

# Store-operated $\text{Ca}^{2+}$ Current in Prostate Cancer Epithelial Cells

ROLE OF ENDOGENOUS  $\text{Ca}^{2+}$  TRANSPORTER TYPE 1\*

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**$\text{Ca}^{2+}$  influx via store-operated channels (SOCs) following stimulation of the plasma membrane receptors is the key event controlling numerous processes in nonexcitable cells. The human transient receptor potential vanilloid type 6 channel, originally termed  $\text{Ca}^{2+}$  transporter type 1 (CaT1) protein, is one of the promising candidates for the role of endogenous SOC, although investigations of its functions have generated considerable controversy. In order to assess the role of CaT1 in generating endogenous store-operated  $\text{Ca}^{2+}$  current ( $I_{\text{SOC}}$ ) in the lymph node carcinoma of the prostate (LNCaP) human prostate cancer epithelial cell line, we manipulated its endogenous levels by means of antisense hybrid depletion or pharmacological up-regulation (antiandrogen treatment) combined with functional evaluation of  $I_{\text{SOC}}$ . Antisense hybrid depletion of CaT1 decreased  $I_{\text{SOC}}$  in LNCaP cells by ~50%, whereas enhancement of CaT1 levels by 60% in response to Casodex treatment potentiated  $I_{\text{SOC}}$  by 30%. The functional characteristics of  $I_{\text{SOC}}$  in LNCaP cells were similar in many respects to those reported for heterologously expressed CaT1, although 2-aminoethoxydiphenyl borate sensitivity and lack of constitutive current highlighted notable departures. Our results suggest that CaT1 is definitely involved in  $I_{\text{SOC}}$ , but it may constitute only a part of the endogenous SOC, which in general may be a heteromultimeric channel composed of homologous CaT1 and other transient receptor potential subunits.**

In a variety of nonexcitable cells,  $\text{Ca}^{2+}$  influx is signaled by the depletion of intracellular  $\text{Ca}^{2+}$  stores, a process initially termed “capacitative calcium entry” or, later, “store-operated calcium entry” (1). This  $\text{Ca}^{2+}$  influx is mediated via plasma membrane store-operated  $\text{Ca}^{2+}$ -permeable channels (SOCs).<sup>1</sup>

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<sup>1</sup> The abbreviations used are: SOC, store-operated channel; ER, endoplasmic reticulum; CRAC, calcium release-activated calcium channel; SOCE, store-operated calcium entry; LNCaP, lymph node carcinoma of the prostate; TRP, transient receptor potential; TRP-L, TRP-like; CaT,  $\text{Ca}^{2+}$  transporter type 1; ECaC, epithelial calcium channel; ODNs, oligodeoxynucleotides; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; TG, thapsigargin; IP<sub>3</sub>, inositol 1,4,5-

Besides playing a major role in replenishing internal  $\text{Ca}^{2+}$  stores in the endoplasmic reticulum (ER) and maintaining ER filling status, these channels are also involved in regulating of numerous important physiological processes, ranging from cell growth and proliferation to apoptosis and cell death.

The best studied SOC in terms of function and biophysical properties are  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  channels (CRACs), which were originally described in T-lymphocytes and mast cells (2, 3). This specific type of SOC is highly selective for  $\text{Ca}^{2+}$  under physiological conditions and has a tiny single-channel conductance, estimated by fluctuation analysis at 24 femto Siemens in 100 mM extracellular  $\text{Ca}^{2+}$  (4).

However, recent electrophysiological studies have clearly established the existence of a number of subtypes of endogenous store-operated  $\text{Ca}^{2+}$  currents in various cells, differentiated by their selectivity, unitary conductance, and pharmacology (for a review, see Ref. 5). Despite considerable efforts in SOC studies, the mechanisms linking store depletion to SOC activation are still unknown, and several fundamentally different hypotheses have been proposed (reviewed in Refs. 6–9): direct coupling of SOC to store proteins (10, 11), diffusible messengers (12), or exocytotic insertion of SOC preformed in vesicles (13). This variety in the biophysical properties and activation mechanisms of SOC reflects the continuing lack of precise knowledge about the molecular identity of these channels.

The widely investigated family of mammalian homologues of the *Drosophila* transient receptor potential (TRP) and TRP-like (TRP-L) channels is considered the most likely source of candidate proteins for the role of SOC (reviewed in Refs. 5 and 14–16). It was recently reported that the epithelial  $\text{Ca}^{2+}$  channel (ECaC)  $\text{Ca}^{2+}$  transporter type 1 (CaT1), a member of the vanilloid subfamily of TRP cationic channels, transient receptor potential vanilloid type 6 (TRPV6) according to the latest nomenclature (16), exhibited the unique biophysical properties of CRACs, which led to the conclusion that CaT1 comprised all or part of the endogenous CRAC pore (17). However, since then, although new reports (18–21) have confirmed the general resemblance between CaT1- and CRAC-transported currents, they also identified a number of major differences challenging the hypothesis that CaT1 *per se* was sufficient to form the endogenous CRAC. Interestingly, Schindl *et al.* (19) recently demonstrated that the regulatory and pharmacological properties of heterologously expressed CaT1 depend on the cell type used for expression and on expression levels, suggesting that it may interact with endogenous TRP channels to produce the resultant heterotetrameric store-dependent channel.

trisphosphate; 2-APB, 2-aminoethoxydiphenyl borate; SK&F 96365, 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenylethyl]-1H-imidazole hydrochloride; TRPV6, transient receptor potential vanilloid type 6; RT, reverse transcription; pF, picofarads.

Beyond its academic interest, the relationship of CaT1 protein to CRAC acquires great potential practical importance as it becomes overexpressed in prostate cancers (22, 23). Overexpression of this protein may represent a novel marker for prostate cancer progression as well as a target for therapeutic strategies. However, functional evidence linking CaT1 to endogenous store-operated Ca<sup>2+</sup> entry (SOCE) in prostate cancer cells is still missing. In our recent work (24), we described SOC-mediated current in LNCaP prostate cancer epithelial cells for the first time. In this study, we used the patch clamp technique combined with interventions that produce controlled alterations in the endogenous CaT1 (antisense depletion and pharmacological up-regulation) to further characterize this current and show the involvement of CaT1 in generating it. This strategy differs fundamentally from most of the previous ones, which were based on overexpression of foreign CaT1 in various cell types, since we manipulated only the endogenous levels of this protein, thus revealing its physiological significance *in situ*. Only recently, a similar strategy, aimed at reducing the number of endogenous channels that presumably include CaT1 as an essential subunit by providing an excess of exogenous CaT1 with a mutated, function-incompatible pore region, was used to demonstrate the involvement of this protein in endogenous SOCE in Jurkat T-lymphocytes (21). Although our data are generally consistent with the functional role of CaT1 in store-operated Ca<sup>2+</sup> entry, they also suggest that this particular TRP channel may represent only a part of the store-operated channel in prostate cancer epithelial cells.

#### EXPERIMENTAL PROCEDURES

**Cell Cultures**—LNCaP cells from the American Type Culture Collection were cultured in RPMI 1740 medium (BioWhittaker, Fontenay sous Bois, France) supplemented with 5 mM L-glutamine (Sigma) and 10% fetal bovine serum (Seromed, Poly-Labo, Strasbourg, France). The culture medium also contained 50,000 IU/liter penicillin and 50 mg/liter streptomycin. Cells were routinely grown in 50-ml flasks (Nunc, Poly-labo) and kept at 37 °C in a humidified incubator in an air/CO<sub>2</sub> (95/5%) atmosphere. For electrophysiological experiments, the cells were subcultured in Petri dishes (Nunc) coated with polyornithine (5 mg/liter; Sigma) and used after 3–6 days.

**Electrophysiology and Solutions**—Macroscopic currents in LNCaP cells were recorded in the whole-cell configuration of the patch clamp technique, using a computer-controlled EPC-9 amplifier (HEKA Electronics). Patch pipettes were made from borosilicate glass capillaries (WPI) on a PIP-5 (HEKA Electronics) puller. The resistance of the pipettes filled with the basic pipette solution (see below) varied from 4 to 6 megaohms. Series resistance compensation was used to improve voltage clamp performance during whole-cell current recordings.

