Differentiation of the human monocytic cell line U937 results in an upregulation of the calcium release-activated current, I_{CRAC}

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1. Single cell fura-2 fluorescence measurements and whole-cell patch clamp recordings were used to investigate the effects of macrophage-like differentiation, induced by dibutyryl cAMP (dbcAMP), on Ca^{2+} influx triggered by Ca^{2+} store depletion in the human monocytic cell line, U937.

2. In differentiated cells, the rise in intracellular [Ca^{2+}] following store depletion by thapsigargin (TG) in nominally Ca^{2+}-free solution was 94% greater and the $[Ca^{2+}]_i$ rise on subsequent re-addition of external Ca^{2+} (2 mM) was 292% greater than in undifferentiated cells.

3. Under conditions where $[Ca^{2+}]_i$ was buffered by BAPTA, TG-induced store depletion failed to activate a detectable inward Ca^{2+} current in undifferentiated U937 cells. Under identical conditions, store depletion of differentiated U937 cells generated an inwardly rectifying Ca^{2+}-selective current which showed no reversal from −140 to +30 mV and was blocked by 1 μM external La^{3+}; characteristics of the calcium release-activated Ca^{2+} current (I_{CRAC}) identified in other cells.

4. We conclude that U937 cells show a differentiation-dependent upregulation of a store-mediated Ca^{2+} entry pathway, identified as I_{CRAC}, which is not correlated with the small associated increase in the size of TG-sensitive Ca^{2+} pools.

The entry of calcium into cells triggered by depletion of inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores, termed 'capacitative Ca^{2+} entry' by Putney (1986), is a major component of agonist-induced Ca^{2+} signalling in non-excitable cells (for review see Sargeant & Sage, 1994) and has been implicated in a number of signalling events including phospholipase D activation (Balsinde & Mollinedo, 1991), cytokine induction (Goldsmith & Weiss, 1988) and generation of Ca^{2+} oscillations (Dolmetch & Lewis, 1994). This Ca^{2+} entry pathway has recently been characterized electrophysiologically in a number of cell types and termed 'calcium release-activated calcium current', I_{CRAC} (Hoth & Penner, 1992; Zweifach & Lewis, 1993; Somasundaram & Mahaut-Smith, 1994; for review see Fasolato, Innocenti & Pozzan, 1994).

The human pre-monocytic cell line, U937, has been widely used as a functional model for monocytes (Harris & Ralph, 1995) and treatment of U937 cells with dibutyryl cAMP (dbcAMP) allows controlled terminal differentiation into a more macrophage-like cell type (Sheth, Dransfield, Partridge, Barker & Burton, 1988). A previous study using stirred suspensions of fura-2-loaded U937 cells (Davis, Halliwell, Sage & Allen, 1995) found that dbcAMP-treated cells showed an increase in the amount of Ca^{2+} influx following store depletion by the Ca^{2+} store Ca^{2+}-ATPase inhibitor thapsigargin (TG). Since differentiation-dependent modulation of capacitative Ca^{2+} entry might potentially provide both a powerful mechanism of modifying cell signalling and also a method for cloning the influx channel, we have further investigated this phenomenon.

The present study was conducted to ascertain firstly whether this dbcAMP-induced enhancement in Ca^{2+} influx was due to an increase in membrane Ca^{2+} permeability or to an increased electrical driving force for Ca^{2+} entry. Secondly, we attempted to determine whether there was any correlation, at the single cell level, between the size of the TG-sensitive Ca^{2+} store and the amount of capacitative Ca^{2+} influx. Finally, using patch clamp recordings, we attempted to directly measure differentiation-dependent changes in Ca^{2+} permeability and to characterize this influx pathway and its inactivation properties.

METHODS

Cell culture and dye loading

U937 cells were cultured in a humidified atmosphere at 37 °C, 6·8% CO_2 in RPMI 1640 medium (Gibco) supplemented with fetal calf serum (10%), glutamine (2 mM), penicillin (10 U ml^{-1}) and streptomycin (10 mg ml^{-1}). Cells were treated with dbcAMP (1 mM) by incubation for 48 h and then left in dbcAMP-free medium for at least 3 h before being harvested by centrifugation, washed and re-suspended in the standard solution (see below) used for fluorescence experiments (with 1 mM $CaCl_2$).

