Activation of a Ca^{2+}-permeable cation channel by two different inducers of apoptosis in a human prostatic cancer cell line

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1. We have combined patch clamp recording with simultaneous $[Ca^{2+}]_i$ measurements in single LNCaP cells (a human prostate cancer cell line), to study the activation of Ca^{2+}-permeable channels by two different inducers of apoptosis, ionomycin and serum deprivation.

2. In perforated patch recording, LNCaP cells had a membrane potential of −40 mV and a resting $[Ca^{2+}]_i$ of 90 nM. Application of ionomycin at levels that induced apoptosis in these cells (10 μM) produced a biphasic increase in $[Ca^{2+}]_i$. The first rise in $[Ca^{2+}]_i$ was due to release of Ca^{2+} from internal stores and it was associated with a membrane hyperpolarization to −77 mV. The latter was probably due to the activation of high conductance, Ca^{2+}- and voltage-dependent K^+ channels (maxi-K). Conversely, the second rise in $[Ca^{2+}]_i$ was always preceded by and strictly associated with membrane depolarization and required external Ca^{2+}. Serum deprivation, another inducer of apoptosis, unmasked a voltage-independent Ca^{2+} permeability as well.

3. A lower concentration of ionomycin (1 μM) did not induce apoptosis, and neither depolarized LNCaP cells nor produced the biphasic increase in $[Ca^{2+}]_i$. However, the first increment in $[Ca^{2+}]_i$ due to release from internal Ca^{2+} stores was evident at this concentration of ionomycin.

4. Simultaneous recordings of $[Ca^{2+}]_i$ and ion channel activity in the cell attached configuration of patch clamp revealed a Ca^{2+}-permeable, Ca^{2+}-independent, non-selective cation channel of 23 pS conductance. This channel was activated only during the second increment in $[Ca^{2+}]_i$ induced by ionomycin. The absence of serum activated the 23 pS channel as well, albeit at a lower frequency than with ionomycin.

5. Thus, the 23 pS channel can be activated by two unrelated inducers of apoptosis and it could be another Ca^{2+} influx mechanism in programmed cell death of LNCaP cells.

Apoptosis, the most common expression of programmed cell death (PCD), is a physiological process that balances cell division in shaping and generating multicellular organisms (Steller, 1995; Thompson, 1995). Both in thymocytes and prostate cells (reviewed by Dowd, 1995), different inducers of apoptosis have been associated with a sustained elevation of intracelullar calcium concentration ($[Ca^{2+}]_i$). Presumably, such an increment in $[Ca^{2+}]_i$ activates different types of Ca^{2+}-dependent effectors via calcium-binding proteins like calmodulin (Dowd *et al.* 1991) or ALG-2 (Vito *et al.* 1996).

However, the nature and regulation of Ca^{2+}-permeable channels in apoptosis remain to be elucidated. Inducers of apoptosis like ionomycin (Martikainen *et al.* 1991), thapsigargin (Furuya *et al.* 1994; Lam *et al.* 1994), H_2O_2 (Distelhorst *et al.* 1996), dexamethasone (Lam *et al.* 1993), and withdrawal of interleukin-3 (Baffy *et al.* 1993) produce partial or complete depletion of internal Ca^{2+} stores. It has been assumed that apoptosis-associated Ca^{2+} influx occurs through the activation of store-operated Ca^{2+} channels (SOC; reviewed by Berridge, 1995) resulting in a sustained elevation in $[Ca^{2+}]_i$ and cell death. In this regard ionomycin, an electroneutral Ca^{2+} ionophore (Erdahl *et al.* 1995), induces an electrophoretic Ca^{2+} influx presumably mediated by the activation of SOC channels (Mason & Grinstein, 1993; Morgan & Jacob, 1994). On the other hand, Ca^{2+}-permeable channels not operated by stores have also been associated with apoptosis including NMDA receptors (Ankarcrona *et al.* 1995) and the type 3 InsP_3 receptor (Khan *et al.* 1996).

In agreement with the importance of Ca^{2+} influx in some cases of cell death, inhibiting the expression of the type 3 $InsP_3$ receptor in the plasma membrane of lymphocytes blocked dexamethasone-induced apoptosis. Furthermore, Antonsson *et al.* (1997) have shown that Bax, an inducer of apoptosis of the Bcl-2 family of proteins, was able to increase plasma membrane permeability in different types of cells, probably by forming non-selective ion channels. This effect was readily blocked by Bcl-2, a typical inhibitor of apoptosis. Thus, a link between pore-forming activity and the induction of apoptosis has been suggested.

Nevertheless, electrophysiological characterization of ion channels associated with apoptosis, particularly Ca^{2+}-permeable channels, has not been addressed before. To study this type of channel, we have combined $[Ca^{2+}]_i$ measurements with patch clamp recordings in a human prostatic cancer cell line (LNCaP). A Ca^{2+}-permeable, non-selective cation channel of 23 pS conductance, unrelated to SOC channels, was activated by two different conditions that trigger apoptosis in these cells. The channel herein described seems to be a good candidate for mediating Ca^{2+} influx in prostatic cells undergoing apoptosis (Furuya *et al.* 1994). A preliminary account of these data has been presented elsewhere (Gutiérrez *et al.* 1997).

METHODS

Cell culture

The human prostate cancer cell line, LNCaP, was obtained from the American Type Culture Collection (ATCC) and used between passages 27 and 32. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin–streptomycin. All tissue culture reagents were from Gibco BRL (Gaithersburg, MD, USA). Electrophysiological and $[Ca^{2+}]_i$ measurements were made with cells bathed in a Hepes-buffered saline (HBS) solution containing (mM): 130 NaCl, 4 KCl, 2 $NaHCO_3$, 1 $MgSO_4$, 2 $CaCl_2$, 10 glucose, 10 Hepes; adjusted to pH 7·4 with NaOH. Ionomycin (Sigma) and thapsigargin (RBI) were prepared as 5 mM and 100 μM stock solutions in dimethyl sulphoxide (Hybri-Max, Sigma), respectively.

Cell viability assay

Cells were trypsinized and resuspended in RPMI 1640 supplemented with FBS (10%) to a final density of 2×10^5 cells ml^{-1}. This suspension (50 μl) was plated in 96 multi-well dishes and allowed to recover for 18 h. Afterwards, cells were either kept in supplemented RPMI or in Hepes-buffered saline (HBS) solution with or without ionomycin. Cell viability was measured using a modified MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide), as previously described (Harris *et al.* 1994) after an incubation period of 24 h.

DNA fragmentation assay

LNCaP cells ($1·5 \times 10^7$ cells) were washed and lysed in 0·5 ml of lysis buffer (140 mM NaCl, 1·5 mM $MgCl_2$, 0·5% NP40 detergent, 10 mM Tris–HCl, pH 7·4) for 30 min at 4 °C after an incubation of 14 h in apoptotic conditions. Nuclei were pelleted at 10 000 *g* for 10 min at 4 °C and resuspended in hypertonic solution (mM: 350 NaCl, 1·5 $MgCl_2$, 1 DTT, 10 Tris–HCl, pH 7·4) for 30 min at 4 °C. Fragmented DNA was separated by centrifugation at 4000 *g* for 10 min at 4 °C and extracted twice from the supernatant. After that, it was separated by electrophoresis in 2% agarose gels and revealed with ethidium bromide.

