Alterations in the regulatory volume decrease (RVD) and swelling-activated Cl\textsuperscript{−} current associated with neuroendocrine differentiation of prostate cancer epithelial cells

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Abstract

Neuroendocrine (NE) differentiation of prostate epithelial/basal cells is a hallmark of advanced, androgen-independent prostate cancer, for which there is no successful therapy. Here we report for the first time on alterations in regulatory volume decrease (RVD) and its key determinant, swelling-activated Cl\textsuperscript{−} current (I\textsubscript{Cl,swell}), associated with NE differentiation of androgen-dependent LNCaP prostate cancer epithelial cells. NE-differentiating regimens, namely, chronic cAMP elevation or androgen deprivation, resulted in generally augmented I\textsubscript{Cl,swell} and enhanced RVD. This occurred as a result of both the increased endogenous expression of ClC-3, which is a volume-sensitive Cl\textsuperscript{−} channel involved, as we show, in I\textsubscript{Cl,swell} in LNCaP (lymph-node carcinoma of the prostate) cells and the weaker negative I\textsubscript{Cl,swell} control from Ca\textsuperscript{2+} entering via store-dependent pathways. The changes in the RVD of NE-differentiated cells generally mimicked those reported for Bcl-2-conferred apoptotic resistance. Our results suggest that strengthening the mechanism that helps to maintain volume constancy may contribute to better survival rates of apoptosis-resistant NE cells.

Endocrine-Related Cancer (2005) 12 335–349

Introduction

Activation of the chloride current in response to cell swelling (I\textsubscript{Cl,swell}) is one of the major mechanisms by which cells tend to restore their volume after hypo-osmotic stress. At normal resting potentials, this current causes the loss of intracellular Cl\textsuperscript{−} and some other anions, thereby decreasing intracellular osmolality to counteract the water inflow that would otherwise lead to increased cell volume. This process is known as regulatory volume decrease (RVD). Extracellular osmotic perturbations are not the only reason for the alterations in the cell volume. At constant extracellular osmolarity, such processes as active solute uptake (MacLeod & Hamilton 1991), hormonal activity (Haussler \textit{et al.} 1994), proliferation (Dubois & Rouzaire-Dubois 1993, Lang \textit{et al.} 2000) and differentiation (Voets \textit{et al.} 1995, 1997) are all associated at some point with changes in the cell volume involving the activation of I\textsubscript{Cl,swell} and its role in their normal progression. Effectively, counteracting the abrupt volume changes and maintaining relative volume constancy, while undergoing all these processes, is one of the most critical prerequisites for cell survival. Indeed, there is strong evidence that disorder or altered cell volume regulation is associated...
with apoptosis (Maeno et al. 2000, Okada & Maeno 2001). Compelling support for such an association has been recently provided by the demonstration of the direct causal link between the apoptotic resistance conferred by such a key survival protein as Bcl-2 and the strengthening of RVD capability by upregulation of \( I_{\text{Cl,swell}} \) (Shen et al. 2002, Lemonnier et al. 2004b).

Malignant neuroendocrine (NE)-differentiated cells, which arise in a variety of tissue carcinomas, represent an apoptosis-resistant cell phenotype, with Bcl-2-independent antiapoptotic mechanisms (Xue et al. 1997, Xing et al. 2001, July et al. 2002). This cell phenotype is especially characteristic of the late, androgen-independent stage of prostate cancer, for which there is currently no successful therapy (e.g. Feldman & Feldman 2001). Tumour enrichment in NE-differentiated cells that not only escape apoptotic death (Fixemer et al. 2002), but also go on to release neurosecretory products with growth-promoting properties in neighbouring cells, contributes to increased malignancy and decreased responsiveness to therapeutic interventions (di Sant’Agnese 1999, 1998, Abrahamsson 1999, Ito et al. 2001). Thus, the study of all factors that underlie and/or contribute to the apoptotic resistance of NE cells is imperative for the development of new therapies for prostate cancer.

Our recent studies have identified and characterized \( I_{\text{Cl,swell}} \)-carrying volume-regulated anion channels (VRACs) in androgen-dependent LNCaP (lymph-node carcinoma of the prostate) (Horoszewicz et al. 1983) prostate cancer epithelial cells (Shuba et al. 2000), and shown their involvement in RVD (Lemonnier et al. 2002b). Given the emerging link between the apoptotic status of the cells and their homeostatic volume mechanisms, as well as the possible implication for prostate cancer, we focused, in the present work, on elucidating how NE differentiation in the model system of LNCaP prostate cancer epithelial cells might have an impact on the RVD process and its major determinant, \( I_{\text{Cl,swell}} \). Our previous data have shown, on the one hand, that NE differentiation of LNCaP cells results in a complex rearrangement of the entire \( \text{Ca}^{2+} \) homeostasis (Vanoverberghe et al. 2004) and, on the other hand, that store-operated \( \text{Ca}^{2+} \) channels (SOCs), which are one of the key determinants of \( \text{Ca}^{2+} \) homeostasis, are functionally coupled with VRACs (Lemonnier et al. 2002b). Taken together, these observations seem to suggest that such an impact may be quite strong, at least via altered \( \text{Ca}^{2+} \) signalling.

Here we show that the NE differentiation of LNCaP cells results in generally augmented \( I_{\text{Cl,swell}} \) and enhanced RVD. We go on to demonstrate in LNCaP cells that this is due to the weaker negative \( I_{\text{Cl,swell}} \) control from SOC-transported \( \text{Ca}^{2+} \) and an increase in endogenous expression of CIC-3 protein, which is one of the molecular candidates for the role of VRAC (Duan et al. 1997). Our results show that strengthening the mechanism that helps to maintain volume constancy contributes to apoptosis-resistant NE cell survival, suggesting that CIC-3 protein, which is one of the contributing factors to such a strengthening, could potentially be considered as a target for future therapeutic interventions in prostate cancer.