In fluorescence experiments, cells were incubated for 30 min at room temperature with 0·5 μM fura-2-AM ester (Molecular Probes) from a 5 mM stock in DMSO. The fura-2-AM ester was mixed with an equal volume of 25% (w/v) Pluronic F-127 (Molecular Probes) in DMSO before addition to the cells. All experiments were carried out at room temperature (20–23 °C).

Solutions and reagents

In fluorescence experiments, the standard external solution contained (mM): NaCl, 145; KCl, 5; $MgSO_4$, 1; glucose, 10; Hepes, 10 (pH 7·35 adjusted with Tris) and 2 mg ml^{-1} bovine serum albumin. In order to maintain filled Ca^{2+} stores, cells were kept in this standard solution with 1 mM added Ca^{2+} and then briefly washed (for 5–10 s) with nominally Ca^{2+}-free solution before beginning an experiment.

In patch clamp experiments, the standard external solution contained (mM): *N*-methyl-D-glucamine ($NMDG^+$) gluconate, 140; $CaCl_2$, 10; $MgCl_2$, 1; glucose, 10; Hepes, 10 (pH 7·3 adjusted with Tris). When Ca^{2+}-free medium was used, divalent ion concentration was kept constant by using 11 mM $MgCl_2$. The patch pipette solution contained (mM): caesium gluconate, 100; Cs_4BAPTA, 10; $MgCl_2$, 2; Na_2GTP, 0·1; Hepes, 15 (pH 7·3, adjusted with Tris). Cs_4BAPTA was from Molecular Probes, all other reagents were from Sigma.

Fluorescence recordings

Single-cell fura-2 fluorescence measurements were made using a Cairn spectrophotometer system, as previously described by Somasundaram & Mahaut-Smith (1994). Excitation light passed through a spinning filter wheel assembly containing four 340 nm and two 380 nm bandpass excitation filters. Emitted light (wavelength, 400–600 nm) was selected by two dichroic filters and further filtered by a 485 nm long-pass gelatin filter. The combined output from all 340 and 380 nm excitation filters provided a 340/380 nm ratio for each revolution of the filter wheel. The signal was then averaged to give a ratio value every 67 ms. Background fluorescence was measured and subtracted from the signal to give fura-2 fluorescence. A variable iris in the emission light path was used to select a region slightly larger than the cell for fluorescence measurement.

The fura-2 signal was calibrated *in vivo* using the equation of Grynkiewicz, Poenie & Tsien (1985):

$$[Ca^{2+}]_i = (S_f/S_b)K_d(R - R_{min})/(R_{max} - R)$$

where S_f and S_b are the proportionality constants for the contribution of the free and bound dye to fluorescence at 380 nm, respectively, K_d is the dissociation constant of fura-2 (taken as 135 nM (Grynkiewicz *et al.* 1985), R is the measured fluorescence ratio 340/380 nm, R_{max} is the ratio value at saturating $[Ca^{2+}]$ and R_{min} is the ratio value under Ca^{2+}-free conditions. R_{min} was measured following addition of 5 μM ionomycin and 10 mM EGTA. R_{max} was measured in the presence of 5 μM ionomycin and 10 mM $CaCl_2$.

The resting membrane potentials of dbcAMP-treated U937 cells were found to be more hyperpolarized than those of untreated cells (R. A. Floto, unpublished observations) and therefore, to control for changes in membrane potential which would modify $[Ca^{2+}]_i$ independently of changes in Ca^{2+} permeability, fluorescence experiments were carried out in the presence of the K^+ ionophore valinomycin (1 μM). This allowed the membrane to be 'clamped' at the reversal potential of K^+ (approximately −90 mV).