Simultaneous patch clamp and $[Ca^{2+}]_i$ measurements in single cells

LNCaP cells (10^6 cells) were trypsinized and loaded either with 0·5 μM fura-2 AM (Molecular Probes) or with fura-2 AM plus 18 μM BAPTA AM in 1 ml of supplemented RPMI medium. After 1 h incubation at room temperature, cells were washed and resuspended in HBS solution and used within a 6 h period. The 340 nm/380 nm fluorescence ratio of LNCaP cells in HBS solution (unless otherwise indicated) was recorded from single cells every 50 ms with a dual wavelength microfluorometer, model RF-F3010 (Photon Technology International, South Brunswick, NJ, USA) mounted on a Nikon TMD microscope. Local application of ionomycin was carried out with a pneumatic picopump PV830 (World Precision Instruments, Sarasota, FL, USA) operated at 4 p.s.i. and using micropipettes of < 4 MΩ resistance (when filled with HBS solution) and placed next to the cell. Microscope calibration and $[Ca^{2+}]_i$ were obtained as described elsewhere (Guerrero *et al.* 1994*a*) with a K_D of 200 nM. Under our recording conditions the parameters of the Grynkiewicz equation were $R_{max} = 4·078 \pm 0·477$, $R_{min} = 0·204 \pm 0·023$ and $\beta = 8·014 \pm 0·117$ for 41 calibrations (Grynkiewicz *et al.* 1985), where R_{max} is the fluorescence ratio in the presence of saturating Ca^{2+} and R_{min} is the fluorescence ratio in the absence of Ca^{2+}. Analysis of the data was based on $[Ca^{2+}]_i$ but the fluorescence ratio was chosen to illustrate the data in most of the figures. Current- and voltage clamp experiments were carried out in the perforated patch configuration simultaneously with $[Ca^{2+}]_i$ measurements of LNCaP cells in HBS at room temperature (Rae *et al.* 1991; Guerrero *et al.* 1994*b*). Borosilicate patch pipettes (WPI) of 4–5 MΩ resistance were used, filled with the following solution (mM): 60 K_2SO_4, 11 NaCl, 30 KCl, 1 $MgSO_4$, 0·1 EGTA, 10 Hepes–NaOH, pH 7·2, for recording membrane potential. A caesium-based pipette solution (mM): 60 Cs_2SO_4, 30 CsCl, 11 NaCl, 1 $MgSO_4$, 0·1 EGTA, 10 Hepes–NaOH, pH 7·2, was used to reduce K^+ conductance in current clamp and for all voltage clamp experiments. Series resistances below 30 MΩ were obtained by using 150 μg ml^{-1} of amphotericin B and 30 μg ml^{-1} of Pluronic F-127 in these pipette solutions (0·3% DMSO). Single channel recordings in the cell attached configuration of the patch clamp technique (Hamill *et al.* 1981) were carried out simultaneously with $[Ca^{2+}]_i$ measurements. Plasma membrane potential was zeroed using a high potassium solution (mM): 8 NaCl, 130 KCl, 2 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 Hepes–NaOH, pH 7·4. Unitary currents were filtered at 200 Hz (4-pole low-pass Bessel filter) and digitized by Digidata 1200 running Axotape (Axon Instruments) at 1 kHz. For recording ionomycin-induced single channel activity, the pipette Na^+–Ca^{2+} solution contained (mM): 140 NaCl, 2 $CaCl_2$, 20 Hepes–NaOH, pH 7·4, and patch potential was held at −100 mV unless otherwise indicated. Ca^{2+} permeability was assessed by having 110 mM of $CaCl_2$ or calcium glutamate as the only permeant ion in the pipette solution. In general, multiple channels were present in the membrane patch so the open probability (P_o) multiplied by the number of channels (N) was obtained by: $NP_o = \Sigma (i_t - i_b)\Delta t / T i_u$, where i_t is the single channel recorded ion current, i_b is baseline current, Δt is sampling time, T is total time (8 s) and i_u is the unitary current calculated from all-points amplitude histograms (Logothetis *et al.* 1987). Results are expressed as means ± S.E.M.; Student's t test was employed for all statistical analysis.

RESULTS

Ionomycin and serum removal induce apoptosis in LNCaP cells

It has previously been demonstrated that a sustained increase in $[Ca^{2+}]_i$ due to Ca^{2+} influx through the plasma membrane is associated with apoptosis of prostate cancer cells (Martikainen *et al.* 1991; Furuya *et al.* 1994). Using DNA ladder fragmentation and MTT assay indicated that ionomycin, a calcium ionophore, and the absence of serum, both induced time-dependent apoptosis. In agreement with previous reports of prostatic cancer cells (Martikainen *et al.* 1991), LNCaP cells required high concentrations of ionomycin (10 μM) to undergo apoptosis (Fig. 1). Lower concentrations (1 μM) could not increase the rate of cell death significantly, either with or without serum. Therefore, comparing the effects of 1 and 10 μM ionomycin should highlight those events associated with apoptosis. Furthermore, cell incubation in the absence of serum triggered apoptosis as well. This condition potentiated the effect of lethal doses of ionomycin (Fig. 1). This is in agreement with the protective role of serum in apoptosis (Shibasaki & McKeon, 1995). Thus, 10 μM ionomycin and the absence of serum induced death by apoptosis of LNCaP cells.

Changes in $[Ca^{2+}]_i$ and membrane potential of LNCaP cells by lethal doses of ionomycin

To study activation of Ca^{2+}-permeable channels during apoptosis of LNCaP cells, we have combined patch clamp recording with simultaneous $[Ca^{2+}]_i$ measurements at the single cell level. LNCaP cells presented a resting $[Ca^{2+}]_i$ of $91{\cdot}5 \pm 7{\cdot}0$ nM ($n = 27$), a stable membrane potential of $-39{\cdot}5 \pm 2$ mV with an input resistance always greater than

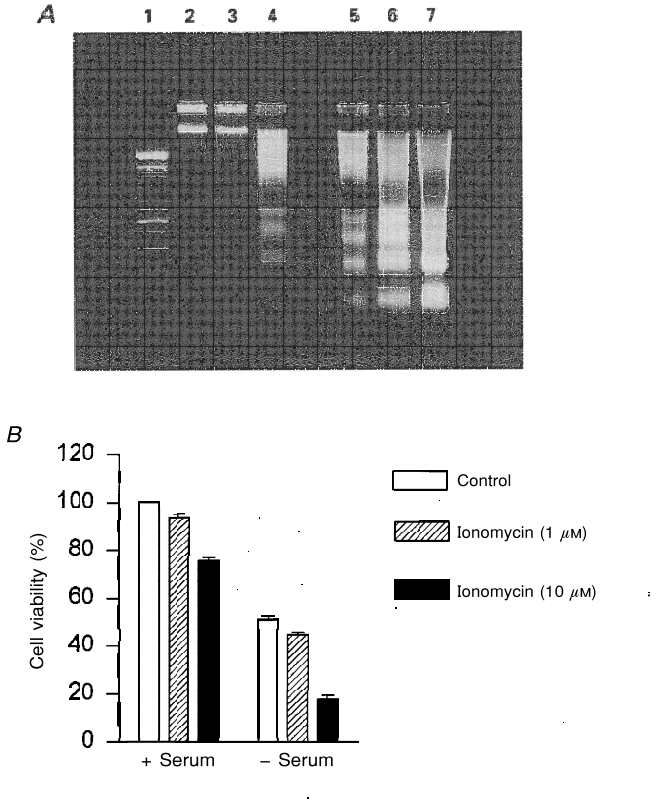

Figure 1. Induction of apoptosis in LNCaP cells by ionomycin or serum removal

A, 2% agarose gel showing DNA degradation in the ladder pattern characteristic of apoptosis. Lane 1, size standards (100 bp ladder). DNA from LNCaP cells in serum-supplemented RPMI medium (lanes 2–4) or HBS (lanes 5–7). Cells were challenged with 0 (lanes 2 and 5), 1 μM (3 and 6) or 10 μM (4 and 7) ionomycin for 14 h prior to the analysis of DNA fragmentation. *B*, MTT assay for cell viability. Cells in the presence (+) or the absence (–) of serum were incubated with ionomycin at the indicated concentrations for 24 h. Cell survival was calculated as the percentage of cells that convert MTT into formazan salts normalized by cells grown in serum and without ionomycin ($n = 6$).

1 GΩ ($n = 27$), and a membrane capacitance of 18·4 ± 1·2 pF ($n = 27$).

Application of 10 μM ionomycin by pressure ejection from a micropipette for 90 s produced a biphasic increase in $[Ca^{2+}]_i$ (Fig. 2*A*). The first rise in $[Ca^{2+}]_i$ was observed almost without delay, reaching 724·4 ± 38·2 nM ($n = 24$) at 12·5 s after the application of ionomycin. This first increase in $[Ca^{2+}]_i$ was associated with membrane hyperpolarization to −77·6 ± 3·4 mV ($n = 18$) which is very close to the equilibrium potential for K^+ (Fig. 2*B*).

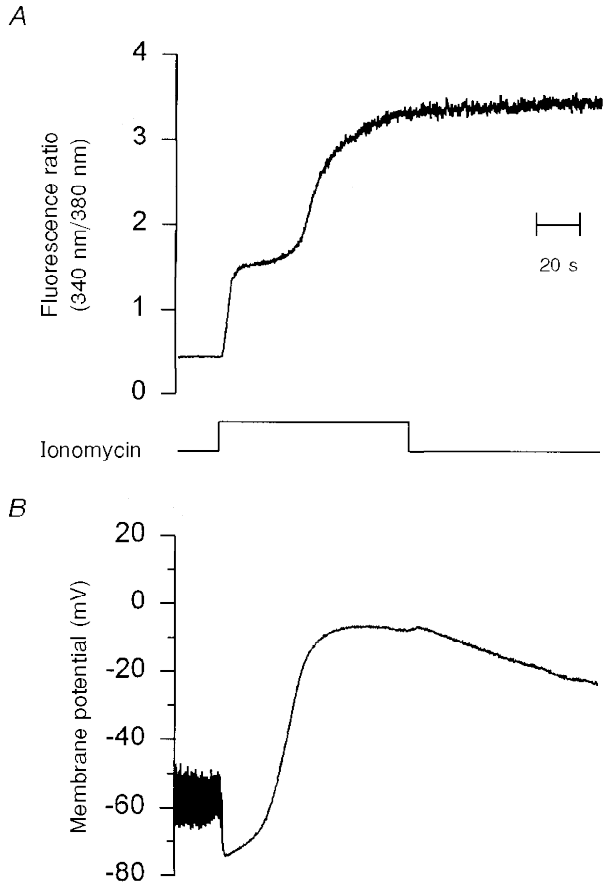

Figure 2. $[Ca^{2+}]_i$ and membrane potential changes induced by lethal doses of ionomycin (10 μM)