**Experimental procedures**

**Cell culture**

The human prostatic carcinoma cell line, LNCaP, was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in RPMI 1640 with 5% decomplemented fetal bovine serum (FBS) (Deutcher, Brumath, France), at 37 °C in a humidified atmosphere containing 5% CO₂. The medium was supplemented with 300 μg/ml t-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Tissue culture media and supplements were obtained from Life Technologies (Cergy Pontoise, France). Cells were seeded in 75 cm² flasks, and the growth medium was renewed every other day. For the experiments, the cells were subcultured in 35 mm petri dishes (Nunc) and used after 3–6 days.

To induce NE differentiation by chronic elevation of intracellular cAMP after subculturing, the medium was supplemented with 1 mM membrane-permeable cAMP analogue, dibutyryl cAMP (db-cAMP) (Sigma) (Afshari et al. 2001, Mariot et al. 2002, Monsul et al. 2004, Vanoverberghe et al. 2004). NE differentiation due to long-term androgen deprivation was achieved with a charcoal-striped culture medium (Mariot et al. 2002, Vanoverberghe et al. 2004). A tube containing charcoal 10% (w/v) and FBS was agitated for 16 h at 4 °C. After 1-h centrifugation at 10 000 g and 4 °C, the supernatant was collected and centrifuged again for 30 min at 27 000 g. The resultant supernatant was run through 0.22 μm filters. Before use, the charcoal-stripped FBS was decomplemented for 30 min at 56 °C. The charcoal-stripped culture medium was obtained with charcoal-stripped FBS.

**Electrophysiology and solutions**

Macroscopic membrane ionic currents were recorded in the whole-cell configuration of the patch-clamp technique. Patch pipettes were fabricated from borosilicate glass capillaries (WPI, Sarasota, FI, USA). The
resistance of the pipettes, filled with the basic pipette solution (in mM): K(OH) –100, KCl –40, MgCl₂ –2, CaCl₂ –3.1, HEPES –10, EGTA –8 (pH 7.3) (adjusted with glutamic acid), varied at 4–6 MΩ. Series resistance compensation was used to improve voltage-clamp performance during the recording of the whole-cell currents. Normal extracellular solution contained (in mM): NaCl –120, KCl – 5, CaCl₂ –2, MgCl₂ – 2, glucose – 5, HEPES –10 (pH 7.3) (adjusted with Na(OH)). For the recording of uncontaminated swelling-activated Cl⁻ current (ICl,swell), we eliminated all other possible currents by using TEA-based isotonic (iso, 300 mosmol/l) and hypotonic (hypo, 170 mosmol/l) extracellular solutions of the following composition (in mM): TEA-Cl – 145 (or 100), CaCl₂ – 2, MgCl₂ – 2, glucose – 10, HEPES – 10 (pH 7.3) (adjusted with TEA(OH)). Necessary supplements, including CIC-3-specific antibody and control antigen (same as used in Western blot experiments; see below), in concentrations that would not significantly change the osmolarity, were added directly to the respective solutions. All chemicals were obtained from Sigma. The external solutions were changed by means of a multibarrel puffing micropipette, with common outflow positioned in close proximity to the cell under study. During the experiment, the cell was continuously superfused with the solution via a puffing pipette to reduce possible artefacts related to the switches from static to moving solution and vice versa. Complete external solution exchange was achieved in less than 1 s.

The currents were analysed off-line with pCLAMP-8 (Axon Instruments, Foster City, CA, USA), Pulse/PulseFit (HEKA Electronic, Lambrecht/Pfalz, Germany) and Origin 6 (Microcal, Northampton, MA, USA) software. Results were expressed as mean ± S.E.M. where appropriate. Each experiment was repeated several times. Student’s t-test was used for statistical comparison among means and differences; a value of P < 0.05 was considered significant. Characteristic times of membrane current response to any intervention were determined as follows. The time intervals from the onset of the intervention until the current reached 0.05 (Amax – Abase) was considered as delay, where Abase is the baseline signal amplitude before the intervention and Amax is the maximal signal amplitude. The time interval between 0.05 (Amax – Abase) and 0.95 (Amax – Abase) was considered to be the response development period.

The estimations of the changes in cell volume during RVD processes were performed on a FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) with the use of Q Cell Quest software for data analysis (Bratosin et al. 1997). The light-scatter channels were set on linear gains. Cells in suspension in normal or Hypo-TEA solutions were gated for forward-angle scatters, and 5000 particles of each gated population were analysed.