Membrane potential measurements

In order to assess the ability of the K^+ ionophore valinomycin to 'clamp' cell membrane potentials during the protocols described above, the potential-sensitive fluorescent dye, 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$; Molecular Probes) was used essentially as described by Larsen, Enelow, Simons & Sullivan (1985). Aliquots of 10^6 cells, resuspended in 2 ml of nominally Ca^{2+}-free standard external saline, were added to glass cuvettes and continually stirred in a Perkin-Elmer spectrofluorimeter set at an excitation wavelength of 620 nm (10 nm slit) and an emission wavelength of 660 nm (10 nm slit). $DiSC_3(5)$ was added at a final concentration of 1 μM and allowed to reach a constant fluorescence level. Valinomycin (1 μM) was then added followed, after 3 min, by (500 nM). This mirrored the average time cells spent in valinomycin prior to addition of TG in single-cell fura-2 experiments. After approximately 6 min, 2 mM Ca^{2+} was added followed 6 min later by 5 μM of the Na^+–K^+ ionophore, gramicidin in order to depolarize the cells to a constant level. The fluorescence value following addition of valinomycin (F_{val}) was subtracted from the maximum fluorescence following gramicidin addition (F_{gram}) and fluorescence values of experiments are shown as a percentage of this maximum fluorescence change.

Electrophysiology

Whole-cell patch clamp experiments were carried out using an Axopatch 200A patch clamp amplifier (Axon Instruments, Foster City, CA, USA). Electronic compensation of capacitance currents and series resistances (10–30 MΩ) was performed. Voltage steps were applied for 1·85 s with a 2 s interval between steps to allow complete recovery of the I_{CRAC}.

Membrane currents during voltage ramps and steps were low-pass filtered at 2 kHz and sampled at 10 kHz using Axon Instruments hardware and pCLAMP6 software (Axon Instruments). Currents were also continuously acquired at 60 Hz (low-pass filtered at 30 Hz) by a Cairn Spectrophotometer system (Cairn Research Ltd, Kent, UK) and also by a VR-10B digital data recorder (Instrutech Corp., NY, USA) acquiring at 37 kHz (low-pass filtered at 2 kHz). Liquid junction potentials were measured by reference to a 3 M KCl bridge and corrected for.

In both fluorescence and patch clamp experiments, TG (1 μM) was applied from a nearby pipette (150 μm from cell) using a pressure injection system (PLI-100, Medical Systems, Greenvale, NY, USA).

Results are expressed as means ± S.D. and significance was assessed using Student's unpaired *t* test.

RESULTS

Single-cell fura-2 fluorimetry

In order to examine the effect of differentiation on the level of Ca^{2+} release from stores and on the degree of capacitative Ca^{2+} entry in individual cells, we investigated the responses of fura-2-loaded cells to TG in nominally Ca^{2+}-free medium

and to the subsequent addition of external Ca^{2+}. To minimize the effect of changes in membrane potential on Ca^{2+} influx, all experiments were carried out in the presence of 1 μM valinomycin. Differentiation did not significantly alter resting $[Ca^{2+}]_i$ in cells (undifferentiated, 64 ± 58 nM; differentiated, 70 ± 55 nM; see Fig. 1*A* and *B*). The mean TG-induced peak $[Ca^{2+}]_i$ in undifferentiated cells was 102 ± 12 nM ($n = 7$) whereas, in cells differentiated by 48 h pretreatment with dbcAMP, this rise was significantly greater (198 ± 71 nM, $P < 0{\cdot}004$, $n = 7$) (Fig. 1*A* and *B*) suggesting an increase in the size of intracellular stores. However, this difference may, in part, reflect changes in the rate of leak of Ca^{2+} from stores or of extrusion of $[Ca^{2+}]_i$. The mean peak $[Ca^{2+}]_i$ following re-addition of external Ca^{2+} in undifferentiated cells was 192 ± 84 nM but was significantly greater in differentiated cells (752 ± 269 nM, $P < 0{\cdot}0001$). This suggests a differentiation-dependent increase in both Ca^{2+} store release and capacitative Ca^{2+} entry. Since membrane potentials were maintained equal and constant by valinomycin (see Fig. 2), the enhanced Ca^{2+}