The composition of the regular bath (extracellular) solution was 120 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 5 mM glucose, 10 mM HEPES, pH 7.3 (adjusted with Na(OH)). The high Ca<sup>2+</sup>, Na<sup>+</sup>-free extracellular solution used for store-operated Ca<sup>2+</sup> current recordings contained 120 mM tetraethyl-ammonium-Cl, 10 mM CaCl<sub>2</sub>, 5 mM glucose, 10 mM HEPES, pH 7.3 (adjusted with tetraethyl-ammonium(OH)). The basic Cs<sup>+</sup>-based, Ca<sup>2+</sup>-free pipette (intracellular) solution included 120 mM CsCl, 1 mM MgCl<sub>2</sub>, 10 mM HEPES, 10 mM BAPTA, pH 7.3 (adjusted with Cs(OH)). Necessary supplements (IP<sub>3</sub>, thapsigargin) were added directly to the respective solutions, from appropriately prepared stock solutions. All chemicals for electrophysiological recordings were from Sigma except for thapsigargin, which was purchased from Calbiochem.

During electrophysiological experiments, cells were maintained in the regular extracellular solution. External solutions were changed using a multibarrel puffing micropipette with common outflow positioned in close proximity to the cell under investigation. During the experiment, the cell was continuously superfused with the solution via puffing pipette to reduce possible artifacts related to the change from static to moving solution and *vice versa*. Complete external solution exchange was achieved in less than 1 s.

Analysis of membrane currents was performed off-line. Usually 3–5 current traces that directly preceded administering store-depleting intervention were averaged to derive mean base-line current, which was then

subtracted from the currents during administration of the intervention. The resultant current was considered as the store-operated current (*I*<sub>SOC</sub>).

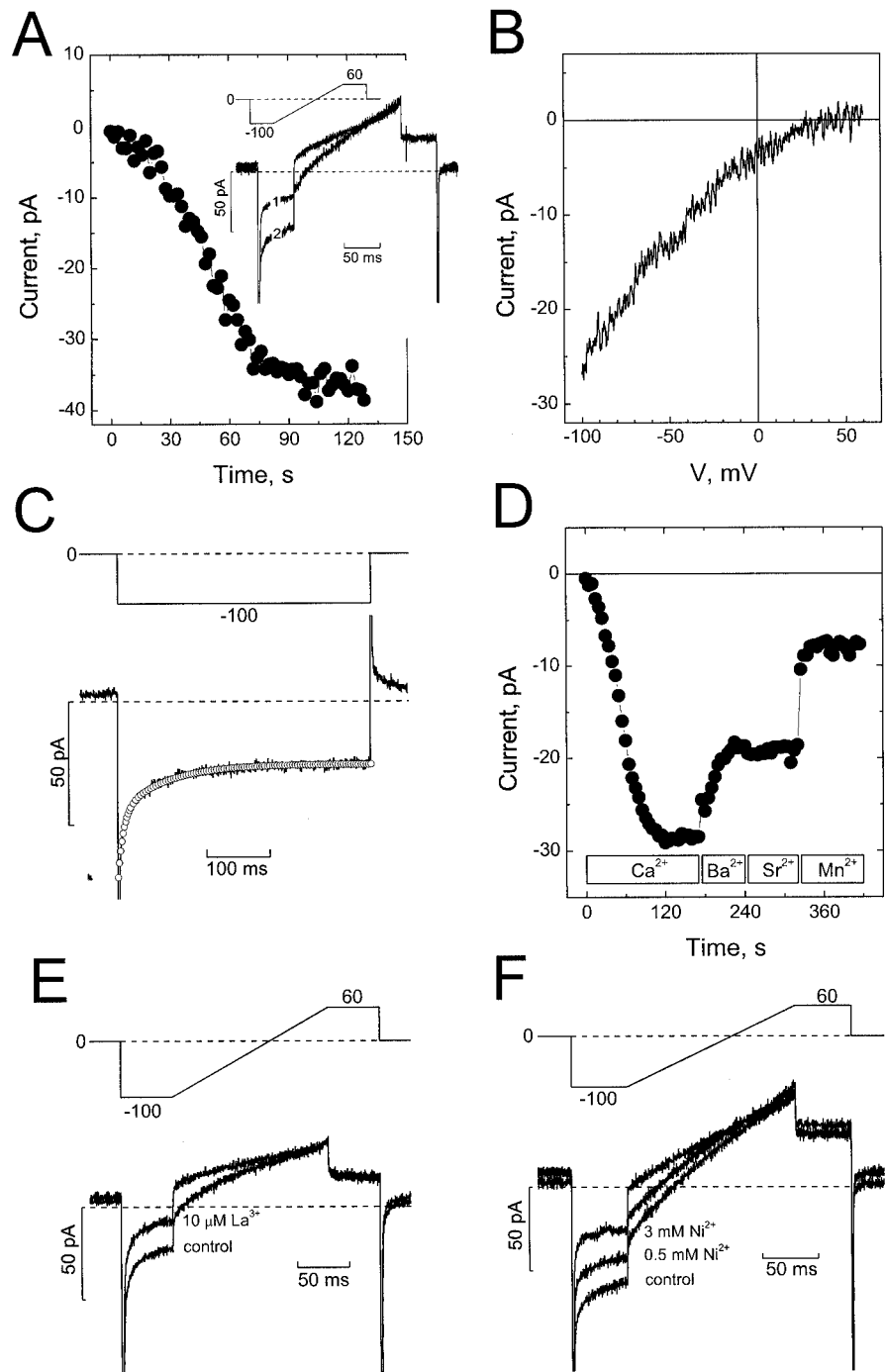
**Analysis of CaT1 Expression (RT-PCR)**—Total RNA was isolated from LNCaP cells using the guanidium thiocyanate/phenol/chloroform extraction procedure (25). After a DNase I (Life Technologies) treatment to eliminate genomic DNA, 2.5 μg of total RNA was reverse transcribed into cDNA at 42 °C using random hexamer primers (PerkinElmer Life Sciences) and murine leukemia virus reverse transcriptase (PerkinElmer Life Sciences) in a 20-μl final volume, followed by PCR as described below. In order to control the amplification of genomic DNA, PCR was also carried out on the nonreverse 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 CaT1, Bcl-2, and β-actin cDNAs were designed on the basis of established GenBank™ sequences. Primers were synthesized by Invitrogen. The primers for human CaT1 cDNA were as follows: 5'-TGAGGATTGCAAGGTGCACCAT-3' (nucleotides 201–222, GenBank™ accession number AF304463) and 5'-CCCCAAAGTAGATGAGGTTGCA-3' (nucleotides 468–490). The expected DNA length of the PCR product generated by these primers was 290 bp. The sense and antisense primers for Bcl-2 were as follows: 5'-GATTGTGCCTTCTTTGAGTTCGGT-3' (nucleotides 469–493, GenBank™ accession number M14745) and 5'-CTACTGCTTTAGTGAACCT-3' (nucleotides 784–803). The expected DNA length of the PCR product generated by these primers was 335 bp. Amplification of the β-actin cDNA was used to control the integrity of the cDNA and also as an internal control to quantify the expression of a given gene in duplex RT-PCR. The following oligonucleotide primers were used to amplify β-actin cDNA (227 bp): 5'-CAGAGCAAGAGAGGCATCCT-3' and 5'-ACGTACATGGCTGGGTGTTGAA-3'. In order to confirm the identity of the amplified products, restriction analysis was carried out on each PCR product using specific restriction enzymes. PCR was performed on the RT-generated cDNA using a GeneAmp PCR System 2400 thermal cycler (PerkinElmer Life Sciences). To detect CaT1 cDNAs, PCR was performed by adding 2 μl of the RT template to the following mixture (final concentrations): 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl<sub>2</sub>, 200 μM each dNTP, 1 μM sense and antisense primers, and 1 unit of AmpliTaq Gold (PerkinElmer Life Sciences) in a final volume of 25 μl. DNA amplification conditions included an initial 10-min denaturation step at 95 °C (which also activates the Gold variant of *Taq* polymerase) and 40 cycles of 30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C, and finally 7 min at 72 °C. The identity of each PCR-amplified product was confirmed by restriction analysis using specific restriction enzymes.