RT-PCR

Total RNA was isolated from LNCaP cells by the guanidium thiocyanate-phenol-chloroform extraction procedure (Chomczynski & Sacchi 1987). After a DNase I (Invitrogen) treatment to eliminate genomic DNA, 2μg total RNA were reverse transcribed into cDNA at 42 °C, using random hexamer primers (Perkin Elmer, Courtaboeuf, France) and MuLV reverse transcriptase (Perkin Elmer) in a final volume of 20μl, followed by PCR, as described below. To control the amplification of genomic DNA, we also performed the PCR on the non-reverse-transcribed RNA, where the reverse transcriptase was omitted in the reverse transcription (RT) mix of each sample. The PCR primers used to amplify the RT-generated CIC-3 cDNAs were designed on the basis of established GenBank sequences. Primers were synthesized by Invitrogen. The primers for human CIC-3 cDNA were as follows: 5'-GGCAGGATACAGCTTCTCACAC-3' (nucleotides 675-696, GenBank accession no. NM_001829) and 5'-TTCCAGAGCCACAGGCA-TATGG-3' (nucleotides 1207-1188). The expected DNA length of the PCR product generated by these primers was 533 bp. For confirmation of the identity of the amplified product, restriction analysis was carried out on PCR products, using specific restriction enzymes. PCR was performed on the RT-generated cDNA with a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer). To detect CIC-3 cDNA, PCR was performed by adding 1μl RT template to a mixture of (final concentrations) 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2.5 mM MgCl₂, 200 μM of each dNTP, 1 μM sense and antisense primers and 1 U AmpliTaq Gold (Perkin Elmer) in a final volume of 25μl. Conditions of DNA amplification included an initial denaturation step of 7 min at 95 °C (which also activated the Gold variant of the Taq Polymerase) and 40 cycles of 30 s at 95 °C, 30 s at 58 °C, 40 s at 72 °C and finally 7 min at 72 °C. Half of the PCR samples were analysed by electrophoresis in a 2% agarose gel, stained with ethidium bromide (0.5 μg/ml) and viewed by Gel Doc 1000 (Bio-Rad).

Western blot

The cells were disrupted in a Kontes glass tissue grinder fitted with a tight pestle. After centrifugation (10000 g), the pellets were fractionated in a standard

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to induce NE differentiation of LNCaP cells: [cAMP]_{in}(NSE). In the present study, we used two regimens and an increased expression of neuron-specific enolase acquire NE characteristics. These include the cessation et al. 2001, Mariot et al. 1999, Deeble et al. 2000). In terms of basic morphological NE characteristics, the two NE-differentiated cell types did not differ from each other and were similar to those described in our previous study (Mariot et al. 2002). Figure 1 presents examples of the typical morphology of LNCaP cells cultured under control conditions and in the presence of 1 mM db-cAMP. Experiments were performed on LNCaP cells subjected to NE differentiation by both regimens for 72–120 h. Experimental results were not dependent on the means of differentiation induction, although we do specify the regimen used in each figure as either−Andr or + db-cAMP for androgen deprivation or db-cAMP treatment respectively. The functional results obtained on NE-differentiated LNCaP cells (NE-LNCaP) were compared with standard androgen-dependent LNCaP cells, which served as a control (ctrl-LNCaP).

NE-differentiated LNCaP cells show upregulated $I_{\text{Cl,swell}}$

In our previous work, we showed that the whole-cell, patch-clamped ctrl-LNCaP cells respond to the decrease in the tonicity of extracellular solution by developing membrane Cl$^-$ current, the magnitude of which correlated with the extent of hypotonicity-induced cell swelling ($I_{\text{Cl,swell}}$, Shuba et al. 2000). We also proved that this current results in the loss of Cl$^-$ at normal resting potential and therefore participates in the RVD process of ctrl-LNCaP cells subjected to hypo-osmotic conditions (Lemonnier et al. 2002b). Exposure to hypotonic solution also caused the activation of a similar current in NE-differentiated LNCaP cells. However, two major differences were immediately noted. First, the current in NE-LNCaP cells had consistently higher amplitude; second, it developed somewhat faster than ctrl-LNCaP cells. The most obvious difference is clearly illustrated by Fig. 2A. This shows representative recordings of the baseline and hypotonicity-activated currents in regular and NE-differentiated LNCaP cells in response to pulse protocols, including voltage-ramp (Fig. 2A, inset). To account for possible differences in cell size, the currents were normalized to cell membrane capacitance and scaled as current densities. Although the baseline currents under isotonic conditions were nearly identical in two cell types, transition to hypotonic solution caused the development of a current almost twice as large in NE-LNCaP cell than that in the ctrl-LNCaP.

Results

When subjected to pharmacological stimulation increasing intracellular cAMP ([cAMP]$_{in}$) levels, or under physiological stimulation using interleukins or long-term androgen deprivation (Bang et al. 1994, Burchardt et al. 1999, Deebel et al. 2001, Zelivianski et al. 2001, Mariot et al. 2002), LNCaP cells rapidly acquire NE characteristics. These include the cessation of mitotic activity, the development of neuritic processes and an increased expression of neuron-specific enolase (NSE). In the present study, we used two regimens to induce NE differentiation of LNCaP cells: [cAMP]$_{in}$ elevation by the use of the membrane-permeable cAMP analogue, dibutyril-cAMP (db-cAMP), and long-term androgen deprivation by means of charcoal-stripped culture medium. The androgen deprivation was indeed previously shown to increase the intracellular cAMP concentration in LNCaP cells (Burchardt et al. 1999). SDS–PAGE gel (15%). After electrophoresis, the proteins were transferred onto a nitrocellulose membrane with a semidyry electrobetterter (BioRad). Once the transfer was completed, the membrane was cut into thin strips that were further processed for Western blot. The strips were blocked for 1 h at room temperature in TNT (15 mM Tris buffer (pH 8), 140 mM NaCl, 0.05% Tween 20 and 5% non-fat dry milk), washed in TNT (three times) and then soaked in primary antibodies (4 μg/ml in TNT: CIC-3 antibody ACL-001 from Alomone Labs, Jerusalem, Israel; 10 μg/ml in TNT: Bcl-2 antibody SC-509 from Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. After thorough washes in TNT, the strips were transferred into the corresponding horse-radish-peroxidase-linked secondary antibodies (Zymed Laboratories, San Francisco, CA, USA) and then diluted in TNT (1/7500) for 1 h. After several washes in TNT without milk, the strips were processed for chemiluminescent detection, using Supersignal West Pico chemiluminescent substrate (Pierce Chemical Company, Rockford, IL, USA) according to the manufacturer’s instructions. The blot was then exposed to X-Omat AR films (Eastman Kodak), and quantification was performed on Quantity-One software (Bio-Rad). Briefly, the intensity of the signal was evaluated by densitometry and was semiquantified, using the relationship between the value of the protein of interest divided by the calnexin value for each experiment. Each experiment presented was repeated at least twice.