A shows the time course of the changes in $[Ca^{2+}]_i$ (340 nm/380 nm fluorescence ratio) elicited by pressure ejection of 10 μM ionomycin from a micropipette for the indicated time (bottom trace). *B* shows the changes in plasma membrane potential and input resistance recorded simultaneously with $[Ca^{2+}]_i$ for the same cell. Input resistance was monitored by applying a 10 pA hyperpolarizing current pulse with a frequency of 1 Hz. Initially, membrane potential and membrane resistance were −50 mV and 1·5 GΩ, respectively. The application of ionomycin produced the first rise in $[Ca^{2+}]_i$ which in turn increased membrane conductance and transiently hyperpolarized the cell to about −75 mV. Later, membrane potential went close to −10 mV in association with the second increment in $[Ca^{2+}]_i$. HBS solution in the bath and K_2SO_4-based solution in the pipette (see Methods). Time scale applies to all traces.

Conceivably, this hyperpolarization was due to the opening of Ca^{2+}- and voltage-dependent, high conductance potassium (maxi-K) channels, present in these cells (Fig. 3). In the cell attached configuration at 0 mV and resting $[Ca^{2+}]_i$, maxi-K channel activity was 0·25 (Fig. 3*A*). This implies a very high level of Ca^{2+} sensitivity for these channels (Carl *et al.* 1996) and, in agreement with that, a steep voltage dependence was obtained (Fig. 3*C*). Furthermore, transient increments in $[Ca^{2+}]_i$ induced by low concentrations of ionomycin resulted in the complete and reversible activation of this channel (Fig. 3*D*). This is expected for high Ca^{2+}-sensitive maxi-K channels, characteristic of secretory cells.

The second increment in $[Ca^{2+}]_i$ occurred 52·7 ± 4·1 s ($n = 24$) after the application of 10 μM ionomycin (Fig. 2*A*). At this time, $[Ca^{2+}]_i$ reached 2·5 μM, and represents the inflection point for the second increment in $[Ca^{2+}]_i$. This second rise eventually produced the virtual saturation of fura-2 by reaching a 340 nm/380 nm ratio of 3·4 ± 0·05 ($n = 22$), very close to our R_{max} of 4. This ratio represents a $[Ca^{2+}]_i$ of 9·9 ± 0·9 μM ($n = 22$) which is clearly beyond the resolution of fura-2.

Membrane depolarization preceded the second phase of the ionomycin-induced increase in $[Ca^{2+}]_i$ by 16·6 ± 3·1 s ($n = 6$). A correlation was observed, where smaller membrane depolarizations were followed by lower $[Ca^{2+}]_i$. This suggests that the occurrence of the second increment in $[Ca^{2+}]_i$ depends on the activation of the depolarizing conductance.

To study such depolarizing conductance, we reduced the contribution of K^+ channels by using Cs^+ in the pipette solution of perforated patch recording (Fig. 4). Resting membrane potential changed to −25·7 ± 2·2 mV ($n = 15$) and the 10 μM ionomycin-induced depolarization was more evident; however, this was still close to 0 mV (Fig. 4*B*). With Cs^+ inside the cell, depolarization peaked slightly above 0 mV, which could be due to a higher permeability for Na^+ than Cs^+; however, this was not tested any further. Nevertheless, the biphasic increase in $[Ca^{2+}]_i$ induced by 10 μM ionomycin was not importantly changed by internal dialysis with Cs^+ in the recording pipette solution (Fig. 4*A*). Thus, the depolarizing conductance was similar with either K^+ or Cs^+ inside the cell, suggesting this was a non-selective conductance and large enough to surpass the hyperpolarizing effect of maxi-K channels.

Conversely, the application of 1 μM ionomycin induced only the first increment in $[Ca^{2+}]_i$ in LNCaP cells (Fig. 5*A*). $[Ca^{2+}]_i$ was elevated from 93·2 ± 11 to 508·4 ± 37·3 nM in 45·9 ± 8·6 s ($n = 12$). This increment was significantly smaller and took longer to peak in comparison to the one produced by 10 μM ionomycin in the application pipette. Besides, no significant activation of a depolarizing conductance was observed when cells were exposed to 1 μM ionomycin, even when K^+ channels were blocked by Cs^+ to facilitate observation of any change in membrane conductance (Fig. 5*A*). The latter implies that this concentration of ionomycin did not induce any significant

activation of an electrophoretic Ca^{2+} influx. To test further the role of Ca^{2+} influx via plasma membrane ion channels, plasma membrane potential was held at +70 mV ($n = 6$), to reduce the driving force for Ca^{2+} influx. In these conditions, 1 μM ionomycin produced a similar change in $[Ca^{2+}]_i$ to that in unclamped cells (Fig. 5*B*). This change in $[Ca^{2+}]_i$ was also similar when 1 μM ionomycin was applied to cells held at −70 mV (not shown). Thus, non-lethal doses of ionomycin did not activate Ca^{2+}-permeable channels. Moreover, these results indicate that the second rise in $[Ca^{2+}]_i$ was strictly

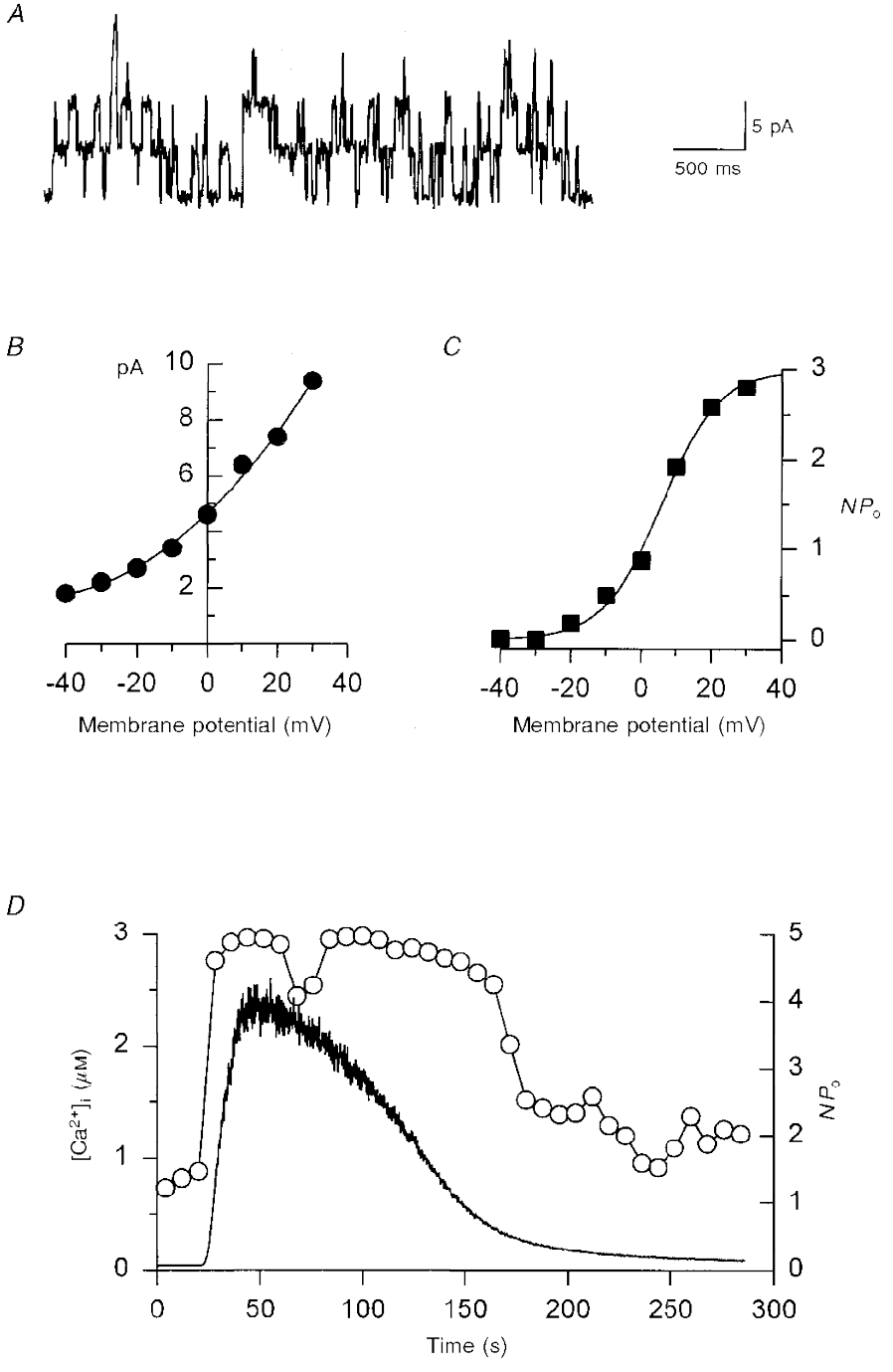

Figure 3. Characterization of a voltage- and Ca^{2+}-dependent, high conductance K^+ channel (maxi-K) of LNCaP cells