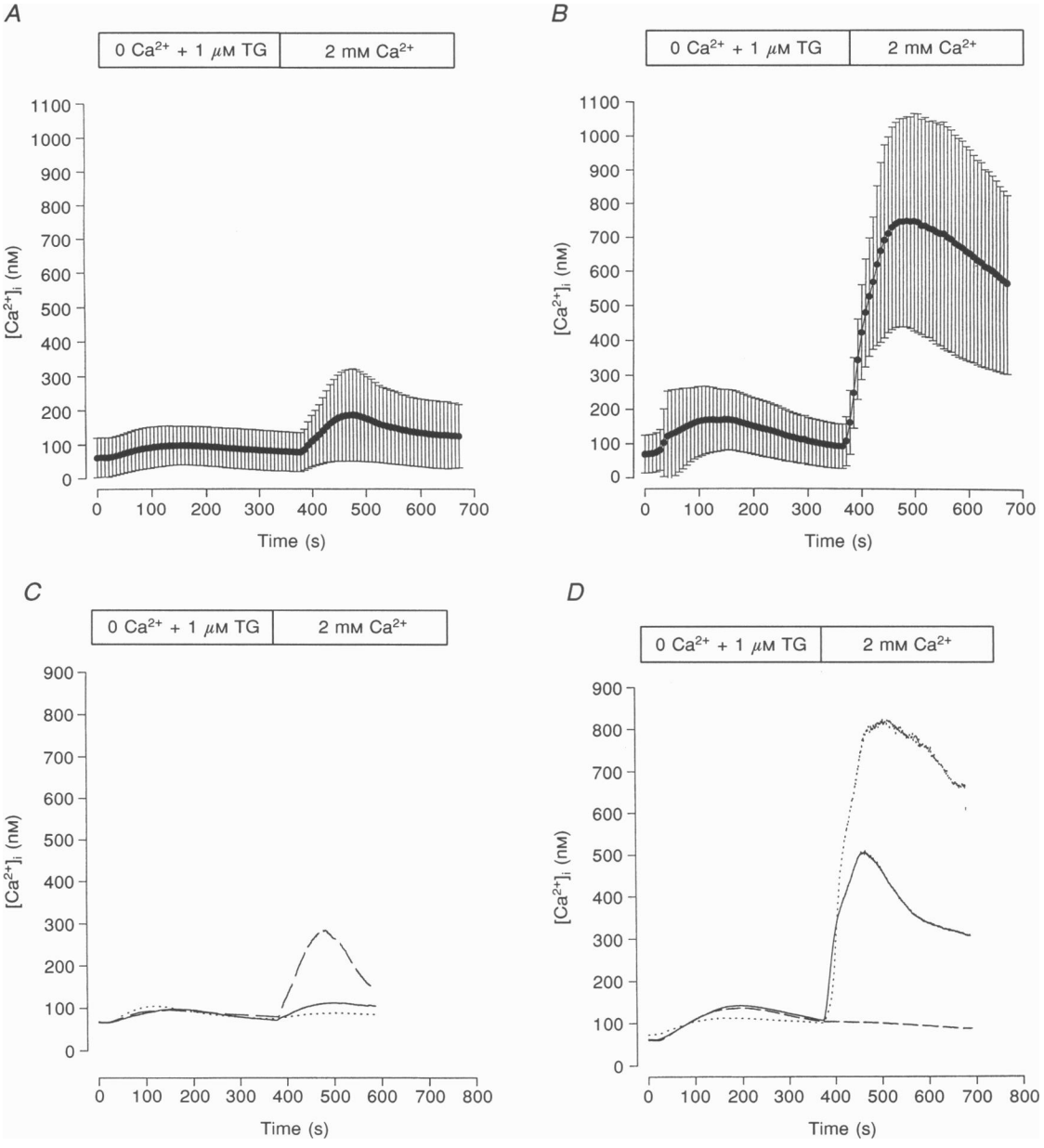

Figure 1. Differentiation-dependent changes in TG-induced store depletion and capacitative Ca^{2+} entry in single cells

