 cells<sup>24,26</sup>. However, the questionability of pharmacological tools (i.e. specificity and side effects) may compromise the conclusions drawn; therefore, other approaches (i.e. siRNAs, overexpression studies) need to be used to conclude on the precise role of Na<sup>+</sup> channels in PCa.

The prostate contains an abundance of high-affinity dihydropyridine (DHP) binding sites<sup>27</sup>. It has also been shown that the percentage of epithelial rat ventral prostate cells undergoing apoptosis in response to androgen ablation is reduced by administering voltage-gated Ca<sup>2+</sup> channel (VGCC) blockers such as nifedipine and verapamil<sup>28,29</sup>. These observations have given rise to the hypothesis that calcium channel blockers, by inhibiting calcium signal-mediated apoptosis, may increase the risk of PCa<sup>23</sup>. Despite this indirect evidence, the presence of VGCC activity has not been detected in PCa epithelial cells by means of electrophysiology. Therefore, as in the case of Na<sup>+</sup> channels, the role of DHP-sensitive Ca<sup>2+</sup> channels in PCa remains questionable until other experimental approaches rather than pharmacological ones, will confirm their expression and activity.

Nevertheless, the progression of prostate cancer to the androgen-insensitivity stage is accompanied by the appearance of new apoptosis-resistant cell phenotypes. The enrichment of androgen-independent tumors with malignant neuroendocrine (NE) cells should especially be noted. Fully differentiated, non-proliferating, neuron-like NE cells are a normal component of the prostate epithelium which, by releasing a variety of neurosecretory products, regulate the development and secretory activity of the prostate in the endocrine/paracrine manner<sup>30,31</sup>. Generally, prostatic NE cells express a variety of membrane ion channels characteristic of

neurons, like TTX-resistant VGSCs, high voltage-activated (HVA)  $\text{Ca}^{2+}$  channels of L- and N-type,  $\text{K}_v$ ,  $\text{K}_{Ca}$ , and  $\text{K}_{ir}$  representatives, and are also able to generate action potentials<sup>32</sup>. However, an expanding population of NE cells beyond normal proportions due to the malignant transformation of epithelial/basal cells is a common characteristic of prostate cancer progression<sup>30</sup>. NE cells lack nuclear AR, thereby representing an androgen-insensitive cell phenotype in the prostate<sup>33</sup>. They also exhibit high apoptosis resistance<sup>34</sup> which, according to existing evidence, is unrelated to the common antiapoptotic Bcl-2 protein<sup>35</sup>, and conferred instead by new survival proteins, survivin<sup>36</sup> and clusterin<sup>37</sup>.

Findings showing the small proportion of undifferentiated LNCaP cells displaying an LVA  $\text{Ca}^{2+}$  current carried by T-type  $\text{Ca}^{2+}$  channels, and the number of cells showing this type of current, as well as the significantly increased current density during the NE differentiation of LNCaP cells induced by either long-term treatments with membrane permeable cAMP analogs or by steroid-deprived culture medium<sup>38</sup> is of special importance. RT-PCR experiments demonstrated that only mRNA for  $\text{Ca}_v3.2$  isoform of T-type  $\text{Ca}^{2+}$  channel  $\alpha 1$  subunit is expressed in LNCaP cells, and becomes highly elevated during NE differentiation<sup>38</sup>. It was also shown that basal  $\text{Ca}^{2+}$  entry through this channel at resting membrane potential due to the presence of a prominent “window current” is likely to facilitate neurite elongation, thereby promoting NE differentiation. It was suggested that this channel could be also involved in the stimulation of mitogenic factor secretion, thus representing an attractive potential target for future therapeutic strategies<sup>38</sup>. However, whether or not these channels contribute to the enhanced anti-apoptotic potential of NE cells is not yet clear.

## **STORE-OPERATED CALCIUM ENTRY AND TRP CHANNELS**

The role of  $\text{Ca}^{2+}$  in the majority of cell signaling pathways involved in carcinogenesis is well established. Calcium homeostasis, the consequences of calcium signaling, is a steady state between influx, efflux and storage of  $\text{Ca}^{2+}$ . From a physiological point of view,  $\text{Ca}^{2+}$  signaling is involved in the manifestation of cell phenotype, proliferation, differentiation, apoptosis, and in cellular activities such as contraction or secretion or cell excitability. Thus, each cellular phenotype, whether normal or pathological, is characterized by a particular “Calcium Signature” reflecting its kinetics, amplitude and sub-cellular localization of the calcium signals. Indeed, if the oscillations of the cytosolic calcium stimulate cell proliferation via activation of the  $\text{Ca}^{2+}$ -dependent transcription factor, NFAT<sup>39</sup>, a sustained elevation in cytosolic  $\text{Ca}^{2+}$  concentration induces apoptosis of cancer cells<sup>40</sup> (Figure 2). Because the problem of  $\text{Ca}^{2+}$  homeostasis in cancer cells is too vast, even in relation to PCa cells, we will limit ourselves to characterizing channels only, and refer the reader to other comprehensive reviews for more in-depth information<sup>41-46</sup>.

In PCa epithelial cells, as in other non-excitabile cell types,  $\text{Ca}^{2+}$  entry from extracellular space is mainly supported by the “capacitative calcium entry” (CCE) mechanism, also known as “store-operated calcium entry” (SOCE) (reviewed in ref. <sup>47</sup>). This mechanism is capable of monitoring endoplasmic reticulum (ER)  $\text{Ca}^{2+}$  filling, enabling influx only when ER content is essentially decreased. It is mediated via specialized plasma membrane store-operated  $\text{Ca}^{2+}$ -permeable channels (SOC). The common physiological trigger for the activation of these channels is inositol trisphosphate-(IP<sub>3</sub>)-induced  $\text{Ca}^{2+}$  release from the ER in response to the stimulation of surface receptors coupled to the phospholipase C-(PLC)-catalyzed inositol phospholipid breakdown signaling pathway. This is why, when these channels have been identified for the first time by patch-clamp experiments they were termed “ $\text{Ca}^{2+}$  release-activated channels” (CRAC)<sup>48</sup>.