Determination of apoptosis

The level of apoptosis was estimated from the number of apoptotic bodies visualized by Hoechst staining, as detailed previously (Skryma et al. 2000).

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Figure 1  Morphological changes of LNCaP prostate cancer cells during dibutyryl cAMP (db-cAMP)-induced neuroendocrine (NE) differentiation. (A) Control LNCaP cells cultured under standard conditions for 2 days (upper panel) and 5 days (lower panel) after passage. (B) Development of NE morphological features in LNCaP cells from the same passage, but cultured for 2 days (upper panel) and 5 days (lower panel) in 1 mM db-cAMP-supplemented medium. 100 μm calibration on all images.

Figure 2  NE differentiation of LNCaP cells augments swelling-activated Cl\(^{-}\) current (I\(_{\text{Cl,swell}}\)). (A) Representative traces of membrane currents under isotonic (iso) and hypotonic (hypo) conditions in the control (ctrl-LNCaP, left panel) and NE-differentiated (NE-LNCaP, 5-day db-cAMP treatment, right panel) LNCaP cells; the voltage ramp-containing pulse protocol used to elicit currents is shown above the control recordings; currents were related to cell membrane capacitance and scaled as current densities (pA/pF). (B) Average i-V relationships of I\(_{\text{Cl,swell}}\) (mean ± S.E.M.) in the control (ctrl-LNCaP, squares, n = 11) and NE-differentiated (NE-LNCaP, circles, n = 10) LNCaP cells exposed to hypotonic solution; the inset shows the same i-Vs after normalization to demonstrate similarity in rectification.
The average current-voltage relationship (I-V) of swelling-activated currents acquired in several ctrl-LNCaP and NE-LNCaP cells, despite reflecting the differences in current amplitudes, showed the same pattern of moderate outward rectification and a reversal potential close to theoretical Cl\(^{-}\) equilibrium (Fig. 2B), typical of the I\(_{\text{Cl,swell}}\) described for LNCaP cells in our previous works (Shuba et al. 2000, Lemonnier et al. 2002b, Vitko et al. 2002). The identical shapes of I-Vs suggested that NE differentiation neither induces the appearance of new types of volume-sensitive channels (i.e. cationic ones) in addition to the original I\(_{\text{Cl,swell}}\)-carrying VRACs nor affects the original VRACs’ permeation properties. However, at +100 mV, the I\(_{\text{Cl,swell}}\) density was twice as high in NE-LNCaP cells (i.e. 88\(\pm\)7 pA/pF, \(n=10\)) as in ctrl-LNCaP ones (i.e. 42\(\pm\)2 pA/pF, \(n=10\)). This ratio between the currents stayed relatively constant over the whole range of membrane potentials tested (i.e. \(\pm100\) mV) (Fig. 2B, inset).

Pharmacological agents that in our previous studies have been shown to exert inhibitory action on I\(_{\text{Cl,swell}}\) in LNCaP cells, such as NPPB, DIDS (Shuba et al. 2000), BAPTA (Lemonnier et al. 2002a) and 2-APB (Lemonnier et al. 2004a), produced the same amount of inhibition of swelling-activated current in both ctrl-LNCaP and NE-LNCaP cells (data not shown). This therefore provides additional confirmation of the common origin of this current and its identification as I\(_{\text{Cl,swell}}\).

In addition to the enhanced density, I\(_{\text{Cl,swell}}\) in NE-differentiated cells was characterized by faster activation in response to the reduction of extracellular tonicity. This is illustrated by the normalized time-courses of I\(_{\text{Cl,swell}}\) activation, presented in Fig. 3A, as well as by the quantification of I\(_{\text{Cl,swell}}\) delay and development periods. As presented in Fig. 3B, the delay before I\(_{\text{Cl,swell}}\) onset was 15\(\pm\)1.7 s, followed by the development period of 90\(\pm\)5.6 s in NE-LNCaP cells, as compared with 24\(\pm\)2.4 s and 110\(\pm\)7.5 s respectively in ctrl-LNCaP cells.

We also examined whether NE differentiation had an impact on I\(_{\text{Cl,swell}}\) voltage-dependent inactivation, which is a common feature of this type of current in many cell models (e.g. Nilius et al. 1997, Shuba et al. 2000). Fig. 4A shows that I\(_{\text{Cl,swell}}\) evoked by rectangular voltage pulses, indeed inactivates at high positive potentials in both ctrl-LNCaP and NE-LNCaP cells. However, the exponential fit of the current’s inactivation phases (\(\tau_{\text{in}}=197\pm31\) ms in ctrl-LNCaP vs \(\tau_{\text{in}}=234\pm38\) ms in NE-LNCaP cells) appeared to be statistically insignificant (Fig. 4B).