Effect of addition of TG (1 μM) in nominally Ca^{2+}-free solution and subsequent re-addition of 2 mM Ca^{2+} on the mean $[Ca^{2+}]_i$ in fura-2-loaded undifferentiated (*A*) and differentiated cells (*B*). The mean $[Ca^{2+}]_i$ (± S.D., $n = 7$) is shown at 7 s intervals during experiments. Traces below are from 3 cells with similar TG-induced Ca^{2+} rises but markedly different levels of Ca^{2+} influx in undifferentiated (*C*) and differentiated cells (*D*). Experiments were carried out at room temperature and in the continuous presence of 1 μM valinomycin.

influx was presumed to be due to an increase in membrane Ca^{2+} permeability.

There was considerable cell-to-cell variation in the size of the capacitative Ca^{2+} influx within both control and treated cell populations (as illustrated by the large error bars in Fig. 1*A* and *B*). To examine whether this variability was related to the size of the TG-sensitive Ca^{2+} pool, we investigated whether there was any correlation, in individual cells, between the peak TG-induced $[Ca^{2+}]_i$ rise and the maximal $[Ca^{2+}]_i$ on re-addition of external Ca^{2+}. We obtained correlation coefficients of 0·05 for undifferentiated cells ($n = 7$) and 0·28 for differentiated cells ($n = 7$), suggesting that there was no significant association of these events. This lack of correlation is illustrated for both undifferentiated and differentiated cells in Fig. 1*C* and *D* where, in each case, traces are shown for three cells with similar TG-induced Ca^{2+} rises but markedly different levels of Ca^{2+} influx.

Effect of valinomycin on cell membrane potential

To confirm that valinomycin maintained cell membrane potentials at a constant and hyperpolarized level for the duration of the fura-2 experiments, we used the fluorescent membrane potential indicator, $DiSC_3(5)$ and repeated the protocol shown in Fig. 1 in the presence and absence of valinomycin. Typical traces are shown for undifferentiated (Fig. 2*A*) and differentiated cells (Fig. 2*B*). Valinomycin caused a rapid and considerable hyperpolarization and subsequent addition of TG and external Ca^{2+} resulted in only a very slight depolarization, never greater than 4% of the total fluorescence range, from this level. In contrast, in the absence of valinomycin both undifferentiated and differentiated cells showed a marked hyperpolarization following addition of both TG and external Ca^{2+} consistent with the activation of Ca^{2+}-activated K^+ channels known to be present in these cells (R. A. Floto, unpublished observations).

Electrophysiological characterization of TG-evoked current

To study directly the calcium release-activated Ca^{2+} current, I_{CRAC}, in U937 cells, we used conditions previously shown to amplify this current and minimize contamination by other currents (Hoth & Penner, 1992; Somasundaram & Mahaut-Smith, 1994; see Methods). Briefly, whole-cell current recordings were made in an extracellular solution containing 10 mM Ca^{2+} and no Na^+ or K^+. Outward K^+ currents were inhibited by substitution of Cs^+ for K^+ in the intracellular solution and $[Ca^{2+}]_i$ was strongly buffered with 10 mM Cs_4BAPTA. Under these ionic conditions in undifferentiated cells, depletion of Ca^{2+} stores by addition of 1 μM TG (for 10 s) did not evoke any detectable currents at a holding potential of −40 mV (Fig. 3*A*). Addition of 1 μM La^{3+}, a potent blocker of I_{CRAC}, did not alter the measured current, confirming no activation of I_{CRAC} in these cells. In