Alterations in calcium homeostasis and in SOC activity seem to play a major role in the establishment of androgen-independent apoptosis-resistant phenotype of PCa. Indeed, the major features of  $\text{Ca}^{2+}$  homeostasis in androgen-independent apoptosis resistant PCa cells (such as LNCaP cells stably transfected with Bcl-2 and neuroendocrine differentiated LNCaP cells) compared to the wild-type androgen-dependent LNCaP cells are: 1) reduced basal  $\text{Ca}^{2+}$  filling of the ER pool, and 2) reduced store-operated  $\text{Ca}^{2+}$  entry<sup>49</sup>. These changes were accompanied by the increased resistance to TG- and  $\text{TNF}\alpha$  induced apoptosis with clear shift to higher importance of  $\text{Ca}^{2+}$  influx vs. ER store depletion in apoptosis induction compared to the wild-type androgen-dependent LNCaP cells<sup>49</sup>. Therefore, identification the molecular nature of SOC and the mechanisms of their activation/regulation is of great importance for understanding of what drives PCa to androgen-independence. However, years of frustration marked the quest for molecular basis of SOC and for molecules underlying the process of capacitative calcium entry. Fortunately, these questions seem to be resolved now due to the very recent series of publications on STIM1 (stromal interaction molecule 1), identified as the mammalian ER  $\text{Ca}^{2+}$  sensor<sup>50,51</sup>, closely followed by identification of Orai1/CRACM1 as a component of the mammalian CRAC channel<sup>52</sup>. The role of these proteins in PCa progression is not yet studied but it is obvious that STIM1 and ORAIs could represent new candidates for PCa research.

Last years, some members of the widely-investigated family of mammalian homologues of the *Drosophila* TRP (Transient Receptor Potential) channel were viewed as being involved in SOC formation (for recent reviews see refs.<sup>47,53,54</sup>). Our own studies conducted on androgen-dependent LNCaP cells have suggested the involvement of the members of the “canonical” TRP subfamily, TRPC1 and TRPC4, in prostate-specific endogenous SOCs<sup>55,56</sup>. However, the expression pattern of TRPC1 and TRPC4 was not modified in androgen-independent apoptosis resistant PCa cells (unpublished results). Interestingly, the activity of a

member of the “vanilloid” TRP subfamily, TRPV6, may also have some relation to the sequence of events following to ER depletion in LNCaP cells, as its antisense knockout decreases endogenous store-operated membrane current ( $I_{SOC}$ )<sup>57,58</sup>, but the mechanisms underlying such TRPV6 activation in LNCaP cells remain elusive. However, in PCa, TRPV6, formerly known as  $Ca^{2+}$  transporter type 1 (CaT1) or epithelial calcium channel 2 (ECaC2)<sup>59</sup>, attracts special attention even beyond its potential role in calcium influx, as its expression was shown to correlate with PCa grade<sup>60-62</sup>. A study conducted on tissue samples from 140 patients with PCa demonstrated the association of TRPV6 with PCa progression and suggested it as a prognostic molecular marker in cancer classification<sup>60</sup>. Moreover, it has been demonstrated that heterologous TRPV6 expression in HEK-293 cells promotes their proliferation in a  $Ca^{2+}$ -dependent manner by increasing  $[Ca^{2+}]_{in}$  levels, which is a prerequisite for its potential role in tumor progression<sup>63</sup>. It seems, however, that the functional role of endogenous TRPV6 in prostatic  $I_{SOC}$  is closely linked to other potential SOC constituents and/or regulators, because heterologous TRPV6 overexpression in LNCaP cells resulted in the appearance of additional membrane current with properties distinct from endogenous  $I_{SOC}$ <sup>64</sup>. In any event, the problem of molecular basis for SOCE in PCa epithelial cells is still far from being resolved.

It is well established that various cellular  $Ca^{2+}$ -dependent processes rely on the specific spatial and temporal patterns of  $Ca^{2+}$  signaling<sup>65</sup>. However, the type and manner of their organization during carcinogenesis is not sufficiently defined. For instance, in PCa epithelial cells, stimulation of two receptors,  $\alpha 1$ -adrenoceptor ( $\alpha 1$ -AR) and metabotropic purinergic receptor (P2Y-R), produce divergent effects on cell proliferation:  $\alpha 1$ -AR stimulation enhances proliferation<sup>39,66</sup>, while P2Y-R stimulation results in growth arrest<sup>40,66</sup>. Such divergent effects on proliferation are quite surprising, given that both receptors act *via* a common PLC-catalyzed inositol phospholipid breakdown signaling pathway that results in the derivation of

IP<sub>3</sub> and diacylglycerol (DAG), two second messengers important for Ca<sup>2+</sup> signaling. Our recent studies on primary human PCa epithelial cells provided some understanding of these puzzling observations<sup>66</sup>. We have shown that Ca<sup>2+</sup> signaling controlled by each receptor relies on different Ca<sup>2+</sup>-entry pathways, ultimately targeting various intracellular effectors. It appeared that stimulation of α1-AR activates plasma membrane non-specific cationic channels via direct DAG gating<sup>39,66</sup> without affecting ER Ca<sup>2+</sup> stores, while P2Y-R stimulation brings about IP<sub>3</sub> receptor-mediated ER store depletion and activation of SOCs<sup>40,66</sup>. Consistent with these peculiarities, the α1-AR agonist, phenylephrin, stimulated oscillatory-type intracellular Ca<sup>2+</sup> signaling involving membrane current through DAG-gated cationic channels (I<sub>DAG</sub>), whereas the P2Y-R agonist, ATP, induced a transient [Ca<sup>2+</sup>]<sub>in</sub> increase, followed by a smaller sustained increase due to store depletion and SOC activation (figure 3). The two Ca<sup>2+</sup> entry pathways also appeared to have a different molecular nature, with the first one mostly relying on a DAG-gated TRP member, TRPC6, and the second one on TRPC1 and TRPC4.