Strongly enhanced I\(_{\text{Cl,swell}}\) amplitude and its faster activation in response to swelling in NE-differentiated cells should theoretically result in a more efficient counteraction to hypotonically induced volume
increase. Figure 5 shows that, as expected, the maximal volume of NE-LNCaP cells increased by only 30% upon hypotonic exposure compared with 50% for ctrl-LNCaP cells, thus suggesting that NE differentiation promotes more effective RVD via I_{Cl,swell} upregulation.

**Molecular basis for I_{Cl,swell} enhancement in NE-differentiated LNCaP cells**

Unfortunately, the molecular origin of endogenous I_{Cl,swell}-carrying VRAC is not known. Of many candidates (for the most recent critical reviews, see Okada 1998, Eggermont et al. 2001, Furst et al. 2002), only CIC-3, a volume-sensitive member of the CIC family of Cl channels, is still considered as being potentially involved in endogenous VRAC, for at least some cell types (Duan et al. 1997, 2001, Wang et al. 2000, 2003, Hermoso et al. 2002). Thus, in trying to find possible reasons for I_{Cl,swell} enhancement in NE-differentiated LNCaP cells, we first wanted to verify the hypothesis of CIC-3 involvement. In doing so, our strategy was firstly to determine whether CIC-3 is expressed in ctrl-LNCaP cells and whether or not its expression is affected by NE differentiation. Secondly, we wished to discover whether CIC-3 impairment had any effect on I_{Cl,swell}.

As documented in Fig. 6, CIC-3 transcript (Fig. 6A) and protein (Fig. 6B) are notably expressed in ctrl-LNCaP cells, and the expression of both considerably augments the following NE differentiation. Parallel
mechanisms underlying the higher apoptotic resistance of NE cells. Such a pattern of CIC-3 expression prompted us to conclude that: 1. CIC-3 may play the role of I_{Cl,swell} in LNCaP cells; 2. the enhancement of I_{Cl,swell} in NE-differentiated LNCaP cells may be at least partially due to increased CIC-3 levels.

To obtain more direct evidence on CIC-3 involvement in I_{Cl,swell}, we used the same anti-CIC-3-specific antibody as employed in Western blot experiments, in the attempt to influence I_{Cl,swell} properties. This antibody has been previously shown to effectively inhibit the function of both heterologously expressed CIC-3 (Duan et al. 2001) and endogenous VRACs in a number of cell types (Duan et al. 2001, Jin et al. 2003, Lemonnier et al. 2004). This antibody was introduced into the cell via a patch pipette during whole-cell, patch-clamp recording, as previously described (Lemonnier et al. 2004). To make sure that the antibody concentration inside the cell balanced the one in the pipette and that the antibody actually interacted with endogenous ClC-3, we dialysed the cell for at least 10 min before administering I_{Cl,swell}-activating hypotonic solution. As a control in this series of experiments, we used cell dialysis with the pipette solution containing inactivated anti-CIC-3, by means of a control antigen (premixed 1 h before the experiment, to ensure complete antibody inactivation). Figure 6C

Figure 5 NE-differentiated LNCaP cells show smaller relative volume increase in response to hypotonic exposure. The bar graph represents relative volume increase in control (ctrl-LNCaP, dark grey bars) and NE-differentiated (NE-LNCaP, light grey bars) cells at different times after application of hypotonic solution (time 0); the height of each bar corresponds to the average percentage of cell volume change at a given time ± S.E.M., measured on a flow cytometer for at least 5000 cells during two distinct experiments.

quantification of the expression of common anti-apoptotic Bcl-2 protein showed no difference (Fig. 6B), consistent with the notion of Bcl-2-independent mechanisms underlying the higher apoptotic resistance of NE cells. Such a pattern of CIC-3 expression prompted us to conclude that: 1. CIC-3 may play the role of I_{Cl,swell} in LNCaP cells; 2. the enhancement of I_{Cl,swell} in NE-differentiated LNCaP cells may be at least partially due to increased CIC-3 levels.

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Figure 6 NE differentiation enhances CIC-3 expression. (A) RT-PCR analysis of the expression of human CIC-3 transcript in control LNCaP cells (ctrl-LNCaP) and its enhancement after NE differentiation (NE-LNCaP) induced by androgen deprivation (−Andr) or db-cAMP treatment (+ db-cAMP). (B) Semiquantitative Western blots with CIC-3- and Bcl-2-specific antibodies showing 10-fold enhancement of CIC-3 protein in NE-LNCaP, compared with ctrl-LNCaP cells and the relative constancy of Bcl-2 protein; the numbers were determined in relation to calnexin expression at each condition, which was used as a control. This experiment was repeated three times. (C) Average time courses of I_{Cl,swell} (mean ± S.E.M.) in response to hypotonic exposure showing diminished I_{Cl,swell} in the NE-LNCaP cells dialysed with CIC-3-specific antibody (0.04 μg/ml, anti-CIC-3, grey squares, n=6) compared with control (cells dialysed with inactivated CIC-3-specific antibody, by means of 0.08 μg/ml antigen, circles, n=8). I_{Cl,swell} amplitudes were measured at +50/−30 mV and normalized to membrane capacitance.
TG in the presence of 5 mM extracellular Ca\(^{2+}\) alters basal store-operated Ca\(^{2+}\) influx (Figure 7). The activation phase and immediate pre-TG value during TG application. (B) Representative traces of I\(_{\text{Cl, swell}}\) (pulse protocol is shown above the traces) recorded in ctrl-LNCaP (left panel) and NE-LNCaP (right panel). (C) Quantification of I\(_{\text{Cl,swell}}\) amplitudes (\(V_t = +100 \text{ mV}\)) at specified recording conditions (relative to the amplitude at \([\text{Ca}^{2+}]_{\text{out}} = 2 \text{ mM}\), \(V_h = -80 \text{ mV}\)). Asterisk denotes statistically significant difference, \(P < 0.05\); see text for details.