In order to study the effects of the CaT1 antisense treatments on the rate of CaT1 and Bcl-2 mRNA synthesis in LNCaP cells, a semiquantitative multiplex PCR was performed using primers amplifying either CaT1 and β-actin (internal standard) or Bcl-2 and β-actin mRNAs. Control experiments using LNCaP cell cDNA showed that the amount of each amplicon obtained in a multiplex PCR was independent of the presence of other primers (cross-correlation analysis), excluding the possibility of strong interference between primers. The number of cycles and the final cDNA concentrations were then adjusted to be in the exponential phase of the amplification of each product. Finally, the amount of each PCR product in multiplex reaction increased linearly with the amount of starting cDNA (5–50 ng of RNA equivalents), ensuring that changes in the ratio of PCR product to control gene product truly reflects a change in mRNA abundance of the gene relative to the control gene. The duplex PCR was then performed using the cDNA (18 ng of RNA equivalent to the RT reaction) of CaT1 sense- and antisense-treated LNCaP cells. The conditions were as follows: 10 min at 95 °C and then 32 cycles of 30-s extension at 72 °C each and a final 7-min extension step. Half of the PCR samples were analyzed by electrophoresis in a 2% agarose gel and stained with ethidium bromide (0.5 μg/ml) and viewed by Gel Doc 1000 (Bio-Rad).

**Antisense Assays**—The LNCaP cells were treated for up to 5 days with either 0.5 μM phosphorothioate antisense oligodeoxynucleotides (ODNs) (Eurogentec) targeted to the coding region of the CaT1 protein and 2.5 μM cytofectin (GS 3815 to 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) at a 2:1 molar ratio, unsized) (Eurogentec) or sense ODNs by adding them directly to the culture medium. The 18-mer ODNs used in these studies had the following sequences: 5'-GGGCAGTGACAAACCCAT-3' for antisense ODN and 5'-ATGGGTTTGTCACTGCCC-3' for sense ODN.

**Data Analysis and Statistics**—Each experiment was repeated several times. The data were analyzed using PulseFit (HEKA Electronics) and Origin 6.0 (Microcal, Northampton, MA) software. Results were expressed as means ± S.E. where appropriate. Student's *t* test was used

**FIG. 1. Whole-cell  $Ca^{2+}$ -carried  $I_{SOC}$  in LNCaP cells.** *A*, the time course of the development of  $I_{SOC, Ca}$  at  $-100$  mV in representative LNCaP cell in response to the dialysis with 10 mM BAPTA-containing pipette solution; time 0 corresponds to the establishment of the whole-cell configuration; the *inset* shows superimposed base-line current just after the rupture of the membrane (1) and fully developed  $I_{SOC, Ca}$  (2) together with the voltage protocol used to elicit the currents ( $n = 9$ );  $I_{SOC, Ca}$  amplitude was measured at  $-100$  mV at the beginning of the hyperpolarization. *B*,  $I$ - $V$  relationship of a fully developed  $I_{SOC, Ca}$  derived from the traces presented in *A* ( $n = 9$ ). *C*, trace of the fully developed  $I_{SOC, Ca}$  (continuous line) in response to the depicted pulse protocol with superimposed fit of the current decay phase with double exponential function (open symbols); parameters of the fit are presented under "Results" ( $n = 4$ ). *D*, time course of the changes of  $I_{SOC}$  at  $-100$  mV in the representative LNCaP cell sequentially exposed to  $Ca^{2+}$ -,  $Ba^{2+}$ -,  $Sr^{2+}$ - and  $Mn^{2+}$ -containing (10 mM) extracellular solutions (marked by horizontal bars); time 0 corresponds to the establishment of the whole-cell configuration ( $n = 5$ ). *E*, superimposed traces from representative cell of the fully developed  $I_{SOC, Ca}$  under control conditions and in the presence of 10  $\mu$ M  $La^{3+}$  ( $n = 3$ ); the pulse protocol used to elicit the currents is shown at the top. *F*, same as in *E* but for another cell sequentially exposed to 0.5 and 3 mM  $Ni^{2+}$  ( $n = 3$ ).



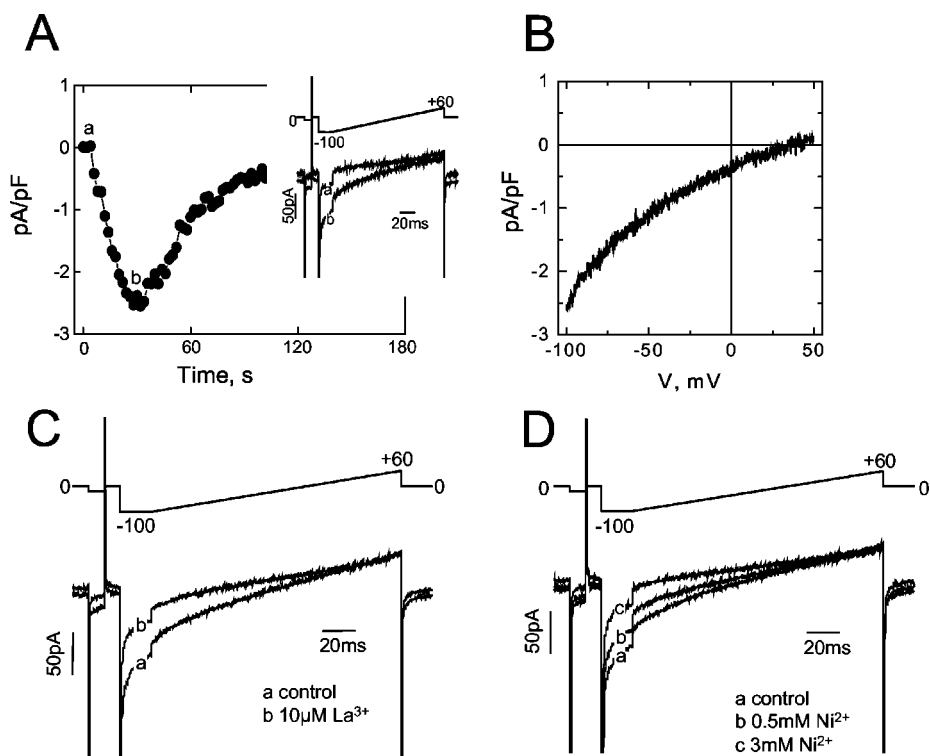
for statistical comparison of the differences, and  $p < 0.05$  was considered significant.

## RESULTS

**Endogenous Whole-cell Store-operated Membrane Currents in LNCaP Cells**—Transmembrane store-operated  $Ca^{2+}$  current ( $I_{SOC, Ca}$ ) can be activated by interventions that lead to the enhanced emptying of ER intracellular  $Ca^{2+}$  stores (26). In this study, two major approaches were used to activate this current in LNCaP prostate cancer epithelial cells: (i) cell dialysis via patch pipette with  $IP_3$  (100  $\mu$ M or 25  $\mu$ M) or (ii) cell dialysis/exposure to the sarcoendoplasmic reticulum calcium ATPase pump inhibitor thapsigargin (TG; 100 nM). Both interventions facilitated the emptying of ER stores, in the first case, by opening  $IP_3$ -sensitive channels in the ER and, in the second case, by inhibiting  $Ca^{2+}$  reuptake into the ER. In addition, in

all experiments, the patch pipette contained high concentrations of the  $Ca^{2+}$  chelator BAPTA (10 mM), which has been shown in numerous studies (e.g. Ref. 27) to be sufficient on its own to deplete the ER stores, due to rapid capture of the  $Ca^{2+}$  that leaks passively from the ER into the cytosol. In keeping with this principle, the LNCaP cells responded to the infusion of 10 mM BAPTA-containing intracellular solution via the patch pipette by generating an inwardly rectifying membrane current (Fig. 1A). With 10 mM  $Ca^{2+}$  in the bath, this current developed to a maximal density of  $1.3 \pm 0.3$  pA/pF (at  $-100$  mV,  $n = 9$ ) in about 90 s following establishment of the whole-cell configuration (Fig. 1A) and then stayed relatively constant or declined slightly over a period of up to 5 min. Current amplitude was measured at the beginning of the hyperpolarization phase to  $-100$  mV (see pulse protocol in Fig. 1A). The