Moreover, our data show that α1-AR stimulation enhances prostate cancer epithelial cell proliferation by inducing store-independent, TRPC6-mediated Ca<sup>2+</sup> entry resulting in the activation of NFAT transcription factor via its Ca<sup>2+</sup>/calmodulin/calcineurin nuclear translocation pathway<sup>66</sup>. TRPC6 antisense knockout exerted effects similar to those of pharmacological α1-AR inhibition, i.e. suppression of agonist-induced Ca<sup>2+</sup> entry, cessation of oscillatory-type Ca<sup>2+</sup> signaling, and consequent termination of cell proliferation. Furthermore, chronic treatment with α1-agonists enhanced TRPC6 protein expression, as well as altered the expression of two cell-cycle regulators, CDK4 and cyclin-dependent kinase inhibitor p27. This provides direct evidence for the α1-AR–TRPC6–NFAT–cell proliferation link. In contrast, Ca<sup>2+</sup> entry associated with P2Y-R stimulation by extracellular ATP and related growth arrest did not involve either TRPC6 channel activation or NFAT translocation.

Our findings demonstrate that the  $\alpha 1$ -AR-dependent  $\text{Ca}^{2+}$  signaling that promotes proliferation of prostate cancer epithelial cells specifically requires the activation of TRPC6 channels coupled to NFAT, thereby suggesting TRPC6 as a promising new target for controlling prostate cancer cell proliferation.

It should be noted that clinical studies also implicate  $\alpha 1$ -AR antagonists as pro-apoptotic agents capable of inducing apoptosis of human prostate cancer epithelial and smooth muscle cells without affecting cellular proliferation<sup>67</sup>. However, these effects seem to be unrelated to  $\alpha 1$ -AR<sup>68</sup> and  $\text{Ca}^{2+}$  signaling associated with it.

Interestingly, the endogenous expression of TRPC1, TRPC3, and TRPV6 proteins per se in LNCaP cells was shown to be controlled by the ER  $\text{Ca}^{2+}$  filling: after a prolonged (24-48 h) depletion of the stores with thapsigargin, a potent pro-apoptotic agent, their expression increased<sup>69</sup>. Enhanced expression of apparently store-dependent TRP members under ER store depletion is difficult to reconcile with the findings that androgen-independent, apoptosis-resistant PCa cell phenotypes, for which chronic underfilling of the ER  $\text{Ca}^{2+}$  pool represents a new level of equilibrium helping them to withstand ER stress-mediated apoptosis, are characterized by reduced SOCE<sup>49,70,71</sup>. It is, therefore, likely that native SOC in prostate cancer epithelial cells is a much more complex entity, whose functional expression cannot be directly correlated with any of the implicated TRP members. In this respect, it is important to assess the role of STIM1 and CRACM1 (Orai1) proteins in PCa cell SOCE.

Cold/menthol-sensitive TRPM8 of the “melastatin” TRP subfamily is yet another TRP member that has recently emerged as an important player in normal and pathological development of the prostate, whose real significance, however, is only beginning to unfold. Although TRPM8 was initially identified as a cold/menthol receptor mediating cold-evoked excitation in sensory neurons<sup>72,73</sup>, in fact, it was first cloned from the human prostate as a prostate-specific gene<sup>74</sup> before its role in cold sensation was established. Our data<sup>75</sup> as well as

those of others<sup>76</sup> indicate that TRPM8 is expressed not only in the plasma membrane of prostate cells, as initially anticipated, but also in the ER membrane, where it operates as an ER Ca<sup>2+</sup> release channel involved in the activation of SOCE in response to cold/menthol stimulus. Moreover, while remaining at moderate levels in a normal prostate, TRPM8 expression strongly increases in prostate cancer. For this reason it has been proposed to be a pro-oncogenic actor in PCa cells<sup>74</sup>. Other non-prostatic primary human tumors (breast, colon, lung, and skin) also become highly enriched in TRPM8, although it is virtually undetectable in corresponding normal tissues<sup>74</sup>. Thus, even this initial information strongly pointed to much broader roles of TRPM8 beyond cold sensation, especially in the prostate and during carcinogenesis. The role of TRPM8 in organs not exposed to ambient temperatures, and especially in prostate gland, remains a gnawing mystery. However, the data accumulated last years allows hypothesizing on that.

In normal prostate, *trpm8* gene expression seems to be directly controlled by AR's<sup>76,77</sup> positioning it as a primary androgen-response gene<sup>77</sup>. Single-cell RT-PCR and immunohistochemical experiments conducted on primary human PCa cells have shown that TRPM8 is mainly expressed in androgen-dependent, apical secretory epithelial cells, and that its expression becomes down-regulated in cells losing the AR activity and regressing to the basal epithelial phenotype<sup>77</sup>. Mature epithelial cells are non-proliferative cells, highly sensitive to apoptotic stimuli (due to the specific regulation of the expression of genes belonging to *Bcl-2* family: anti-apoptotic *Bcl-2* gene expression is repressed whereas pro-apoptotic *Bax* gene expression is stimulated by AR)<sup>78,79,80</sup>. Nevertheless, the secretion of products (including citric acid, prostate-specific antigen (PSA), acid phosphatase, several enzymes and lipids) is the major function of apical epithelial prostate cells. Therefore, considering the specific TRPM8 expression in these cells, we suggested the potential role of this channel in secretion.