Figure 7B shows that the dialysis of NE-LNCaP cells with active anti-ClC-3 greatly impaired I\(_{\text{Cl,swell}}\) activation in response to hypotonic exposure, whereas dialysis with inactivated antibody did not.

Altogether, these results prompted us to conclude that CIC-3 is definitely involved in I\(_{\text{Cl,swell}}\) in LNCaP cells, and that I\(_{\text{Cl,swell}}\) enhancement following NE differentiation can at least be partially explained by the increase in endogenous ClC-3 levels.

**Ca\(^{2+}\)-dependent regulation of I\(_{\text{Cl,swell}}\) in NE-differentiated LNCaP cells**

An additional factor that may influence the magnitude of I\(_{\text{Cl,swell}}\) is altered Ca\(^{2+}\)-dependent VRAC regulation. Indeed, our two recent studies have shown that: 1. I\(_{\text{Cl,swell}}\)-carrying VRACs in LNCaP cells are effectively inhibited by Ca\(^{2+}\) entering the cell via store-operated Ca\(^{2+}\) channels (SOCs), an effect which implies spatial co-localization of two channel types in the plasma membrane (Lemonnier et al. 2002b); 2. NE differentiation of LNCaP cells is accompanied by downregulation of store-operated current (I\(_{\text{SOC}}\)), most probably due to a reduction in the number of active SOCs (Vanoverberghe et al. 2004). Taken together, these results suggest that NE differentiation may also alter the mode of Ca\(^{2+}\)-dependent regulation of I\(_{\text{Cl,swell}}\). To verify this, we used the same experimental approach as in the original study (Lemonnier et al. 2002b), namely, exposure of the cell generating I\(_{\text{Cl,swell}}\) to SERCA pump inhibitor thapsigargin (TG), which facilitates the depletion of the intracellular Ca\(^{2+}\) store and thereby induces Ca\(^{2+}\) entry from extracellular space via activated SOCs.

Figure 7A shows that, in agreement with the previously postulated mechanism of Ca\(^{2+}\)-dependent regulation (Lemonnier et al. 2002b), exposure of ctrl-LNCaP to 0.1 \(\mu\)M TG in the presence of 5 mM \([\text{Ca}^{2+}]_{\text{out}}\) caused close to 50% (51.6 ± 3.2; \(n = 11\)) I\(_{\text{Cl,swell}}\) inhibition. In contrast, similar experiments on NE-LNCaP cells provided almost no current inhibition (2.7 ± 2.1%; \(n = 11\)). The virtual elimination of TG effects on I\(_{\text{Cl,swell}}\) in NE-LNCaP cells is consistent with our previous finding that NE differentiation down-regulates store-operated Ca\(^{2+}\) influx (Vanoverberghe et al. 2004).

Thus, it is quite plausible that augmented I\(_{\text{Cl,swell}}\) in NE-differentiated LNCaP cells may result not only...
from upregulated endogenous ClC-3, but also from weaker tonic Ca\(^{2+}\)-dependent VRAC inhibition due to generally smaller background store-operated Ca\(^{2+}\) influx under basal, non-stimulated conditions compared with ctrl-LNCaP cells. Figure 7B shows that, consistent with such a possibility, the changes of I\(_{\text{Cl,swell}}\) in response to manipulations that alter the magnitude of store-operated Ca\(^{2+}\) influx are much more pronounced in ctrl-LNCaP cells than NE-LNCaP cells. These manipulations were as follows: 1. shift of holding potential (V\(_{h}\)) from –50 to –80 mV (at normal [Ca\(^{2+}\)]\(_{\text{out}}\) = 2 mM), which brings about the increase of I\(_{\text{SOC}}\) due to increase of driving force for Ca\(^{2+}\); 2. removal of extracellular Ca\(^{2+}\) (at V\(_{h}\) = –80 mV), which abolishes I\(_{\text{SOC}}\). The virtual insensitivity of I\(_{\text{Cl,swell}}\) to these interventions in NE-LNCaP cells suggests much weaker VRAC negative control from SOC-transported Ca\(^{2+}\) under basal conditions, which thus may be considered as a contributing factor to I\(_{\text{Cl,swell}}\) enhancement in these cells.

**Upregulated I\(_{\text{Cl,swell}}\) contributes to the enhanced apoptotic resistance of NE-differentiated LNCaP cells**

In order to verify that I\(_{\text{Cl,swell}}\) is indeed involved in regulation of the apoptotic potential of LNCaP cells and that its augmentation during NE differentiation can give the cells some protection against programmed death, we conducted a series of experiments in which we measured tumour necrosis factor (TNF)\(\alpha\)-induced apoptosis of ctrl-LNCaP and NE-LNCaP cells in the absence and in the presence of the I\(_{\text{Cl,swell}}\) blocker, NPPB. Figure 8 shows that exposure to TNF\(\alpha\) (10 ng/ml) for 48 h resulted in more than twofold higher percentage of ctrl-LNCaP cell apoptosis (11.75 ± 0.23%; n = 4) than with NE-LNCaP cells (4.7 ± 0.79%; n = 4), a result which well agrees with documented enhancement of the survival after NE differentiation (Fixemer et al. 2002). Addition of the I\(_{\text{Cl,swell}}\) blocker NPPB to the culture medium strongly promoted (in a concentration-dependent manner) TNF\(\alpha\)-induced apoptosis of both cell types, suggesting that functional I\(_{\text{Cl,swell}}\) even under normotonic conditions is important for sustaining generally lower susceptibility to apoptotic stimuli. However, if in the population of ctrl-LNCaP cells the percentage of apoptosis (induced by 100 \(\mu\)M NPPB) increased by a factor of 4 (to 62.2 ± 3.84%; n = 4), then in NE-LNCaP cells it increased by a factor of 10 (to 51.1 ± 2.04%; n = 4), indicating higher sensitivity of NE-LNCaP cell apoptosis to I\(_{\text{Cl,swell}}\) blockade. Such a difference may be explained by a more prominent role of I\(_{\text{Cl,swell}}\) in the protection against apoptosis of particularly NE-differentiated cells, an effect probably due to the augmented amplitude of this current.