**FIG. 2. Intracellular  $IP_3$  changes the time course of  $Ca^{2+}$ -carried  $I_{SOC}$  in LNCaP cells.** *A*, the time course of the development of  $I_{SOC, Ca}$  at  $-100$  mV in a representative LNCaP cell in response to the dialysis with 10 mM BAPTA plus 100  $\mu$ M  $IP_3$ -containing pipette solution; time 0 corresponds to the establishment of the whole-cell configuration ( $n = 7$ ). *B*,  $I$ - $V$  relationship of  $IP_3$ -evoked  $I_{SOC, Ca}$  acquired at the maximum of the time course of *A* ( $n = 7$ ). *C*, superimposed traces from a representative cell of the maximal  $IP_3$ -evoked  $I_{SOC, Ca}$  (control) following application of 10  $\mu$ M  $La^{3+}$ ; the pulse protocol is shown at the top ( $n = 4$ ). *D*, same as in *C*, but for another cell sequentially exposed to 0.5 and 3 mM  $Ni^{2+}$  ( $n = 4$ ).



$I$ - $V$  relationship of the current showed strong inward rectification and an apparent reversal potential at  $+43 \pm 1.1$  mV ( $n = 9$ ) (Fig. 1*B*). The current was dependent on extracellular  $Ca^{2+}$  concentration and disappeared completely in nominally  $Ca^{2+}$ -free extracellular solution, suggesting that it is carried solely by  $Ca^{2+}$  ions.

Exposure of the cell to 100 nM TG after full development of the  $Ca^{2+}$ -dependent inward current in response to BAPTA did not produce any further enhancement of the current amplitude (data not shown), suggesting that sarcoendoplasmic reticulum calcium ATPase pump inhibition did not cause any additional ER  $Ca^{2+}$  store depletion beyond that produced by high cytosolic BAPTA alone and that the observed  $Ca^{2+}$  current is transferred via activated SOCs (*i.e.* is indeed  $I_{SOC, Ca}$ ). Inclusion of 100 nM TG in the patch pipette together with 10 mM BAPTA slightly accelerated the  $I_{SOC, Ca}$  development time course (to about 60 s) but did not alter its maximal amplitude compared with BAPTA alone (see, for example, Fig. 3). We therefore used dialysis with 10 mM BAPTA as the only intervention to activate  $I_{SOC, Ca}$  in subsequent experiments unless otherwise specified. It should be noted that our experimental conditions did not favor the activation of  $Mg^{2+}$ -sensitive cationic channels (known as MIC or MagNum), easily confused with SOCs under certain conditions (28–30), so we were absolutely confident that the only current we were dealing with was transferred through the SOCs.

Application of a prolonged hyperpolarizing pulse to  $-100$  mV revealed time-dependent inactivation of the inward  $I_{SOC, Ca}$ , which could be described by the sum of two exponential functions ( $\tau_{fast} = 9.1 \pm 1.8$  ms and  $\tau_{slow} = 55.7 \pm 7.8$  ms,  $n = 4$ ) and a steady-state level (Fig. 1*C*). Substituting 10 mM  $Ba^{2+}$  for 10 mM  $Ca^{2+}$  reduced current amplitude by about 35%. Further equimolar replacement of  $Ba^{2+}$  with  $Sr^{2+}$  produced almost no change in current, whereas using  $Mn^{2+}$  instead of  $Sr^{2+}$  caused a strong current decrease. Quantification of  $Ca^{2+}$ -,  $Sr^{2+}$ -,  $Ba^{2+}$ - and  $Mn^{2+}$ -carried  $I_{SOC}$  (Fig. 1*D*) in five cells yielded the sequence of the relative channel conductance:  $Ca^{2+}$  (1) >  $Sr^{2+}$  (0.69)  $\approx$   $Ba^{2+}$  (0.65)  $\gg$   $Mn^{2+}$  (0.23), very similar to the analog

sequences found for native SOCs in other preparations (3, 4, 31) as well as that of heterologously expressed CaT1 protein, a likely molecular candidate for the role of native SOCs (17).

We also examined the sensitivity of  $I_{SOC, Ca}$  in LNCaP cells to the polyvalent cations  $La^{3+}$  and  $Ni^{2+}$ , which had been shown to block native CRAC and expressed CaT1 currents (3, 17). Fig. 4, *E* and *F*, presents  $I_{SOC, Ca}$  recordings from two representative cells exposed to  $La^{3+}$  and  $Ni^{2+}$ , respectively. Inspection of these recordings shows that  $La^{3+}$  appears to be a much more potent blocker than  $Ni^{2+}$ . A 10  $\mu$ M concentration of  $La^{3+}$  reduced the current virtually to its base-line level, indicating complete inhibition, whereas  $Ni^{2+}$ , even at 0.5 mM, was able to block 40% at most, and as much as 3 mM  $Ni^{2+}$  was required for complete current inhibition (Fig. 1*F*). This high sensitivity of  $I_{SOC, Ca}$  in LNCaP cells to  $La^{3+}$  is characteristic of CaT1-mediated current (17).

Next, we investigated whether inclusion of  $IP_3$  in the patch pipette had any impact on the properties of  $I_{SOC, Ca}$  in LNCaP cells. Fig. 2*A* shows that, with 100  $\mu$ M  $IP_3$  in the BAPTA-containing pipette solution, the inward  $Ca^{2+}$  current developed considerably faster and to about a 2-fold higher maximal amplitude of nearly 3 pA/pF ( $n = 7$ ) compared with BAPTA alone (see Fig. 1*A*) and then declined quite rapidly. This increase in  $I_{SOC, Ca}$  amplitude and the dramatic change in its development kinetics and rundown are not surprising, considering that  $IP_3$  produces active store depletion by opening  $IP_3$ -sensitive  $Ca^{2+}$ -permeable channels in the ER, which also leads to facilitated  $Ca^{2+}$ -dependent inactivation of SOCs (*e.g.* Refs. 26 and 32). Nevertheless, despite the dramatic change in kinetics,  $I_{SOC, Ca}$  evoked by the combined action of BAPTA plus  $IP_3$  still exhibited an  $I$ - $V$  relationship ( $n = 7$ ) (Fig. 2*B*) nearly identical to that observed with BAPTA alone (see Fig. 1*B*), suggesting that both interventions recruit the same store-operated channels. This conclusion is further validated by the fact that the  $IP_3$ -induced  $I_{SOC, Ca}$  showed similar sensitivity to the polyvalent cations  $La^{3+}$  and  $Ni^{2+}$  ( $n = 4$ ) (Fig. 2, *C* and *D*).

*Pharmacology of the Endogenous  $I_{SOC}$  in LNCaP Cells—* Pharmacological modulation of endogenous  $I_{SOC, Ca}$  in LNCaP