In PCa tumors, a significant difference in mRNA expression level of TRPM8 between malignant and non-malignant tissue specimens has been detected<sup>81</sup>. This was comparable to the currently used PCa marker, PSA, thus, qualifying TRPM8 as its potential competitor in PCa diagnosis and staging. A significant difference in TRPM8 expression between human benign prostate hyperplasia (BPH) and PCa tissues is also obvious at protein level (figure 4). According to Tsavaler's hypothesis defining *trpm8* as an oncogene<sup>74</sup>, TRPM8 overexpression and overactivity in circumscribed, androgen-dependent PCa may be correlated to the higher rate of growth of these cells compared to normal ones<sup>82,83</sup>. During the transition to androgen independence, TRPM8 is lost in xenograft model and also in PCA tissue from patients treated preoperatively with antiandrogen therapy, suggesting that its loss may be associated with a more advanced form of the disease<sup>84</sup>. According to a clinical study describing that androgen-independent PCa metastasis proliferate more slowly than the androgen-dependent ones<sup>83</sup>, our unpublished results demonstrated that LNCaP cells resistant to anti-androgen bicalutamide treatment (LNCaP-bic<sup>R</sup>) displayed a reduced doubling time. This is correlated to a decreased expression of AR, TRPM8 and the Proliferating Cell Nuclear Antigen (PCNA) mRNAs, while anti-apoptotic Bcl-2 mRNA expression is increased (figure 5). All these data reinforce the putative pro-proliferative role of TRPM8 in androgen-dependent PCa cells.

Finally, Barritt's group has demonstrated that both pharmacological activation of TRPM8 and siRNA-mediated TRPM8 silencing in LNCaP cells can decrease the cell viability<sup>76</sup>, probably by perturbing the TRPM8-dependent intracellular Ca<sup>2+</sup> homeostasis. However, it is still not clear whether TRPM8 involvement in cell viability is carried out through a pro-proliferative and/or an anti-apoptotic mechanism.

## CHLORIDE CHANNELS

Activation of the chloride current through specialized volume-regulated anion channels (VRACs) in response to cell swelling ( $I_{Cl,swell}$ ) is one of the major mechanisms by which cells tend to restore their volume following hypo-osmotic stress – a process known as regulatory volume decrease (RVD) (reviewed in refs. <sup>85,86</sup>). Extracellular osmotic perturbations are not the only reason for alterations in cell volume. Effectively counteracting abrupt volume changes and maintaining relative volume constancy during active solute uptake, exocytosis, proliferation and differentiation are major prerequisites for cell survival. Indeed, there is strong evidence that disordered or altered cell volume regulation is associated with apoptosis<sup>86</sup>. Compelling support for such an association has been provided by demonstrating the direct causal link between apoptotic resistance conferred by antiapoptotic Bcl-2 protein and the strengthening of RVD capability due to upregulation of  $I_{Cl,swell}$ <sup>87,88</sup>.

Using LNCaP cells, we have shown that PCa cells are endowed with the powerful  $I_{Cl,swell}$ , which provides an effective RVD under hypoosmotic stress<sup>89,90</sup>, and that the magnitude of this current, as well as the capability of an even further increase in RVD with cell transition to androgen-independence and apoptosis resistance<sup>88,91</sup>. Moreover, the enhancement of  $I_{Cl,swell}$  and the related strengthening of RVD appeared to be independent on the specific reasons for such a transition: overexpression of the common anti-apoptotic Bcl-2 protein or NE differentiation, suggesting that it represents a general phenomenon.

Although the molecular nature of native  $I_{Cl,swell}$ -carrying VRACs is not known, and several membrane proteins are considered as potential candidates<sup>85,86</sup>, our data are also consistent with CIC-3 protein<sup>92</sup> involvement in prostate-specific VRAC, as well as with its upregulation in androgen-independent PCa cell phenotypes<sup>88,91</sup>. Importantly, it appears that  $Ca^{2+}$  homeostasis and volume homeostasis of PCa cells are interrelated due to functional coupling of SOCs and VRACs in confined plasma membrane caveolae microdomains<sup>56</sup>, enabling  $Ca^{2+}$  entering the cell *via* SOCs to exert inhibitory action on VRACs<sup>90</sup>. Such coupling is partly

responsible for the upregulation of  $I_{Cl,swell}$  in androgen-insensitive, apoptosis-resistant PCa cell phenotypes. Indeed, these cells are generally characterized by reduced  $I_{SOC}$ , most likely due to the diminished number of functional SOCs<sup>70,49,71</sup>. Therefore, less baseline inhibition of VRACs is expected. In turn, downregulation of SOCs and SOCE in androgen-insensitive, apoptosis-resistant PCa cells apparently represents an adaptive response to chronic underfilling of their ER  $Ca^{2+}$  pools resulting from enhanced ER leak, accompanied by the decreased expression of ER luminal  $Ca^{2+}$  binding protein, calreticulin, and SERCA2b  $Ca^{2+}$  pump isoform<sup>70,49</sup>.

## CONCLUSIONS

Figure 6 presents a summary of various membrane currents and associated ion channels identified in PCa cells, and their possible involvement in metastatic behavior. All of these channels potentially represent attractive targets for diagnosis, staging and/or treatment of PCa. However, more studies are needed, especially in *in vivo* systems, before any of them will result in practical implications.