A, single channel recording obtained in the cell attached configuration with high K^+ solution in the bath and Na^+–Ca^{2+} solution in the pipette (see Methods) at a holding potential of 0 mV and resting $[Ca^{2+}]_i$. Openings are in the upward direction. *B* and *C*, current–voltage curves of single channel current (*B*) and steady-state voltage dependency (*C*) for ion channels depicted in *A*. Boltzmann equation fitting is shown as a continuous line with a $V_{½}$ of 5·8 mV and a slope factor, k of 8·3 mV. *D*, simultaneous recording of maxi-K channel activity in the cell attached configuration (NP_o, right axis, ○) and the change in $[Ca^{2+}]_i$ (left axis, continuous line), induced by the application of 1 μM ionomycin for 30 s (beginning at 20 s).

associated with the appearance of a non-selective conductance, which was activated by ionomycin, but only at concentrations that induce apoptosis of LNCaP cells.

Different sources of Ca^{2+} are involved in the ionomycin-induced increment in $[Ca^{2+}]_i$

To provide evidence for calcium release from internal stores being the source of Ca^{2+} for the first rise in $[Ca^{2+}]_i$, 10 μM ionomycin was applied to cells in the absence of external Ca^{2+} (Fig. 6*A*). The initial rise in $[Ca^{2+}]_i$ was not affected ($n = 6$). However, the second increment was completely abolished. Internal Ca^{2+} stores were also depleted by incubating cells with 100 nM thapsigargin for 30 min. The treatment with thapsigargin elevated basal $[Ca^{2+}]_i$ to 275·9 ± 42·4 nM ($n = 10$; Fig. 6*A*), probably by the activation of capacitative Ca^{2+} influx. In these thapsigargin-treated cells, 10 μM ionomycin increased $[Ca^{2+}]_i$ by only 215·3 ± 64·8 nM in the first phase ($n = 4$), whereas $[Ca^{2+}]_i$ rose by 633 ± 38·6 nM ($n = 24$) in cells not exposed to thapsigargin. In thapsigargin-treated cells the second increment in $[Ca^{2+}]_i$ reached 7·9 ± 0·9 μM ($n = 4$) and was observed at 52·2 ± 8·2 s (Fig. 6*A*). The second increment in $[Ca^{2+}]_i$ was therefore not significantly affected by depleting internal stores in comparison to non-treated cells.

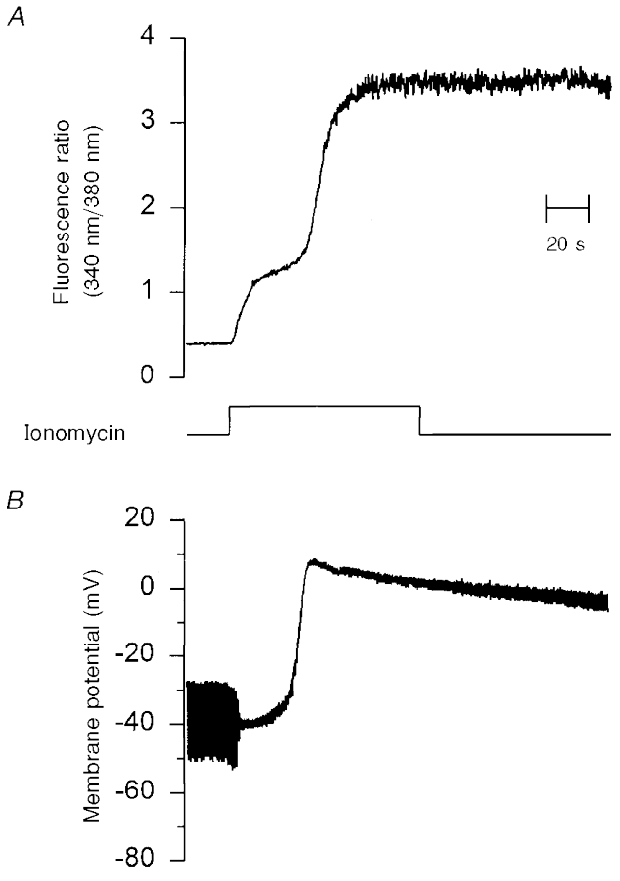

Figure 4. Changes in $[Ca^{2+}]_i$ and membrane potential induced by 10 μM ionomycin in Cs^+-loaded LNCaP cells

Similar conditions to the experiment shown in Fig. 2, except that Cs_2SO_4 solution was used in the recording pipette (see Methods). *A* shows the ionomycin-induced increase in $[Ca^{2+}]_i$ indicated by the fura-2 fluorescence ratio (340 nm/380 nm). *B* is the simultaneous recording of plasma membrane potential and input resistance of the same cell. Resting membrane potential and input resistance were −30 mV and 2 GΩ, respectively. The application of 10 μM ionomycin produced a slight hyperpolarization to −40 mV and also an increase in membrane conductance followed by a transient depolarization to +5 mV. A gradual reduction in the membrane conductance is evident by the end of the trace. This suggests that the non-selective cation channel opens only transiently. Time scale is the same for all traces.

As expected, the second rise in $[Ca^{2+}]_i$ induced by ionomycin was the only phase sensitive to changes in plasma membrane potential to alter the driving force for Ca^{2+} influx (Fig. 6*B*). At +70 mV, the first increase in $[Ca^{2+}]_i$ (710·6 ± 38·2 nM, $n = 8$) was not affected; however, the second increment in $[Ca^{2+}]_i$ (1·5 ± 0·3 μM, $n = 8$) was substantially smaller. Conversely, when the membrane potential was held at −70 mV ($n = 6$), the ionomycin-induced changes in $[Ca^{2+}]_i$ were similar to those observed in current clamp conditions (Fig. 6*B*). These results suggest that the first rise in $[Ca^{2+}]_i$ induced by ionomycin has an important component due to Ca^{2+} release from thapsigargin-sensitive internal Ca^{2+} stores. This would explain why the first increment in $[Ca^{2+}]_i$ was not affected by changing the plasma membrane potential. The second increase in $[Ca^{2+}]_i$ required external calcium, was reduced by holding the membrane potential at +70 mV (which strongly lowers the driving force for Ca^{2+} influx) and finally, was not affected by previously depleting internal Ca^{2+} stores with thapsigargin. Consequently, the second rise in $[Ca^{2+}]_i$ was probably due to the activation of a Ca^{2+}-permeable ion channel at the plasma membrane.

Accordingly, 1 μM ionomycin could not increase $[Ca^{2+}]_i$ (−6·5 ± 25·8 nM, $n = 4$) if internal stores had previously been depleted with 100 nM thapsigargin (not shown). The latter and the data shown in Fig. 5 indicate that non-lethal concentrations of ionomycin increase $[Ca^{2+}]_i$, almost exclusively, by mobilizing internal Ca^{2+} stores since no evidence was found for the activation of an electrophoretic Ca^{2+} influx mechanism at the plasma membrane.

Thus, the capability of ionomycin to induce apoptosis appears to correlate with the activation of Ca^{2+} influx through the plasma membrane, most probably by inducing an endogenous non-selective, voltage-independent, Ca^{2+}-permeable cation channel.

Serum removal increases Ca^{2+} permeability in LNCaP cells

LNCaP cells die by apoptosis in the absence of serum (Fig. 1). The effect of membrane potential on $[Ca^{2+}]_i$ was studied to assess the Ca^{2+} permeability of serum-deprived cells (Fig. 7). Approximately two-thirds of cells maintained in the absence of serum for 8 h had resting $[Ca^{2+}]_i$ higher than 300 nM. Although this suggests an increased Ca^{2+} permeability, this was difficult to verify because unreliable perforated patch recordings were obtained in these cells due

to bad seals or extremely high leak current. Therefore, only serum-deprived cells with basal $[Ca^{2+}]_i$ near 100 nM could be studied (Fig. 7*B*). In the latter cells, cycling of the membrane potential between −70 and +70 mV unmasked an increased Ca^{2+} permeability ($n = 10$). This was evident as membrane hyperpolarization elevated the initial rate of rise in $[Ca^{2+}]_i$, while membrane depolarization was associated with a reduction in $[Ca^{2+}]_i$. The gradual increment in the rate of rise of $[Ca^{2+}]_i$ was apparent when Ca^{2+} transients were fitted by single exponentials; the kinetic constant of the fourth Ca^{2+} transient was 86% faster compared with the second one (Fig. 7*B*). Nevertheless, the same type of analysis applied to the falling phase showed that the rate of Ca^{2+} extrusion was very similar among the different Ca^{2+} transients (Fig. 7*B*). It is not clear how oscillations of membrane potential promote the appearance of this Ca^{2+} permeability. However, they do not seem to be necessary because this Ca^{2+} conductance could also be activated when membrane potential was held constant at negative values (not shown). Conversely, this increased Ca^{2+} permeability of serum-deprived cells was never observed in those cells kept in serum (Fig. 7*A*). Thus, the absence of serum induces a Ca^{2+}-permeable ionic conductance in LNCaP cells.