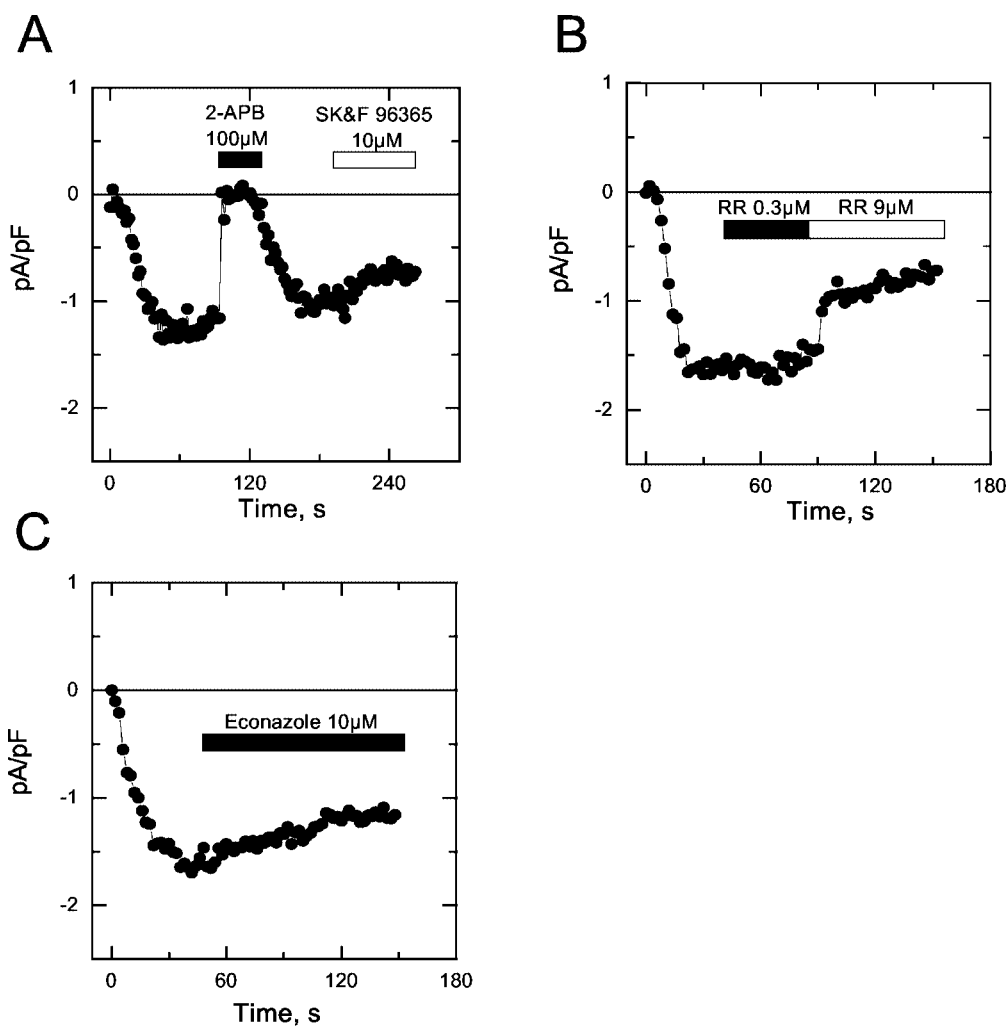


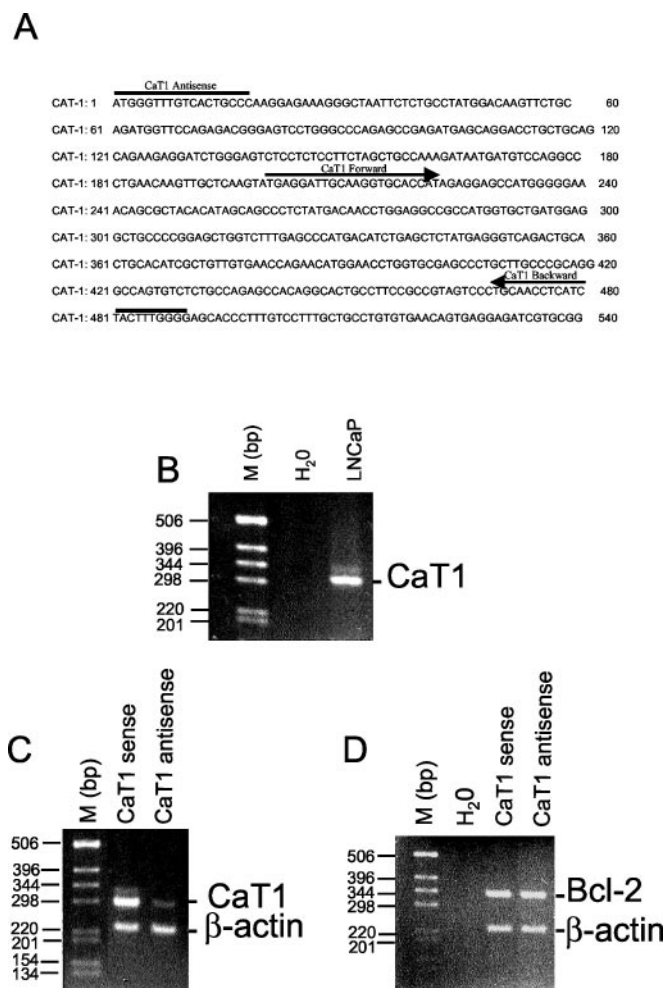
FIG. 3. **Pharmacology of  $I_{SOC,Ca}$  in LNCaP cells.** A, the effects of 100  $\mu$ M 2-APB ( $n = 5$ ) and 10  $\mu$ M SK&F 96365 ( $n = 5$ ) on the whole-cell  $I_{SOC,Ca}$  (measured at  $-100$  mV) elicited in the representative regular LNCaP cell by the dialysis with 10 mM BAPTA plus 100 nM TG-supplemented pipette solution; the periods of drug application are marked by horizontal bars. B and C, same as in A, but for the effects of 0.3 and 9  $\mu$ M of ruthenium red (RR) ( $n = 5$ ) (B) and 10  $\mu$ M econazole ( $n = 3$ ) (C) in two other LNCaP cells.

cells was studied on currents activated by intracellular BAPTA (10 mM) plus TG (100 nM) to ensure reliable store depletion and to ensure that the action of the compounds would less likely be distorted by the rundown process seen when  $IP_3$  was used in the pipette.

The following organic compounds were used: 2-APB, an  $IP_3$  receptor antagonist and inhibitor of "classic"  $I_{CRAC}$  (18, 33); SK&F 96365, an SOC- and TRP-mediated  $Ca^{2+}$  entry blocker (34, 35); econazole, an antimycotic drug; and ruthenium red, a polycationic dye. The last two have been shown to be quite potent inhibitors of ECaC1/CaT2-mediated current (33). Among these drugs, 2-APB appeared to be the most effective  $I_{SOC,Ca}$  inhibitor in LNCaP cells. At its effective concentration (100  $\mu$ M according to previous studies), it caused almost 100% current inhibition, which was completely reversible upon drug withdrawal (Fig. 3A). 50  $\mu$ M 2-APB also caused 100% current inhibition. Ruthenium red at the 0.3  $\mu$ M concentration required to block ECaC1/CaT2 current completely (36) produced 15%  $I_{SOC,Ca}$  inhibition at most in our experiments, and as much as 9  $\mu$ M was required to increase the blockade to 50% (Fig. 3B). It is noteworthy that ECaC2/CaT1 has been shown to have about 100-fold lower affinity for ruthenium red ( $IC_{50} = 9 \pm 1$   $\mu$ M) compared with ECaC1/CaT2 ( $IC_{50} = 121 \pm 13$  nM) (37). With the other two drugs, SK&F 96365 (10  $\mu$ M) inhibited  $I_{SOC,Ca}$  by 22%, but econazole (10  $\mu$ M) apparently had no effect (Fig. 3, A and C).

**CaT1 Expression in LNCaP Cells**—To date, the human TRPV6 channel, originally termed CaT1, is considered the most likely molecular candidate for the role of native SOCs (17, 19, 21). Peng *et al.* (22) already reported high expression levels of CaT1 transcripts in LNCaP cells, although no functional evidence has been yet presented for the role of the corresponding channel-forming protein in the endogenous SOCE in these cells. To determine whether or not CaT1 is directly involved in generating  $Ca^{2+}$  current in response to the depletion of intracellular  $Ca^{2+}$  stores in LNCaP cells, we altered the levels of its transcripts by either suppressing them via antisense ODN technology or enhancing them with a specific pharmacological treatment (the antiandrogen, Casodex, generic name bicalutamide), while simultaneously monitoring the changes in store-operated  $Ca^{2+}$  current. Prior to carrying out this type of manipulation, we initially tested CaT1 mRNA expression in LNCaP cells by RT-PCR, using specific primers that only amplify CaT1 mRNA (Fig. 4A). As shown in Fig. 4B, the CaT1-specific primers generated a PCR product of the expected size in LNCaP cells, suggesting that CaT1 protein was expressed in these cells.