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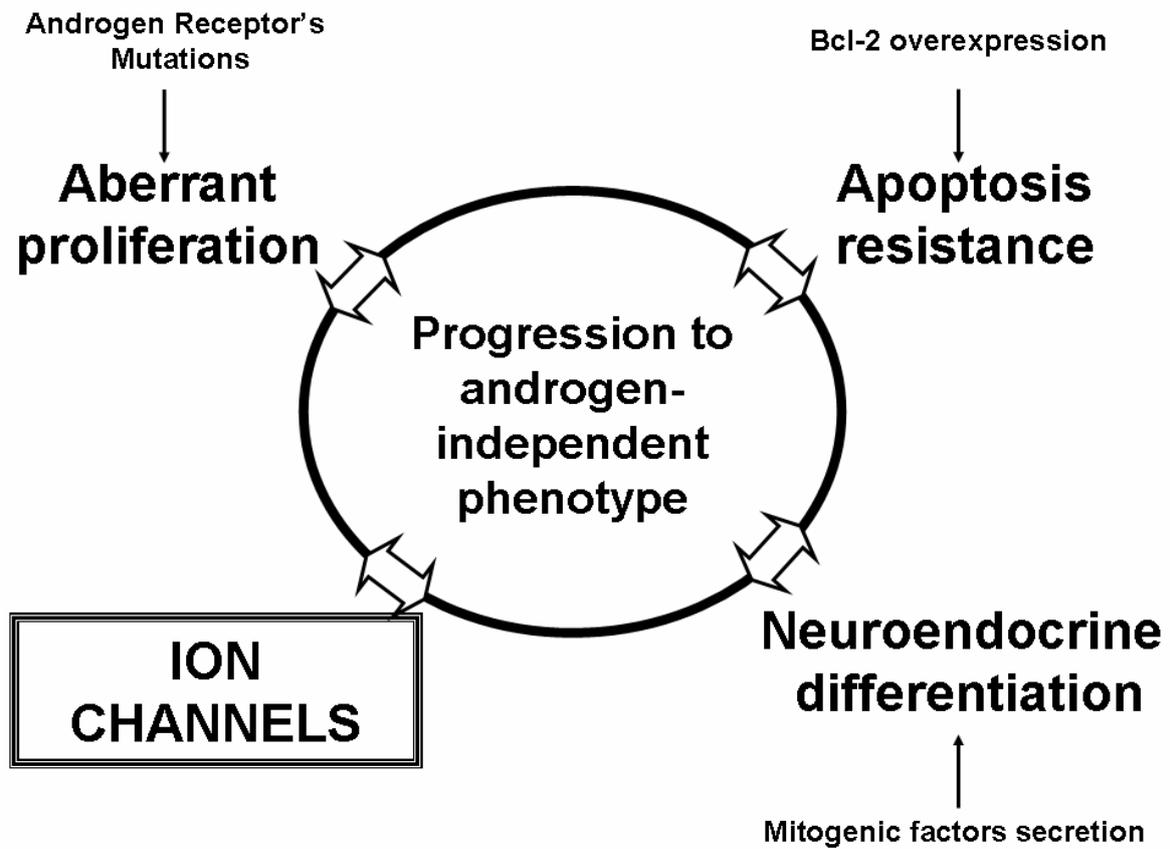
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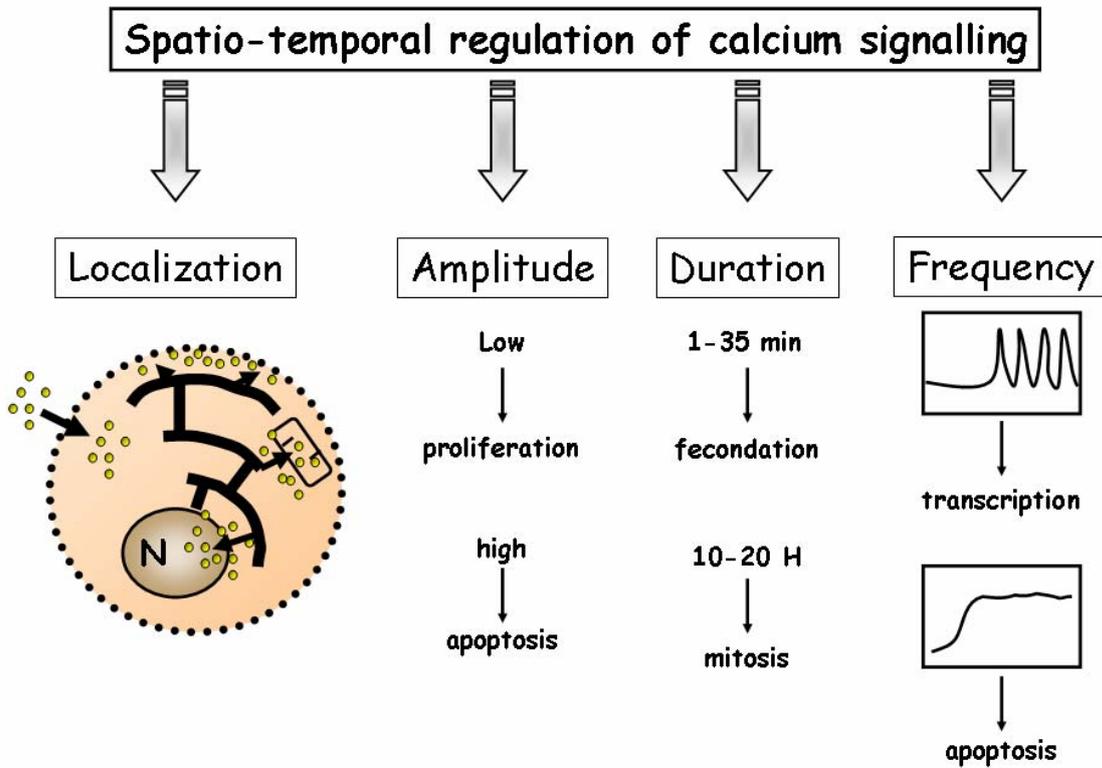
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**Fig. 1**