Characterization of Ca^{2+}-permeable channels activated by inducers of apoptosis

To identify the channel responsible for the second rise in $[Ca^{2+}]_i$, single channel recordings in the cell attached configuration were carried out during the application of 10 μM ionomycin. High external K^+ concentration was used to avoid interference by changes in the plasma membrane potential. Resting $[Ca^{2+}]_i$ was higher (171·7 ± 19·8 nM, $n = 16$) than in control cells. However, the effect of 10 μM ionomycin on $[Ca^{2+}]_i$ was similar to those cells kept in HBS (first rise, 664·8 ± 53·7 nM; second rise, 10 ± 2·1 μM; $n = 16$). Interestingly, the second rise in $[Ca^{2+}]_i$ in the presence of high K^+ was significantly delayed (87·5 ± 8·3 s, $n = 14$; $P < 0·001$) with respect to that obtained in HBS (see inset in Fig. 10).

Ionomycin (10 μM) induced the activation of a non-selective cation channel (Fig. 8*A* and *B*) recorded in the cell-atttached configuration of patch clamp. With 140 mM NaCl and 2 mM $CaCl_2$ in the pipette solution, the slope conductance of this channel was 23·5 ± 2 pS ($n = 4$) and the extrapolated reversal potential was 0·03 ± 2 mV, (Fig. 8*C*). An inward single channel current was also activated by 10 μM ionomycin

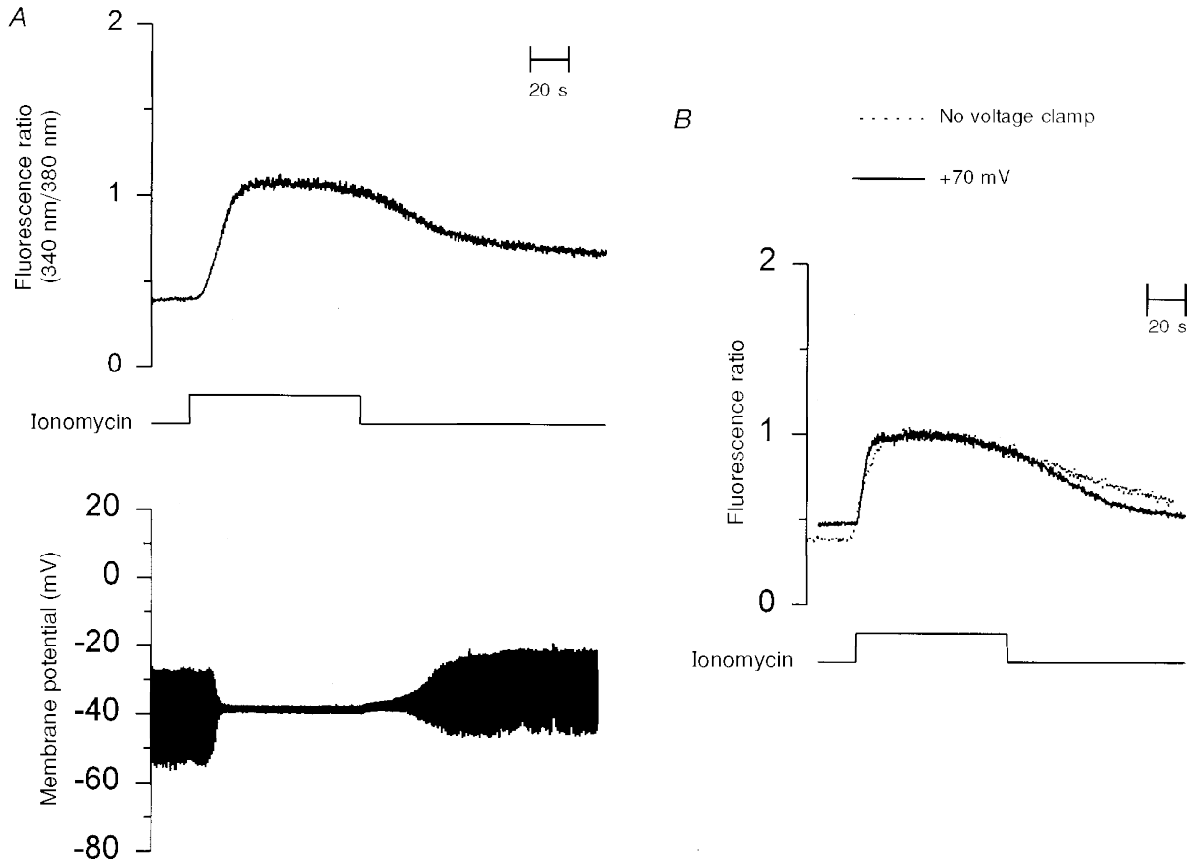

Figure 5. A non-lethal dose of ionomycin (1 μM) cannot increase electrophoretic Ca^{2+} influx

Caesium-loaded LNCaP cells in HBS solution under current clamp (*A*) or voltage clamp (*B*, continuous line) were exposed to 1 μM ionomycin for the time indicated. Input resistance was measured by applying 10 pA hyperpolarizing pulses at a frequency of 1 Hz. In *B*, changes in $[Ca^{2+}]_i$ induced by ionomycin (1 μM) of a cell held at +70 mV throughout recording (continuous line) are compared with those from a non-voltage clamped cell (dotted line). Membrane potential was close to −80 mV as long as $[Ca^{2+}]_i$ was elevated (not shown).

if Ca^{2+} was the only permeant cation in the pipette solution (Fig. 8*B*); however, the slope conductance was reduced to 12 ± 1 pS in this case ($n = 3$). This implies a small but finite Ca^{2+} permeability for this channel. Membrane depolarization slightly increased the steady-state activity of the 23 pS channel (Fig. 8*D*). However, it is unlikely that this channel is directly gated by voltage because important activation can be induced by ionomycin even at −100 mV membrane potential (Fig. 10).

Three different approaches were carried out to test whether the 23 pS channel was Ca^{2+} dependent. One was to load cells heavily with BAPTA, a Ca^{2+} chelator, by incubating them with 18 μM BAPTA AM (Fig. 9). In these cells, ionomycin could not produce the initial increment in $[Ca^{2+}]_i$ (Fig. 9*A*); however, the steady-state activity of the 23 pS channel was similar (compare 0·388 in Fig. 8*D* with 0·342 in Fig. 9*B*).

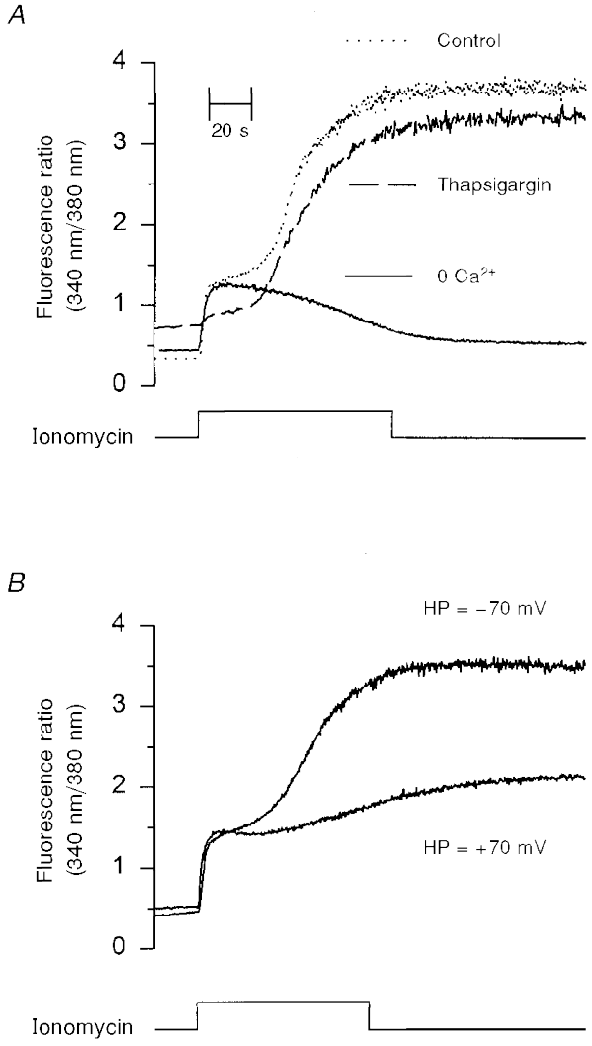

Figure 6. Sources of Ca^{2+} utilized by ionomycin to increase $[Ca^{2+}]_i$ in LNCaP cells