The CaT1 antisense ODN was designed in the ATG region of the CaT1 mRNA sequence to inhibit endogenous CaT1 expression (Fig. 4A). The exact mechanism, by which the antisense ODNs are able to block protein translation, is not fully understood. One hypothesis implies activation of the RNase H en-



**FIG. 4. Antisense treatment reduces CaT1 mRNA in LNCaP cells.** *A*, CaT1 mRNA nucleotide sequences showing the CaT1 regions targeted by CaT1-specific forward and backward primers used for RT-PCR as well as by CaT1 antisense oligonucleotide used for hybrid depletion. *B*, RT-PCR analysis of the CaT1 mRNA (290 bp) expression in LNCaP cells with the use of CaT1-specific primers. *C*, RT-PCR analysis demonstrating the reduction of the CaT1 mRNA (290 bp) in LNCaP cells treated for 72 h with antisense oligonucleotide compared with the cells treated for the same period of time with sense oligonucleotide. *D*, RT-PCR analysis demonstrating that treatment of LNCaP cells with CaT1 antisense or sense oligonucleotides does not affect the mRNA content for CaT1-unrelated Bcl-2 protein (335 bp); in each case, a duplex PCR was performed with  $\beta$ -actin (227 bp) used as an internal standard of the total mRNA of each sample. H<sub>2</sub>O was used as a negative control. PCR-amplified products were analyzed by a 2% agarose gel electrophoresis and visualized by EtBr staining. *M*, molecular weight marker. Each experiment was repeated three times.

zyme (38–40), which cleaves RNA-DNA complexes. The formation of an antisense-mRNA hybrid activates this enzyme, resulting in the cleavage of both the antisense and mRNA strands and leading to the down-regulation of the specific mRNA. Thus, we tested the efficiency of the CaT1 antisense ODNs treatment in reducing the CaT1 mRNA content in LNCaP cells by RT-PCR. Consistent with the above mentioned hypothesis, Fig. 4C shows a significant reduction in the amount of CaT1 PCR product after 72-h treatment with 0.5  $\mu$ M antisense ODNs compared with the control (*i.e.* LNCaP cells treated under the same conditions with sense ODNs). At the same time, the antisense treatment did not affect the mRNA content of the CaT1-unrelated antiapoptotic oncoprotein Bcl-2 (Fig. 4, *C* and *D*), suggesting that the action of CaT1 antisense ODNs is highly selective.

#### Antisense Hybrid Depletion of CaT1 Suppressed Store-operated

Ca<sup>2+</sup> Current in LNCaP Cells—Maximal effects of antisense ODNs treatment on cell responses to Ca<sup>2+</sup> store-depleting interventions were observed after a 72-h incubation, with almost no change over the next 2 days. Immediately after the whole-cell configuration was established, using 10 mM BAPTA plus 100  $\mu$ M IP<sub>3</sub> pipette solution, both CaT1 sense- and antisense-treated LNCaP cells showed nearly identical base-line current with linear *I-V* and small amplitude. At  $-100$  mV, the current was  $1.0 \pm 0.1$  pA/pF and  $0.9 \pm 0.1$  pA/pF, respectively. This base-line current was considered to reflect a passive leak and was, therefore, subtracted from the currents achieved at later in cell dialysis.

Fig. 5A compares averaged time courses of the development of inward  $I_{SOC, Ca}$  in CaT1 sense- and antisense-treated LNCaP cells in response to dialysis with BAPTA plus IP<sub>3</sub> intracellular solution. Progression of the dialysis was accompanied by a relatively rapid increase in  $I_{SOC, Ca}$  density in both cell types (Fig. 5A), due to depletion of intracellular Ca<sup>2+</sup> stores associated under the combined action of BAPTA plus IP<sub>3</sub>. However, the maximal density of  $I_{SOC, Ca}$  in CaT1-depleted cells ( $1.4 \pm 0.36$  pA/pF) was about half that in the control ( $2.8 \pm 0.34$  pA/pF) (Fig. 5, *A* and *D*). Inspection of the time courses of Fig. 5A also indicated significantly slower current development in CaT1-deficient cells in response to BAPTA plus IP<sub>3</sub>, with virtually no rundown after it reached maximal amplitude. This observation was further validated by quantification of the first derivatives of the time courses, which showed that CaT1 depletion slowed the current development rate from  $0.18 \pm 0.03$  pA/pF/s to  $0.08 \pm 0.02$  pA/pF/s and the current rundown from  $0.04 \pm 0.01$  pA/pF/s to  $0.01 \pm 0.006$  pA/pF/s. Reducing the IP<sub>3</sub> concentration in the pipette to 25  $\mu$ M resulted in a considerably longer delay in  $I_{SOC, Ca}$  activation in both sense- and antisense-treated LNCaP cells (Fig. 5B), which is qualitatively consistent with previous observations in RBL-1 cells (26). This treatment also had much less effect on the maximal current amplitudes (Fig. 5D), as well as development and rundown kinetics (Fig. 5B), than 100  $\mu$ M IP<sub>3</sub>. Moreover, the increase in the delay period was much more pronounced for CaT1-depleted cells (Fig. 5B). Collectively these results prove not only that CaT1 is directly involved in transferring a substantial portion of  $I_{SOC}$  in LNCaP cells but also that it determines the store sensitivity and Ca<sup>2+</sup>-dependent inactivation properties of endogenous SOCs.

Ca<sup>2+</sup>-carried  $I_{SOC}$  activated in control and CaT1-depleted LNCaP cells in response to dialysis with the Ca<sup>2+</sup> chelator BAPTA (10 mM) plus TG (100 nM) was characterized by almost 2-fold lower maximal current densities and noticeably slower development time courses (Fig. 5, *C* and *D*), compared with currents activated by BAPTA plus IP<sub>3</sub>. These differences are generally consistent with previous observations showing that the magnitude of  $I_{SOC}$  as well as the temporal parameters of its development depend on the tools used to deplete intracellular calcium stores (26). Still, like BAPTA plus IP<sub>3</sub>-activated currents, the  $I_{SOC, Ca}$  evoked in response to BAPTA plus TG dialysis was also nearly 50% lower in CaT1-depleted cells than control (Fig. 5, *C* and *D*), suggesting that the decrease in current amplitude following CaT1 depletion was associated with a change in the number of underlying channels or their intrinsic properties, irrespective of the means used to activate them.

To determine whether CaT1 depletion altered the pharmacological sensitivity of the residual store-dependent current, we also compared the action of 2-APB (100  $\mu$ M), SK&F 96365 (10  $\mu$ M), ruthenium red (9  $\mu$ M), and econazole (10  $\mu$ M) on  $I_{SOC, Ca}$  in CaT1 sense- and antisense-treated LNCaP cells. A summary of these results is presented in Fig. 5E. The most notable differ-