**Discussion**

In the present paper, we report on three major findings: 1. NE differentiation of androgen-dependent prostate cancer epithelial cells results in the increase of I\(_{\text{Cl,swell}}\)-enhanced RVD capability and the upregulation of endogenous expression of ClC-3 protein; 2. ClC-3 protein participates in the generation of I\(_{\text{Cl,swell}}\) in LNCaP prostate cancer epithelial cells; 3. NE differentiation weakens SOC-mediated, Ca\(^{2+}\)-dependent inhibition of VRACs underlying I\(_{\text{Cl,swell}}\). All three findings are of utmost physiological importance, as they establish new mechanisms in cell volume regulation associated with the acquisition of androgen-independence and apoptotic resistance during the NE differentiation of prostate epithelial cells.

**NE differentiation and membrane ion channels**

NE differentiation, which is characterized by a cessation of proliferative activity and a formation of neuronal-like morphological features, is also known to induce changes in membrane conductances towards the phenotype characteristic of excitability. For instance, NE cells, which in limited and controlled proportions are normal components of the prostate, contributing to the regulation of its growth and secretory activity in the endocrine/paracrine fashion, have been shown to express various types of voltage-gated Ca\(^{2+}\), Na\(^{+}\) and...
K⁺ channels as well as fire action potentials upon stimulation (Kim et al. 2003). One of the hallmark features of malignant NE differentiation is enhanced expression of different types of high-voltage-activated Ca²⁺ channels (e.g. Mergler 2003), which, in addition to their role in excitability, may also take part in supporting the hypersecretory activity of mitogenic factors as well as in aggressive neuronal outgrowth.

Our recent study has shown that LNCaP cells induced to differentiate into NE phenotype also develop a robust low-voltage-activated Ca²⁺ conductance supported by the expression of T-type Ca²⁺-channel α₁H subunits (Mariot et al. 2002). By providing for basal calcium entry at resting membrane potential, this conductance plays a role in the facilitation of neurite lengthening. Overexpression of α₁H was only a part of a complex modification of the whole calcium homeostasis in NE-differentiated LNCaP cells, which we also showed to feature a reduced filling of the endoplasmic reticulum (ER) Ca²⁺ store, a decreased expression of endolamelar SERCA 2b Ca²⁺ ATPase and luminal Ca²⁺ binding/storage chaperone calreticulin, and a substantial downregulation of store-operated Ca²⁺ current (I_{SOC}) (Vanoverberghe et al. 2004). We suggested that such modifications to calcium homeostasis were part of the mechanism underlying the enhanced apoptotic resistance of androgen-independent, NE-differentiated prostate cancer cells, since exactly the same modifications were also associated with the apoptotic resistance conferred by an overexpression of anti-apoptotic Bcl-2 protein (Vanden Abeele et al. 2002).

NE differentiation and volume homeostasis

As far as volume homeostasis is concerned, our present study is the only one to describe alterations during one of its key processes, RVD, and to uncover underlying mechanisms during NE differentiation. We have found that NE differentiation of LNCaP prostate cancer epithelial cells considerably enhances I_{Cl,swell} and the RVD associated with it. Moreover, we identify CIC-3 protein, which is a volume-dependent member of the CIC family of CT channels, as a likely molecular substrate underlying I_{Cl,swell} enhancement. Our experiments showed that not only is CIC-3 notably expressed on protein level in LNCaP cells, but also that it has a functional implication for I_{Cl,swell} as intracellularly applied CIC-3-specific antibody reduces by twofold the I_{Cl,swell} amplitude in response to hypotonicity. These results prompted us to conclude that CIC-3 may be a part of endogenous VRAC per se or, given the evidence on possible intracellular CIC-3 location (e.g. Li et al. 2002, Gentzsch et al. 2003), a part of the molecular machinery involved in VRAC activation/regulation in LNCaP cells. Moreover, the fact that the elevation of endogenous levels of CIC-3 protein in response to NE-differentiating regimens was paralleled by the enhancement of I_{Cl,swell} not only provided an additional strong argument for a causal link between CIC-3 expression and VRAC function, but also suggested that NE differentiation modulates I_{Cl,swell} by affecting CIC-3 content.

Another mechanism by which NE differentiation may contribute to the upregulation of I_{Cl,swell} is by weakening its Ca²⁺-dependent inhibition. Indeed, we showed in our recent study, NE differentiation of LNCaP cells is associated with the decrease in I_{SOC}, which is likely to be due to the diminished number of functional SOCs, occurring as an adaptive response to the long-term reduction in the ER Ca²⁺ content (Vanoverberghe et al. 2004). Consistent with this and with the inhibitory action of SOC-transported Ca²⁺ on VRACs recently demonstrated by us (Lemonnier et al. 2002b), we find here that TG-conferred Ca²⁺-dependent inhibition of I_{Cl,swell} in NE-LNCaP cells is considerably weaker than in the ctrl-LNCaP cells. As SOCs may have some background activity, this may set some basal level of VRAC inhibition, which, because of the smaller number of SOCs in NE differentiated cells, would result in generally augmented I_{Cl,swell}.