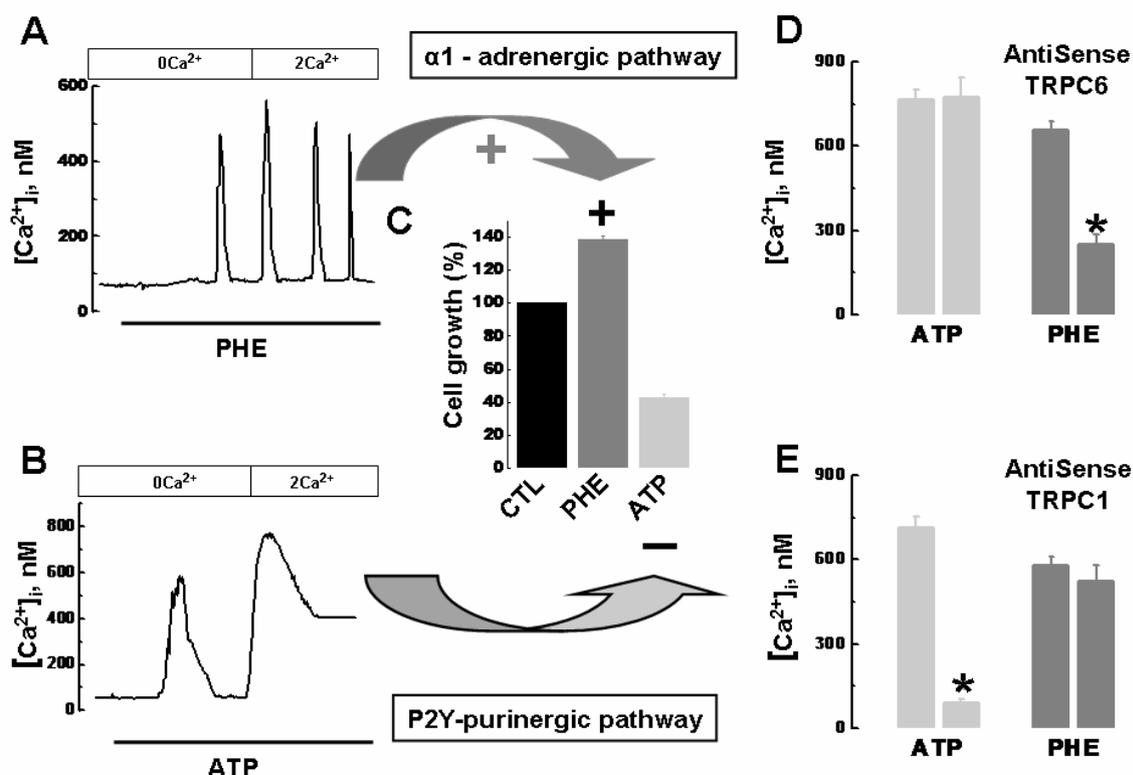
**Fig. 1. Schematic representation of the malignant transformation of prostatic cells** resulting from enhanced proliferation, neuroendocrine differentiation, and apoptosis resistance. These are the prime reasons for abnormal tissue growth, which can eventually turn into uncontrolled expansion and invasion, characteristic of cancer. Such transformation is often accompanied by changes in ion channel expression and, consequently, by abnormal cellular responses.

**Fig. 2**



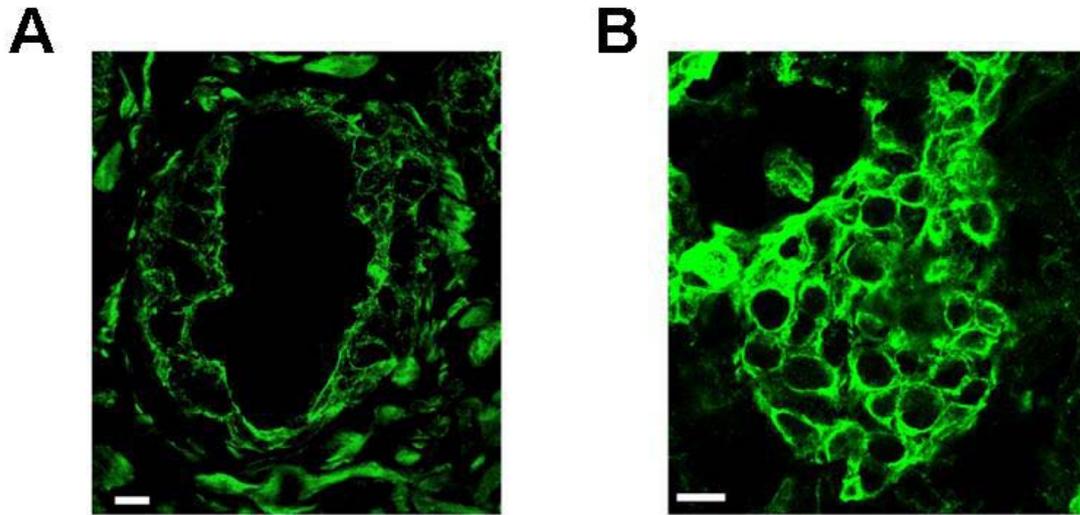
**Fig. 2. Spatio-temporal regulation of  $\text{Ca}^{2+}$  signaling.** This regulation is characterized by a particular “Calcium Signature” reflecting its kinetics (duration and frequency), amplitude and sub-cellular localization of the calcium signals. For example, if the oscillations of the cytosolic  $\text{Ca}^{2+}$  stimulate cell proliferation via activation of the  $\text{Ca}^{2+}$ -dependent transcription factor, NFAT, a sustained elevation in cytosolic  $\text{Ca}^{2+}$  concentration induces apoptosis of cancer cells.