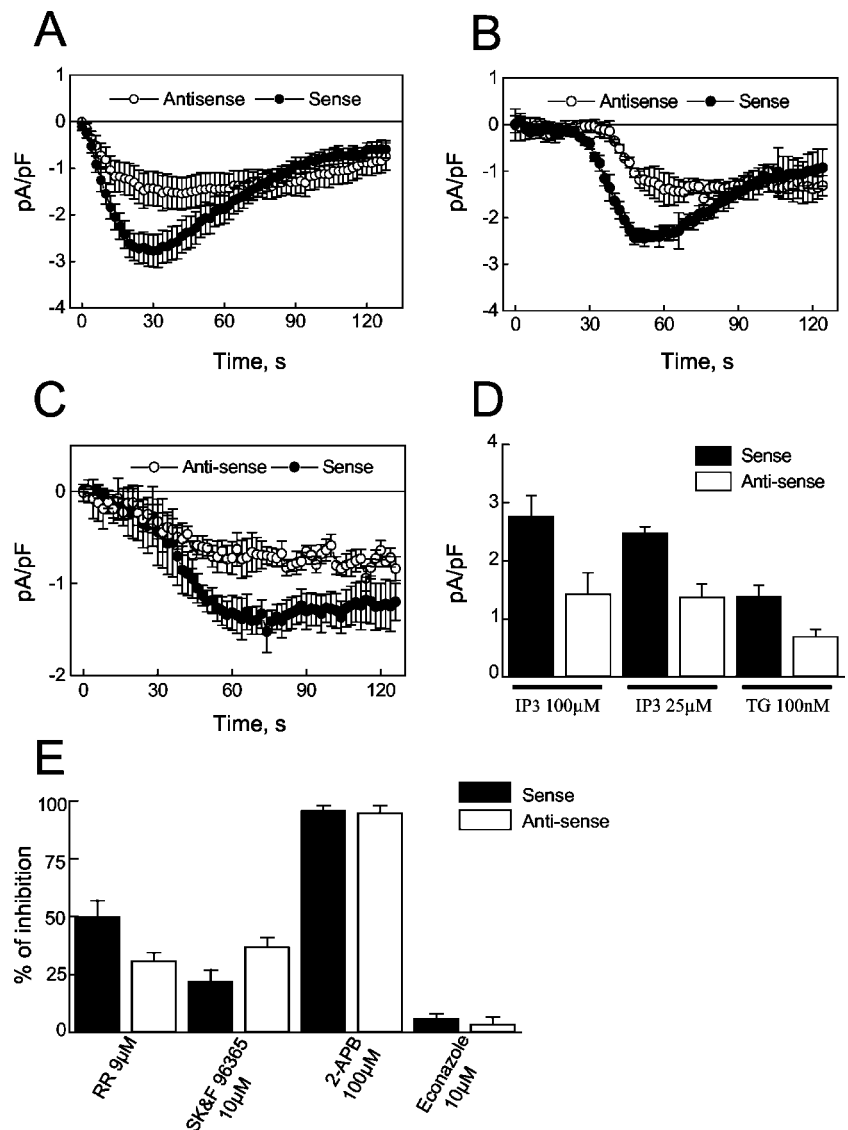


FIG. 5. CaT1 depletion reduces  $I_{SOC}$  in LNCaP cells. A and B, averaged time courses of the development of  $I_{SOC,Ca}$  (at  $-100$  mV) in LNCaP cells treated for 72 h with CaT1 sense (filled circles) and antisense (open circles) oligonucleotides (mean  $\pm$  S.E.,  $n = 12$  for each cell type) in response to the dialysis with 100  $\mu$ M (A) or 25  $\mu$ M (B)  $IP_3$ -supplemented BAPTA-containing pipette solution. C, same as in A and B, but in response to the dialysis with BAPTA plus TG (100 nM). D, quantification of the differences in the maximal  $I_{SOC,Ca}$  density in CaT1 sense-treated (black column) and antisense-treated LNCaP cells in response to  $IP_3$  (100 and 25  $\mu$ M) and TG (100 nM). E, comparison of the effects of 2-APB (100  $\mu$ M), SK&F 96365 (10  $\mu$ M), ruthenium red (9  $\mu$ M), and econazole (10  $\mu$ M) on  $I_{SOC,Ca}$  in CaT1 sense- and antisense-treated LNCaP cells (mean  $\pm$  S.E.,  $n = 4-6$ ).

ences were noted in the action of SK&F 96365 and ruthenium red. The first agent blocked the residual current to a greater extent in antisense-treated cells, whereas the second was more effective with respect to the original  $I_{SOC,Ca}$  in sense-treated cells. These results suggest that most of the current that “goes away” upon CaT1 depletion is ruthenium red-sensitive but insensitive to SK&F 96365.

**Pharmacological Up-regulation of CaT1 Levels Potentiates Store-operated  $Ca^{2+}$  Current in LNCaP Cells**—It has been reported that the level of CaT1 transcripts in LNCaP cells is negatively controlled by androgen receptors (22). Therefore, to further demonstrate the correlation between CaT1 transcript expression and the magnitude of  $I_{SOC,Ca}$ , we compared this current in regular LNCaP cells and LNCaP cells treated for 72 h with Casodex (1  $\mu$ M), an androgen receptor antagonist (22). Fig. 6A shows that, during Casodex treatment, the level of CaT1 mRNA in LNCaP cells gradually increased, exceeding the base line by almost 60% after 72 h. This enhancement was accompanied by an  $\sim 30\%$  up-regulation of  $I_{SOC,Ca}$  evoked by BAPTA plus  $IP_3$  or BAPTA plus TG infusion (Fig. 6, B and C), providing additional support for the hypothesis that endogenous CaT1 is involved in generating this current.

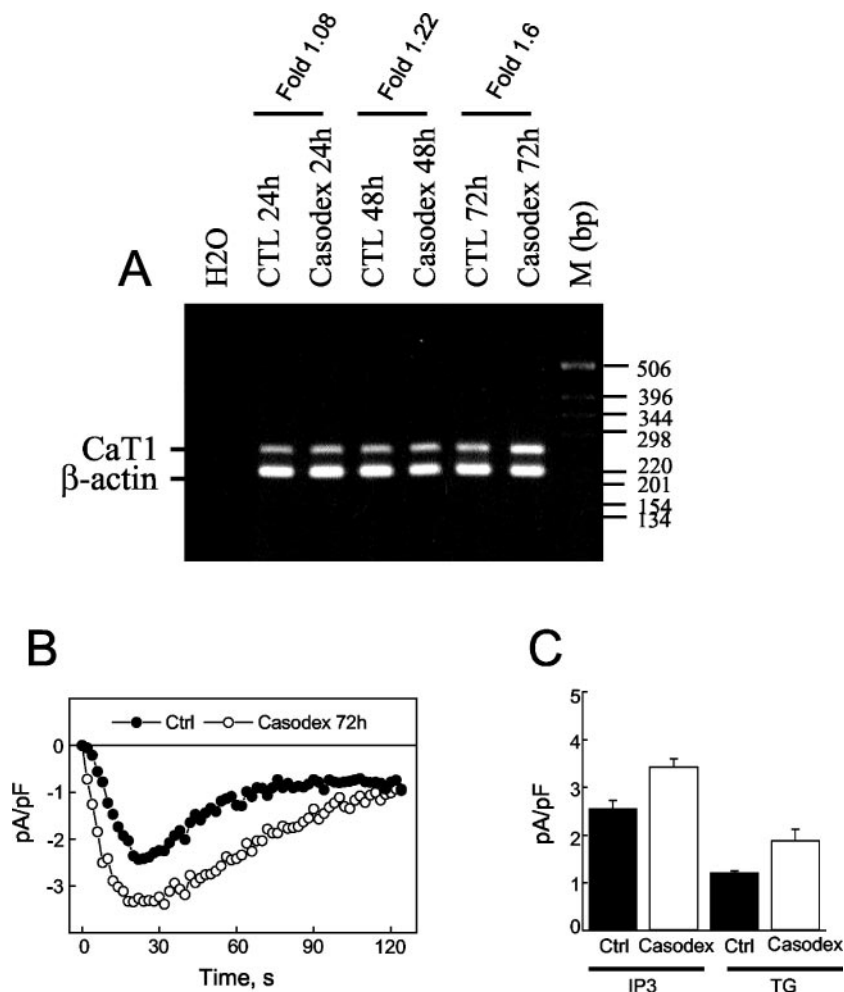
#### DISCUSSION

The major objective of this study was to characterize endogenous store-operated  $Ca^{2+}$  current in LNCaP prostate cancer

epithelial cells and test the hypothesis that the channel-forming CaT1 protein contributed to generation of this current. Although there is substantial agreement between the biophysical properties of CaT1-mediated current in a heterologous expression system and endogenous  $I_{CRAC}$  (17, 18), there were no previous data demonstrating a direct correlation between endogenous expression of CaT1 protein and the function of endogenous SOCE in the same cells. We used an approach consisting of manipulating endogenous CaT1 levels by means of antisense hybrid depletion or pharmacological up-regulation, combined with functional evaluation of  $I_{SOC,Ca}$ , to obtain this data for LNCaP prostate cancer epithelial cells, which have been shown, in recent independent studies, to possess robust capacitative  $Ca^{2+}$  entry (24) and express quite high levels of CaT1-specific mRNA (22). This approach shows that CaT1 is definitely involved in SOCE in LNCaP cells, but it may constitute only a part of the endogenous store-operated channel.