A, changes in $[Ca^{2+}]_i$ in response to 10 μM ionomycin in control cells (dotted line), in the absence of extracellular Ca^{2+} using a calcium-free HBS solution supplemented with 0·5 mM EGTA (continuous line) and in cells treated with thapsigargin (100 nM) to deplete internal Ca^{2+} stores (dashed line) are compared in this panel. The first increment in $[Ca^{2+}]_i$ was not affected by the absence of external Ca^{2+}; however, it was virtually abolished by treating cells with thapsigargin. The second increment in $[Ca^{2+}]_i$ required external Ca^{2+} and it was not significantly reduced by thapsigargin. *B*, LNCaP cells under voltage clamp at the indicated holding membrane potential (HP) were exposed to 10 μM ionomycin for the time indicated. In agreement with *A*, reducing the driving force for Ca^{2+} influx greatly attenuated the second increment in $[Ca^{2+}]_i$ without affecting the first rise in $[Ca^{2+}]_i$. All traces are representative of at least three experiments (see text). The time scale applies to all traces.

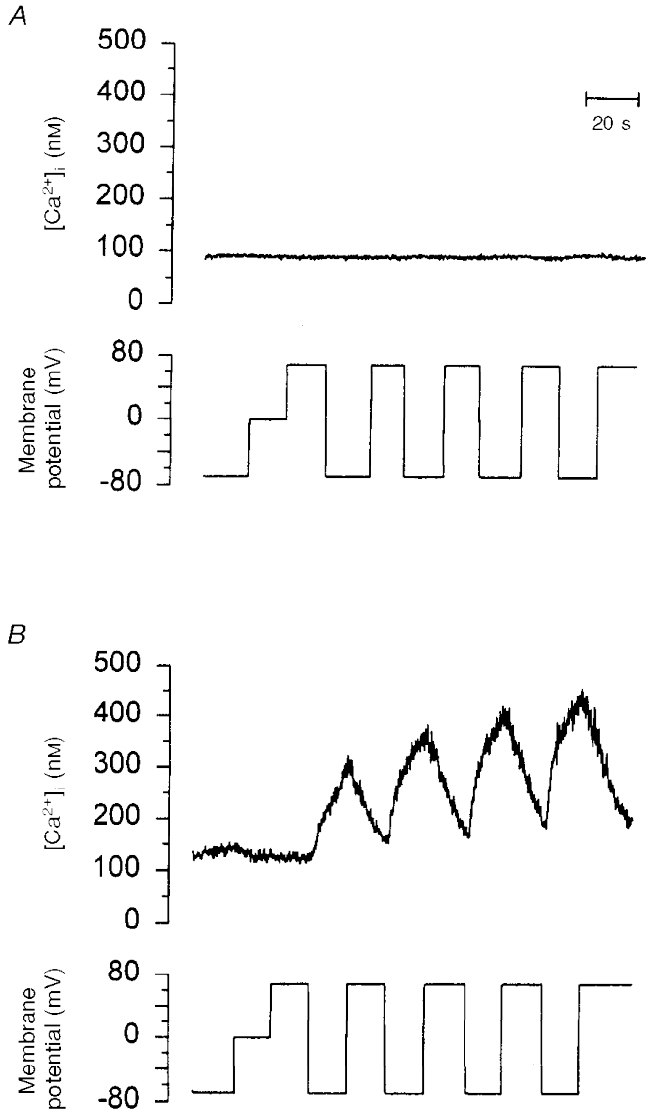

Figure 7. Serum removal increases Ca^{2+} permeability in LNCaP cells

A, Ca^{2+} permeability of LNCaP cells cultured in the presence of serum was studied by recording the effect on $[Ca^{2+}]_i$ of the indicated changes in membrane potential. *B*, LNCaP cells deprived of serum for 8 h were loaded with fura-2 and voltage clamped similarly to those cells in *A*, to assess the effect of membrane potential variations on $[Ca^{2+}]_i$. Note that $[Ca^{2+}]_i$ increases only during hyperpolarization.

Additionally, the activity of the 23 pS channel was not increased by exposing excised membrane patches to 2 mM Ca^{2+} in the bath solution ($n = 5$). Finally, excised membrane patches in 2 mM Ca^{2+} bathing solution from cells that had not been exposed to ionomycin did not show any 23 pS channel activity ($n = 15$). All these data argue against the 23 pS channel being directly activated by an increment in $[Ca^{2+}]_i$.

The 23 pS channel was not open at the resting $[Ca^{2+}]_i$ nor during the ionomycin-induced first increment in $[Ca^{2+}]_i$ ($n = 24$, Fig. 10). The application of 1 μM ionomcyin did not activate the channel either ($n = 5$, not shown). However, the activation of a 23 pS channel was evident during the second rise in $[Ca^{2+}]_i$ (Fig. 10). This was true in 18 out of 24 cells studied, where at least one channel was activated in the patch. It is possible that in the remaining 6 cells we had just patched plasma membrane regions devoid of the 23 pS channel. Current clamp experiments showed that input resistance was transiently reduced by lethal doses of ionomycin (Fig. 4). This suggests that the non-selective ion channel does not activate permanently. Accordingly, the 23 pS channel was also transiently activated as shown in the two examples of Fig. 10.

A 23 pS channel was activated in 9 out of 15 cells that were held in the absence of serum for at least 8 h. However, in this case the ion channel activity was lower than that induced by 10 μM ionomycin. This could be the explanation for the smaller Ca^{2+} transients obtained in serum-deprived cells (Fig. 7).

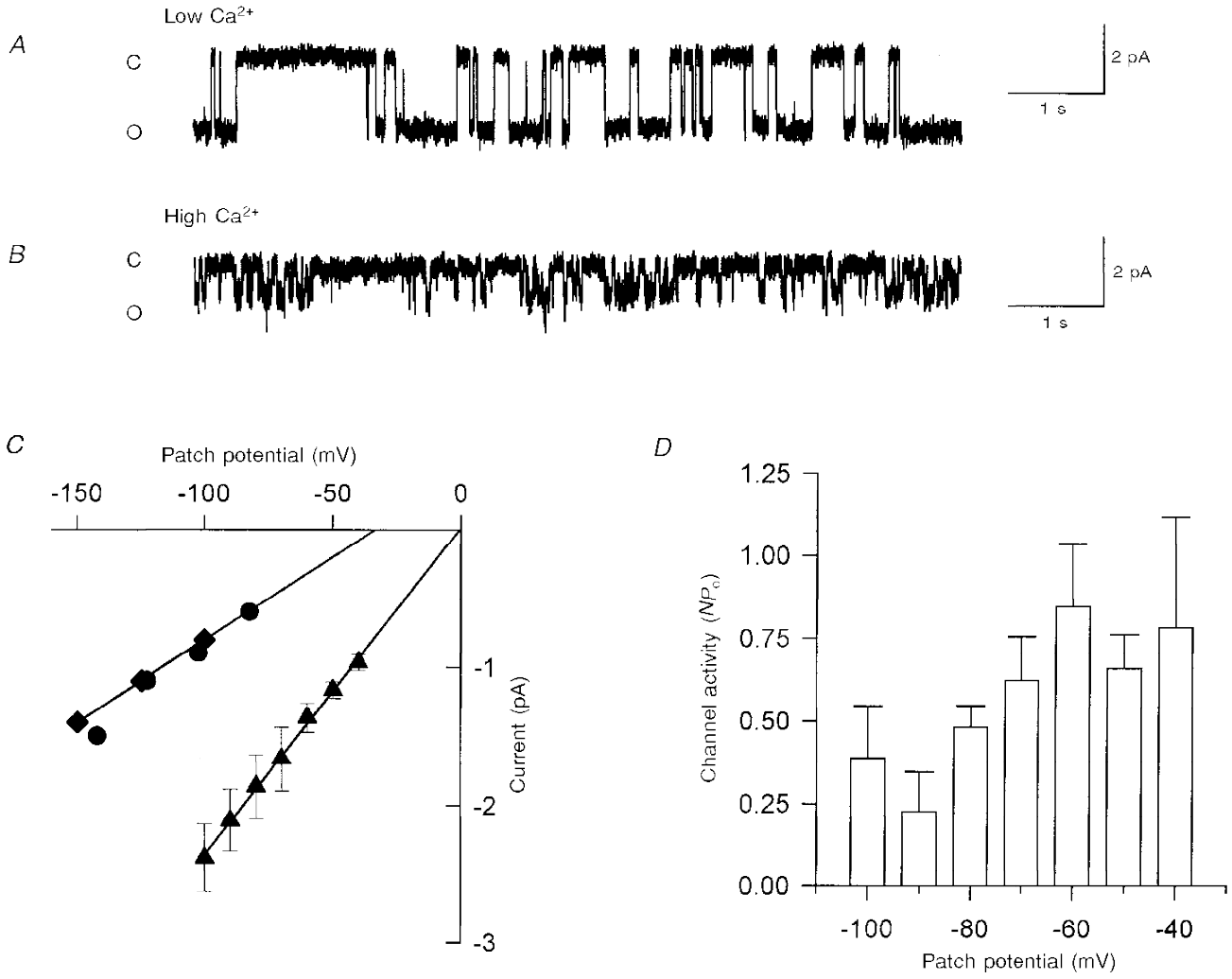

Figure 8. Characterization of ion channels associated with the second rise in $[Ca^{2+}]_i$