Fig. 3



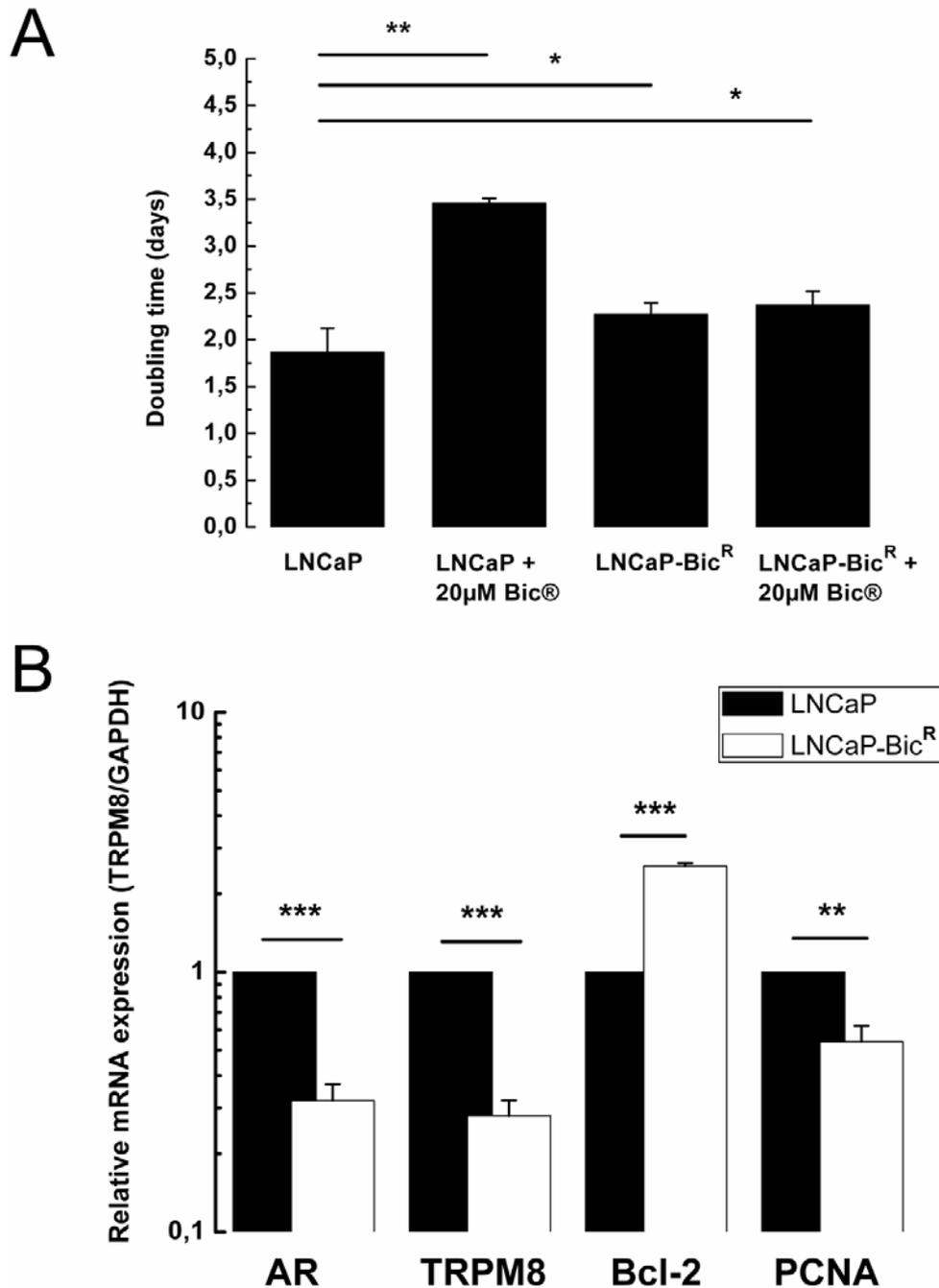
**Fig. 3. Differential role of TRP channels in agonist-stimulated calcium signaling and PCa cell proliferation.** **A:** Typical pattern of cytosolic  $\text{Ca}^{2+}$  oscillations induced in primary cultured human PCa epithelial cells by the agonist of  $\alpha 1$ -adrenergic receptors ( $\alpha 1$ -AR), phenylephrine (PHE, 10  $\mu\text{M}$ ); the importance of  $\text{Ca}^{2+}$  entry for supporting oscillatory activity and the inability of PHE-to induced  $\text{Ca}^{2+}$  release is evidenced by the lack of any signal in the absence of extracellular  $\text{Ca}^{2+}$  ( $0\text{Ca}^{2+}$ ); cells incubated in the presence of PHE (10  $\mu\text{M}$ ) for 2 days enhanced proliferation by almost 40% compared to control conditions (CTL) (**C**). **B:** The shape of cytosolic  $\text{Ca}^{2+}$  signal in primary cultured human PCa epithelial cells induced by the purinergic receptor (P2Y-R) agonist, ATP (10  $\mu\text{M}$ ); in the absence of extracellular  $\text{Ca}^{2+}$  ( $0\text{Ca}^{2+}$ ), ATP mobilizes of intracellularly stored  $\text{Ca}^{2+}$ , followed by quasi-sustained store-operated  $\text{Ca}^{2+}$  entry upon re-exposure to  $\text{Ca}^{2+}$  ( $2\text{Ca}^{2+}$ ); cell culturing in the presence of ATP (100  $\mu\text{M}$ ) for 2 days delayed proliferation by 60% (**C**). **C:** The effects of two agonists on human PCa epithelial cell proliferation. **D, E:** Antisense knockout of store-independent, DAG-gated TRP member, TRPC6, does not affect ATP-induced  $\text{Ca}^{2+}$  signals (measured as maximal  $[\text{Ca}^{2+}]_i$  increase in the presence of 2 mM  $\text{Ca}^{2+}$ ), but greatly attenuates PHE-induced ones (because of its oscillatory nature measured as an integral of  $[\text{Ca}^{2+}]_i$  over a 30 min period) (**D**), and *vice versa*, similar knockout of a store-dependent TRP member, TRPC1, significantly reduces the ATP-evoked  $\text{Ca}^{2+}$  signal, but leaving the PHE-induced one unaffected (**E**).