**Hybrid Depletion Experiments**—Functional scrutiny of CaT1 protein heterologously expressed in CHO-K1 cells led to the initial conclusion that it comprised all or part of the endogenous CRAC pore (17). Recently, however, this conclusion was seriously challenged, since a direct step-by-step comparison of the properties of heterologously expressed CaT1 in HEK 293 cells with the “classic” endogenous CRAC in RBL-2H3 cells under identical conditions revealed substantial differences (18,

**FIG. 6. Antiandrogen treatment enhances CaT1 expression and  $I_{SOC}$  in LNCaP cells.** *A*, RT-PCR analysis with the use of CaT1-specific primers demonstrating gradual enhancement of the CaT1 mRNA (290 bp) with time of LNCaP cell treatment with specific androgen receptor antagonist, Casodex ( $1 \mu M$ ). This experiment was repeated three times. *B*, time courses of the development of  $I_{SOC, Ca}$  (at  $-100$  mV) in representative control (filled circles) and Casodex-treated (72 h, open circles) LNCaP cells in response to the dialysis with  $100 \mu M$   $IP_3$ -supplemented BAPTA-containing pipette solution. *C*, quantification of the differences in the maximal  $I_{SOC, Ca}$  density in the control (black column) ( $n = 8$ ) and Casodex-treated (72 h) ( $n = 9$ ) LNCaP cells in response to  $IP_3$  ( $100 \mu M$ ) and TG ( $100$  nM).



20). Although there may be a number of reasons for this inconsistency, many of which are undoubtedly related to the channel expression system and cell-specific modulation of its function (19), CaT1 represents one of the best suited candidates for the role of SOC discovered to date. This is especially true, since “classic” CRAC represents only one phenotypic manifestation of SOCs, which are, in general, characterized by a whole range of cell-specific properties.

We used a different approach to assess the role of CaT1 in store-operated  $Ca^{2+}$  entry in LNCaP prostate cancer cells, which are known to have an up-regulated gene for this protein (22), by developing antisense oligonucleotides to inhibit expression of endogenous CaT1 protein selectively without affecting any of the other components in endogenous SOC. These nucleotides certainly reduced the CaT1 mRNA content in LNCaP cells to a significant extent, suggesting that protein levels are likely to be reduced as well. Knockout of CaT1 mRNA was accompanied by a decrease of  $\sim 50\%$  in  $I_{SOC}$  density.

The slower development of residual store-dependent current, which accompanied a general  $I_{SOC}$  reduction in CaT1-deficient cells, highlights the role of CaT1 not only in transferring this current but also in establishing the coupling efficacy of endogenous SOCs to the filling status of the stores and  $Ca^{2+}$ -dependent inactivation. Moreover, inhibition of androgen receptors, conditions that favor overexpression of CaT1 in LNCaP cells (22), potentiated  $I_{SOC}$ . Together with the fact that CaT1 depletion had the opposite effect on the current, this suggests a direct correlation between endogenous CaT1 levels and the size of the store-operated current. These findings are consistent with the hypothesis that CaT1 either represents a functionally

significant subunit in a homo- or heteromeric assembly of endogenous SOC or is an important regulator of this channel.

Since CaT1 *per se* is a channel-forming protein rather than a channel regulator (17, 18), the results of the hybrid depletion experiments led us to conclude that it either forms a monomeric SOC channel in LNCaP prostate cancer cells or is an integral part of the heteromeric SOC channel, which loses and/or alters its function when CaT1 is removed.

*How Similar Is Endogenous SOC in LNCaP Cells to CaT1?*—Our functional studies of endogenous SOCs in LNCaP cells favor the second possibility. Indeed, the properties of the endogenous  $I_{SOC}$  described in this study are quite similar, in many respects, to the ones reported for heterologously expressed CaT1 in CHO-K1 cells (see Ref. 17). First, the endogenous SOCs in LNCaP cells and CaT1 channels have the same unique activation mechanism (*i.e.* dependence on the ER and cytoplasmic  $Ca^{2+}$  levels). Second, macroscopic current properties such as sharp inward rectification,  $Ca^{2+}$ -dependent inactivation, high  $Ca^{2+}$  selectivity under normal conditions, the order of relative conductance for divalent cations  $Ca^{2+} > Sr^{2+} \approx Ba^{2+} \gg Mn^{2+}$ , and sensitivity to blockade by  $Ni^{2+}$  and  $La^{3+}$  are also nearly identical.

Despite these similarities, there are also notable differences, primarily relating to the lack of the significant constitutively active current characteristic of heterologously expressed CaT1 and the lack of slight potentiation by 2-APB of constitutively active CaT1 currents observed in both RBL and HEK 293 cells (17, 19). On the contrary, our results show strong inhibition of endogenous SOC similar to that of the store-dependent CaT1 currents of RBL-cells by 2-APB (19). It is important to note that

most of the distinctive properties of the original  $I_{SOC}$  in LNCaP cells compared with CaT1-induced current were also maintained in the residual  $I_{SOC}$  following CaT1 depletion. This is apparently due to the fact that CaT1 may only be a part of the endogenous SOC in LNCaP cells, whereas, for CaT1-specific properties to predominate, CaT1 must form a monotetrameric channel as a result of its strong overexpression (19). However, the stronger inhibition of control *versus* residual  $I_{SOC}$  by ruthenium red is consistent with the CaT1 nature of the portion of the current eliminated by antisense, since CaT1 is known to be quite sensitive to this drug (37).

*What Are the Other Possible Candidates?*—Thus, on the basis of our functional data, it seems unlikely that CaT1 is the only Ca<sup>2+</sup> transport protein involved in endogenous  $I_{SOC}$  in LNCaP cells. Recent studies have shown that, in addition to CaT1, these cells also express high levels of mRNA for two other members of the rapidly growing family of structurally related Ca<sup>2+</sup> transport proteins, CaT2 (also termed ECaC1 or TRPV5 (see Ref. 16) and CaT-L (also known as htrp8A and htrp8B), which has subsequently been shown to be encoded by the same gene as CaT1, but for consistency with original literature we keep the initial designation (22, 23)). Although CaT2 and CaT-L channels are Ca<sup>2+</sup>-selective, as well as showing inward rectification and Ca<sup>2+</sup>-dependent feedback inhibition, their functional properties generally seem to be even more dissimilar to those of endogenous SOCs in LNCaP cells. Heterologously expressed CaT-L protein, for instance, induces a constitutive Ca<sup>2+</sup> current (23), which, as has already been pointed out, is not detectable in LNCaP cells. In addition, such specific organic inhibitors of ECaC1/CaT2-mediated current as econazole and ruthenium red (36) had little or no effect on endogenous  $I_{SOC}$  in LNCaP cells. On the other hand, 2-APB, an IP<sub>3</sub> receptor antagonist and “classic” endogenous CRAC currents inhibitor (30), which has no effect on ECaC1/CaT2 current at 100 μM (36), totally blocked endogenous  $I_{SOC}$  in LNCaP cells. Collectively, these data support the hypothesis that none of the endogenously expressed Ca<sup>2+</sup> transport proteins, CaT1, CaT2, or CaT-L alone can be responsible for the endogenous  $I_{SOC}$  in LNCaP cells.

It should be noted that, in addition to the Ca<sup>2+</sup>-transporting proteins mentioned above, all members of the vanilloid family of TRP channels, TRPV (see Ref. 16), LNCaP cells are also likely to express other TRP channels as well. Indeed, it has been shown that the gene for Trp-p8 in the melastatine family, TRPM (16), is up-regulated in prostate cancer cells (41). Although the functional properties of Trp-p8 in prostate cells have not yet been elucidated, in general, this fact suggests that various TRP channel proteins, many of which have indeed been implicated in store-operated Ca<sup>2+</sup> entry, may take part in the formation of functional store-operated channels in prostate cancer cells. As was suggested, SOC may have either a hetero- or homotetrameric TRP-based structure, which may account for its varying cell-specific properties (15, 42). Moreover, depending on the endogenous expression of other store-dependent TRP channels in the particular cell type, an artificially produced excess or deficiency of CaT1 may impact the natural stoichiometry of SOCs, yielding channels with diverse biophys-

ical properties (19). Our data suggest that the most likely endogenous SOC in LNCaP cells is a heterotetramer composed of several TRP channel subunits, including endogenous CaT1, which, however, does not play a dominant role in defining the main properties of the resultant channel.

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