A, single channel current records in the cell attached configuration with a patch potential of −100 mV and Na^+–Ca^{2+} solution (see Methods). *B*, single channel recordings with 110 mM $CaCl_2$ in the pipette solution and at −100 mV patch potential. Closed (C) and open (O) state levels are indicated on the left side of the record. *C*, current–voltage curves obtained in Na^+–Ca^{2+} solution (▲), 110 mM $CaCl_2$ (◆) or 110 mM calcium glutamate (●) as charge carriers. The extrapolated reversal potential is indicated assuming no rectification occurred. *D*, mean ± standard deviation of the steady-state activity of the 23 pS channel is shown at the indicated membrane potentials ($n = 4$).

Thapsigargin, another inducer of apoptosis in prostate cells (Furuya *et al.* 1994), induced the same 23 pS channel, although at a very low level of activity and in only 5 out of 14 cells that were exposed to 1 μM thapsigargin for at least 2 h. Thapsigargin (100 nM), which still induces apoptosis in LNCaP cells (not shown), failed to activate the 23 pS channel. Whether this is due to our recording conditions (i.e. high K^+ solution delays the activation of Ca^{2+} influx by ionomycin, Fig. 10) or that the 23 pS channel does not play a role in thapsigargin-induced cell death, needs further studies. Nevertheless, the latter possibility is in agreement with recent evidence that Ca^{2+} influx does not seem to be important for thapsigargin-induced apoptosis (Bian *et al.* 1997).

Thus, the 23 pS ion channel activated by ionomycin and serum removal is a Ca^{2+}-permeable, Ca^{2+}-independent, non-selective cation channel, which does not require membrane depolarization to open. This channel could participate as a Ca^{2+} influx mechanism in apoptosis and is not controlled by depletion of internal Ca^{2+} stores.

DISCUSSION

This study demonstrates the activation of a Ca^{2+}-permeable, non-selective cation channel of 23 pS conductance in prostatic cancer cells (LNCaP) by two different inducers of apoptosis, ionomycin and serum removal. This channel appears to be activated by a novel, but still undefined, mechanism.

Membrane depolarization preceded and was strictly associated with the second rise in $[Ca^{2+}]_i$ induced by lethal doses of ionomycin. This could imply that a membrane depolarization is needed for the 23 pS channel to open. The latter does not seem to be the case, since Ca^{2+} influx triggered by 10 μM ionomycin was not inhibited by holding membrane potential at −70 mV and the 23 pS channel could be activated at −100 mV and showed only a slight increase in NP_o with membrane depolarization. It seems then, that the 23 pS channel is not directly gated by membrane depolarization. However, it could be that membrane potential modulates the appearance of the 23 pS channel. Membrane depolarization by bathing cells in a high K^+ solution greatly delayed the second increment in $[Ca^{2+}]_i$,

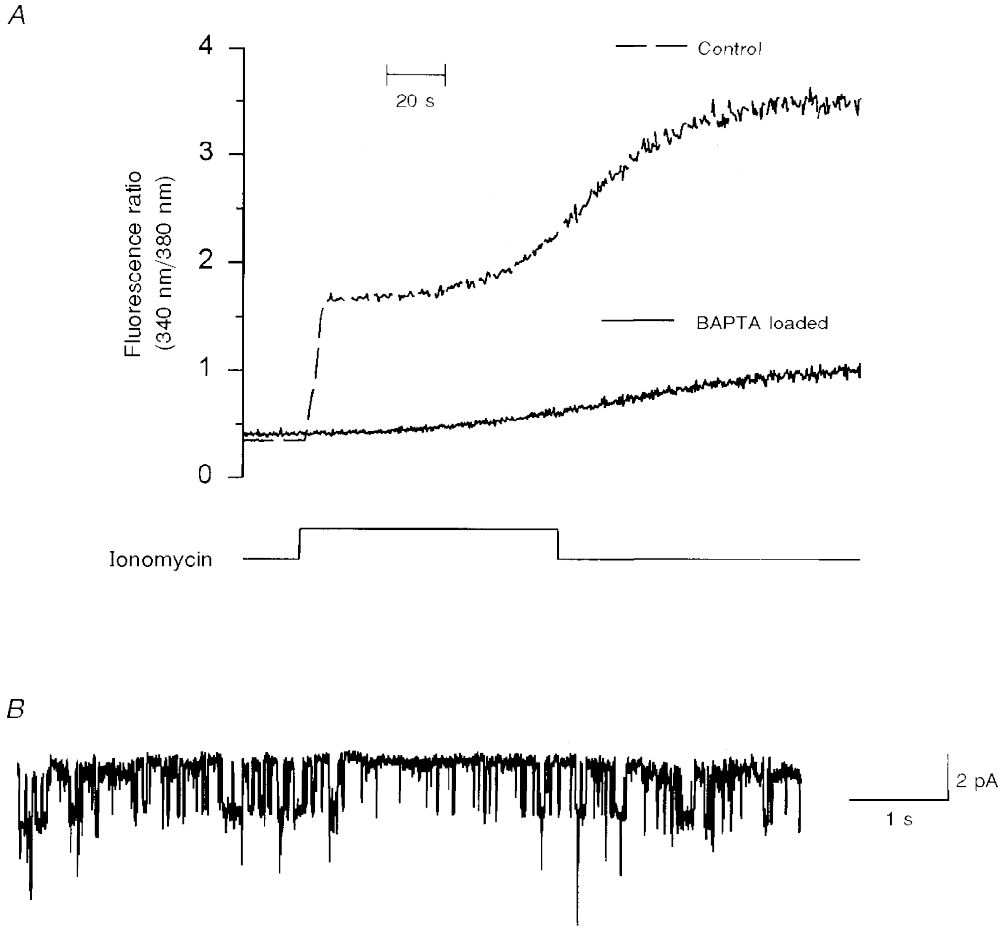

Figure 9. Heavy loading of LNCaP cells with a Ca^{2+} chelator (BAPTA) does not inhibit the activation of the 23 pS channel by ionomycin

A, LNCaP cells, incubated either with 0·5 μM fura-2 AM alone (dashed line) or together with 18 μM BAPTA AM (continuous line) were exposed to 10 μM ionomycin for the time indicated. *B*, single channel current record from a BAPTA-loaded cell showing a 23 pS channel with a steady-state activity (0·342 at −100 mV) similar to the activity shown in Fig. 8*D*. Two more cells loaded with BAPTA gave similar results.

that associated with apoptosis. Moreover, membrane hyperpolarization manifested the activation of Ca^{2+} permeability in serum-deprived cells. Interestingly, it has been shown that high K^+ solutions improve the survival of cells (Isenberg & Klockner, 1982) and that membrane depolarization might reduce the assembly of Bax channels (Antonsson *et al.* 1997). Whether there is a connection between these observations and how membrane depolarization delays the activation of the 23 pS channel remain to be demonstrated.

Activation by intracellular Ca^{2+} is a common feature among non-selective cation channels (reviewed in Siemen, 1993). The following evidence indicates that the 23 pS channel is not directly activated by an increment in $[Ca^{2+}]_i$, as opposed to a high conductance K^+ channel, present in LNCaP cells, which can be reversibly activated by elevating $[Ca^{2+}]_i$ with ionomycin. The exposure of excised patches to 2 mM Ca^{2+} neither activated the 23 pS channel when patches came from control cells nor changed the NP_o in previously ionomycin-activated channels in the cell attached configuration. Further, the 23 pS channel activation, despite the ionomycin-induced increment in $[Ca^{2+}]_i$, was strongly curtailed by BAPTA. Nevertheless, it can still be argued that local increments in $[Ca^{2+}]_i$ at the plasma membrane could be indirectly promoting the appearance of the 23 pS channel. This may not be the case with ionomycin since it produces slow and homogeneous increments in $[Ca^{2+}]_i$ (Williams *et al.* 1985; Badminton *et al.* 1996; Muñoz *et al.* 1998).