**Fig. 4**



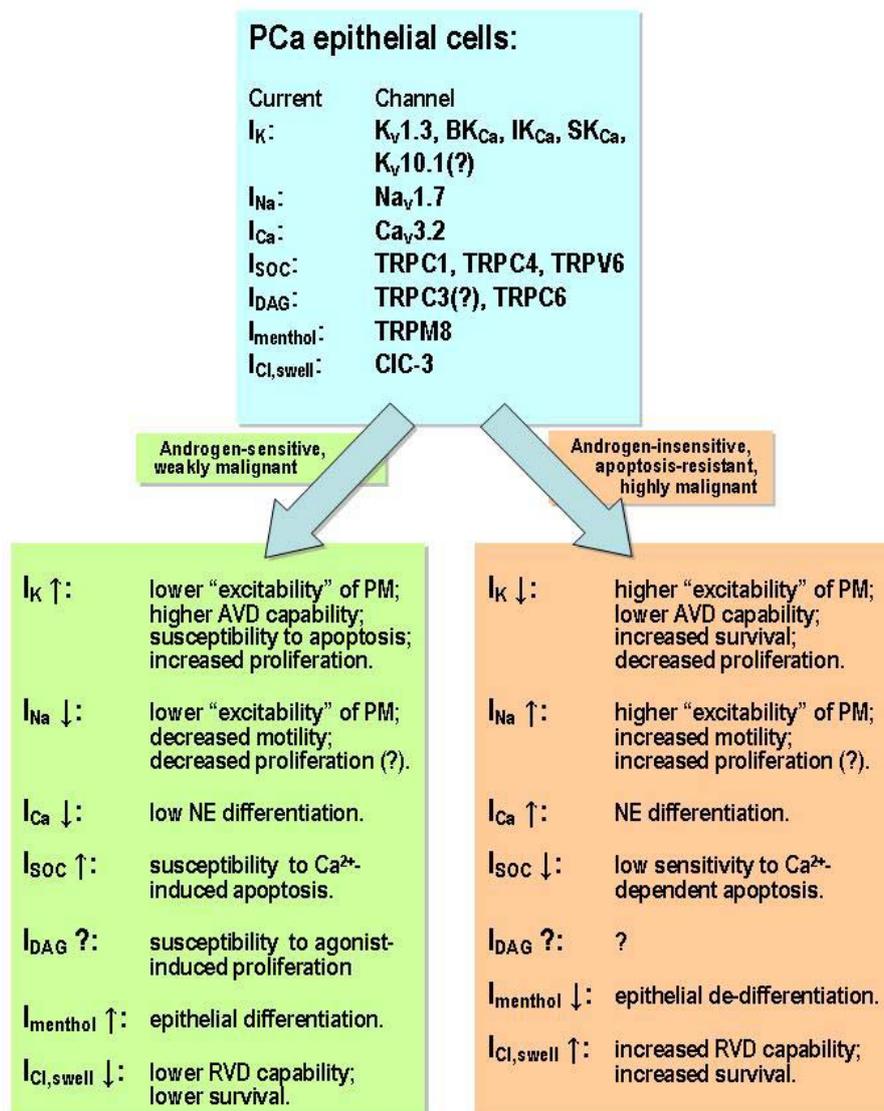
**Fig. 4. PCa-specific enhancement of cold/menthol sensitive TRPM8 channel expression.**  
**A, B:** Confocal images of human benign prostate hyperplasia (BPH, **A**) and PCa (**B**) tissue samples stained with TRPM8-specific antibody (code: ab3243, Abcam, Cambridge, UK) showing much higher TRPM8 protein expression in PCa.

Fig. 5



**Fig. 5. Transition of metastatic PCa cells to androgen-independence leads to a decreased proliferation and strong shifts in gene expression. A,** Comparison of doubling time of LNCaP cell line and of an LNCaP sub-clone resistant to bicalutamide treatment (LNCaP-bic<sup>R</sup>, obtained after a 10-month 100 µM bicalutamide selection). Addition of 20 µM bicalutamide increased the doubling time of LNCaP cells, but do not affect the proliferation of LNCaP-bic<sup>R</sup>. **B,** Normalized expression of AR, TRPM8, Bcl-2 and PCNA mRNA in both LNCaP cells and LNCaP-bic<sup>R</sup> cells figured out with PCR experiment. LNCaP-bic<sup>R</sup> cells displayed a significant decrease of AR, TRPM8 and cell cycle-associated PCNA expression, though anti-apoptotic Bcl-2 mRNA level was increased.

**Fig. 6**



**Fig. 6. Summary of membrane currents and respective ion channels in prostate cancer (PCa) epithelial cells and their involvement in metastatic behaviors of androgen-sensitive and androgen-insensitive cell phenotypes.** "↑" denotes upregulation, "↓" – downregulation, "?" – information is not available; channel designations correspond to accepted nomenclatures; all other abbreviations are presented in the abbreviations list.