The fact that I_{Cl,swell} in NE-LNCaP cells is activated somewhat faster in response to lowered tonicity than in the ctrl-LNCaP cells suggests that VRACs may be subject to the additional regulatory influences after NE differentiation. As, on the one hand, differentiation is accompanied by substantial cytoskeletal rearrangements (Peters et al. 1995, Chauhan et al. 2004) and, on the other hand, actin-based cytoskeleton has been implicated in VRAC function (Levitan et al. 1995, Shen et al. 1999), one might speculate that the observed changes in volume sensitivity and voltage-dependent inactivation can be explained by cytoskeletal factors.

NE differentiation, RVD and apoptotic resistance: what is the link?

Given that malignant NE differentiation is associated with enhanced apoptotic resistance (Fixemer et al. 2002), it is tempting to speculate on how upregulated I_{Cl,swell} and strengthened RVD may translate into a higher resistance of NE-differentiated prostate cancer epithelial cells to apoptosis.

The two hallmark features of apoptotic cell death, in which membrane ion channels are most likely to play a part, are cell shrinkage (Lang et al. 2000) and decay of
the resting membrane potential (e.g. Dallaporta et al. 1999). Cl channels in general and volume-regulated ones in particular have already been implicated both in the modulation of resting membrane potential in some cell types (Jentsch et al. 2002) and in normotonic apoptotic volume decrease (AVD) (Lang et al. 2000, Maeno et al. 2000, Okada & Maeno 2001, Okada et al. 2001). Moreover, there is strong evidence that over-expression of a common anti-apoptotic Bcl-2 protein and, apparently, the apoptotic resistance associated with it directly affect I\text{Cl,swell} and RVD (Shen et al. 2001). Interestingly, the features of I\text{Cl,swell} and RVD reported for Bcl-2-conferred apoptotic resistance seem to be exactly the same as those detailed herein for NE differentiation, namely, augmented I\text{Cl,swell} and enhanced RVD capability. Since the NE phenotype represents qualitatively different anti-apoptotic mechanisms from Bcl-2-mediated ones (Xue et al. 1997, Xing et al. 2001, July et al. 2002), these results, taken together, suggest that strengthening RVD may be a common prerequisite for better survival.

A strengthening of I\text{Cl,swell}-mediated RVD during the acquisition of apoptotic resistance would be evidence against the RVD and AVD coupling hypothesis, at least in terms of VRAC involvement (Maeno et al. 2000). Indeed, since both RVD and AVD are associated with the loss of solutes, a strengthening of RVD mechanisms during the acquisition of apoptotic resistance would result in strengthening of AVD, too, thereby promoting apoptosis instead of preventing it. The coupling hypothesis relies solely on the fact that VRAC inhibitors are able to prevent apoptotic events (Maeno et al. 2000). However, these inhibitors have been shown to reduce K\textsuperscript{+} efflux and the AVD associated with it (Trimarchi et al. 2002), suggesting the likelihood of non-specific actions of these agents.

Our own data show that I\text{Cl,swell} blockers do not prevent, but rather promote, apoptosis, suggesting that this current is an important determinant in improving cell survival, and that the survival potential is proportional to I\text{Cl,swell} magnitude. The fact that the I\text{Cl,swell} blocker, NPPB, influences apoptosis under normotonic conditions suggests some background I\text{Cl,swell} activation, which, if indeed present, theoretically should lead to cell shrinkage and eventually assist apoptosis. The fact that this does not happen favours the idea that RVD and AVD are supported by distinct mechanisms and also that the enhanced RVD capability of apoptosis-resistant cell phenotypes is probably just a reflection of their better ability to maintain relative volume constancy by counteracting negative influences of different origins, thereby promoting cell survival. One cannot also exclude that VRAC may be somehow coupled to apoptosis machinery independent of its ability to carry I\text{Cl,swell}. In this case enhanced I\text{Cl,swell} and RVD may be just a reflection of the increased levels of putative VRAC protein(s) during acquisition of apoptosis resistance.

**Potential implications for androgen-independent prostate cancer**

Apoptosis is essential in maintaining tissue homeostasis. The acquisition of resistance to apoptosis plays a pivotal role in tumour genesis by disrupting the balance between cell proliferation and cell destruction, and by allowing cancer cells to escape radiation and chemotherapy. Androgen-independent prostate cancer is characterized by tumour enrichment in apoptosis-resistant cell phenotypes. These cell types, although differing in specific anti-apoptotic mechanisms, seem nevertheless to share the same basic changes in volume homeostasis. Consequently, it would seem that targeting the key players involved in maintenance of volume homeostasis, in the attempt to enhance the pro-apoptotic potential of malignant cells, may prove to be a useful strategy in the treatment of advanced prostate cancer. In the present work, we have identified a molecular entity, namely, CIC-3 protein, the expression of which increases during NE differentiation of prostate cancer epithelial cells. This consequently helps them to better maintain volume constancy and therefore enhances their anti-apoptotic potential. Thus, CIC-3 may be considered as a potential target for influencing the apoptotic status of NE cells in advanced, androgen-independent prostate cancer.

**Acknowledgements**

This work was supported by grants from INSERM, La Ligue Nationale Contre le Cancer, l’ARC (France) and INTAS-99-01248.

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