The third possible mechanism for activation of non-selective channels is depletion of internal Ca^{2+} stores, as has been proposed recently as a mechanism of activation for this type of channel (e.g. Krause *et al.* 1996). In this respect, it has been suggested that ionomycin increases Ca^{2+} influx by activation of store-operated Ca^{2+}-permeable channels (Mason & Grinstein, 1993; Morgan & Jacob, 1994). However, it does not seem that the 23 pS channel is a store-operated channel. Both 1 and 10 μM ionomycin in the application pipette produced the initial rise in $[Ca^{2+}]_i$. This came from internal Ca^{2+} stores, based on the following evidence: (1) the absence

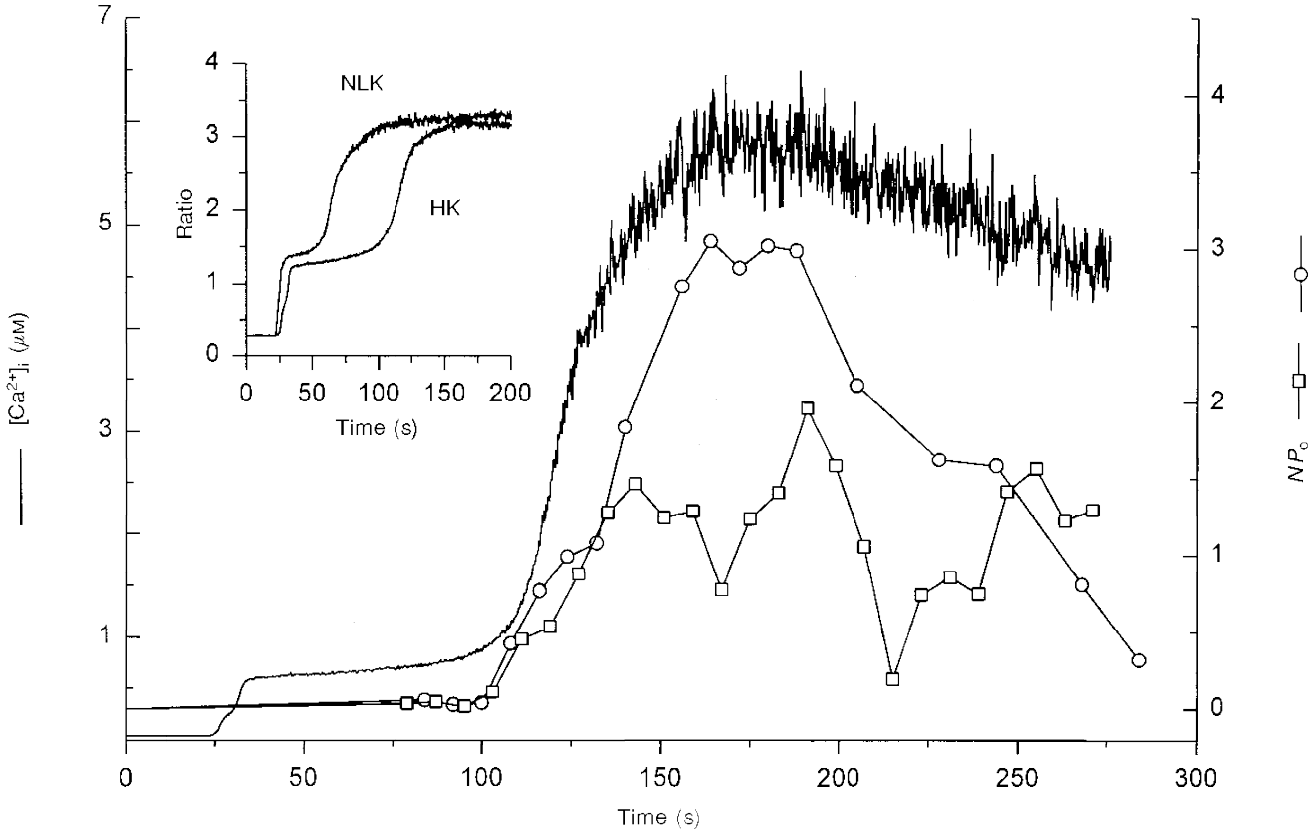

Figure 10. Time course of activation of non-selective cation channels (NP_o) compared with ionomycin-induced rise in $[Ca^{2+}]_i$

Ion channel activities of a 23 pS channel (○ and □, right axis) from two different cells recorded in the cell attached configuration (−100 mV patch potential and in high K^+ external solution) are compared with a typical $[Ca^{2+}]_i$ response to the application of 10 μM ionomycin for 90 s (beginning at 20 s). $[Ca^{2+}]_i$ trace and the 23 pS channel activity (□) are from the same cell. Note that the channel is not open at rest or during the initial rise in $[Ca^{2+}]_i$ (indicated by horizontal lines); 23 pS channels in the membrane patch opened just before the second rise in $[Ca^{2+}]_i$. Inset compares the time course of 10 μM ionomycin-induced increase in $[Ca^{2+}]_i$ for cells bathed in HBS (NLK) or in high K^+ solution (HK). The main difference was the time taken by the second increment in $[Ca^{2+}]_i$ to occur in high K^+ solution.

of extracellular calcium did not abrogate the first rise in $[Ca^{2+}]_i$ induced by 10 μM ionomycin; (2) this rise in $[Ca^{2+}]_i$ was not modified by changing plasma membrane potential and (3) this first increment in $[Ca^{2+}]_i$ was virtually abolished by depleting internal Ca^{2+} stores with thapsigargin. These data imply that both ionomycin concentrations were able to empty internal Ca^{2+} stores, while only 10 μM ionomycin produced an important Ca^{2+} influx through the plasma membrane when external calcium was present. Additionally, ionomycin still activated Ca^{2+} influx in cells where store-operated Ca^{2+} entry was present (based on the elevated resting $[Ca^{2+}]_i$ in the presence of thapsigargin, dashed line in Fig. 6*A*). Altogether, these data indicate that the 23 pS channel is *not* operated by depletion of internal Ca^{2+} stores.

Free radicals can increase $[Ca^{2+}]_i$ in association with apoptosis (Fernandez *et al.* 1995) and recently, it has been demonstrated that oxidative stress activates a Ca^{2+}-permeable, non-selective cation channel of 30 pS conductance in endothelial cells (Koliwad *et al.* 1996). Further, there are studies suggesting a role for oxidative stress in apoptosis induced by either ionomycin (Hatanaka *et al.* 1996) or serum removal (Atabay *et al.* 1996). Whether reactive oxygen species are involved in the activation of the 23 pS in these cells remains to be demonstrated.

A positive correlation was evident between membrane depolarization and the rate of rise of $[Ca^{2+}]_i$ during the current clamp and Ca^{2+} measurement experiments. These data suggested the activation of a Ca^{2+}-permeable non-selective cation channel to increase $[Ca^{2+}]_i$. The evidence for the 23 pS channel being such a Ca^{2+} entry mechanism in apoptosis can be summarized as follows: (1) this channel was not active at all in resting, healthy cells, nor in those exposed to non-lethal doses of ionomycin; (2) the activation of the 23 pS channel correlated with the rise in $[Ca^{2+}]_i$ due to Ca^{2+} influx through the plasma membrane; (3) culturing cells in the absence of serum produced both an increased Ca^{2+} permeability and the apperance of a 23 pS channel. In conclusion, the 23 pS channel is a good candidate for a Ca^{2+} entry pathway that could be involved in apoptotic cell death of LNCaP cells. Indeed, Ca^{2+} influx has previously been shown to be essential for apoptosis of prostatic cancer cells (Martikainen *et al.* 1991; Furuya *et al.* 1994). However, this study does not rule out the participation of store-operated Ca^{2+} channels in apoptosis as well. Moreover, the nature of the Ca^{2+}-permeable channel does not seem to be as critical for triggering apoptotic cell death as calcium influx itself. In this regard, increased activity of voltage-gated L type Ca^{2+} channels has been associated with ageing of mammalian hippocampal neurons (Thibault & Landfield, 1996). Likewise, neuronal cell death can be induced by activation of NMDA receptors (Choi, 1992) or the presence of β amyloid (Arispe *et al.* 1994). Apparently, all these proteins induce cell death due to an increased Ca^{2+} entry. Thus Ca^{2+} influx seems to be the key event in the activation of different Ca^{2+}-dependent effectors of apoptosis for instance, tissue transglutaminase and some endonucleases (reviewed in Guerrero & Arias, 1998). Recently, Bax, the typical inducer of apoptosis of the Bcl-2 family of proteins, has been shown to present a pore-forming activity which can be linked to apoptosis of neuronal cells. Bcl-2 blocked the Bax-induced increase in membrane permeability and it also inhibited apoptosis in these cells (Antonsson *et al.* 1997). Thus, the 23 pS channel is a Ca^{2+}-permeable channel that could provide another Ca^{2+} entry mechanism during cell death of LNCaP cells. Nevertheless, a selective inhibitor or a molecular identification of this channel will be required to establish its role in the induction and progression of apoptosis.

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