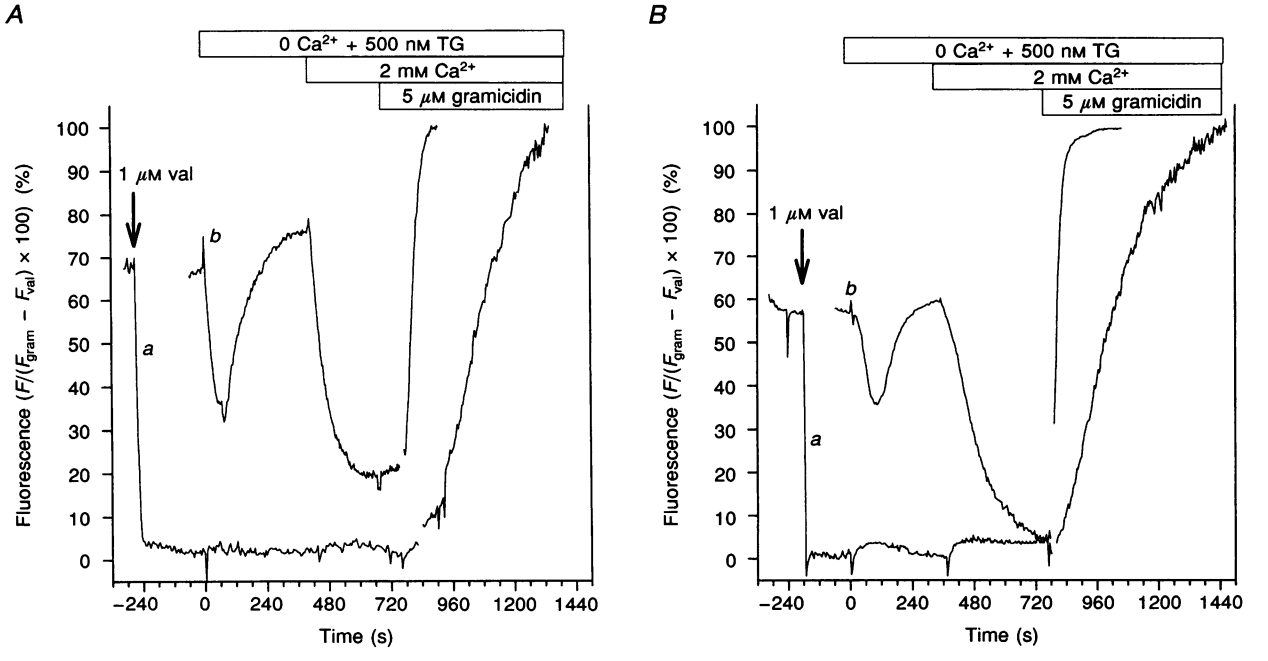

Figure 2. Effect of valinomycin on cell membrane potential

$DiSC_3(5)$ fluorescence traces of undifferentiated (*A*) and differentiated cells (*B*), in nominally Ca^{2+}-free solution, showing response to addition of TG (500 nM), 2 mM Ca^{2+} and gramicidin (5 μM) with (*a*) and without (*b*) pretreatment with valinomycin (1 μM). Fluorescence values are plotted as a percentage of the total fluorescence range (see Methods), calculated by subtracting the fluorescence value following valinomycin (F_{val}) from that following addition of gramicidin (F_{gram}). A drop in $DiSC_3(5)$ fluorescence indicates membrane hyperpolarization.

order to measure instantaneous current–voltage (I–V) relationships, voltage ramps from −140 to +30 mV were applied before and 2 min after TG application and subtracted from ramps given after addition of La^{3+}. In all (21/21) undifferentiated cells tested, no detectable current was observed in the subtracted voltage ramps (Fig. 3*B*).

In U937 cells, differentiated by a 48 h period of pretreatment with dbcAMP, an identical protocol resulted in the development of a small inward current 10–30 s after TG application in 20/23 cells, which was completely blocked by 1 μM La^{3+} (Fig. 3*C*). Further applications of TG failed to elicit any additional current (data not shown) suggesting maximal current activation was achieved. Differentiated cells gave maximal current densities of 0·12 ± 0·02 pA pF^{-1} (at −40 mV holding potential). The I–V relationship of the La^{3+}-sensitive current, shown in Fig. 3*D*, indicates the development of an inwardly rectifying conductance displaying no reversal over the membrane potential range studied and showing no current noise attributable to single-channel events. This observed inward current, once fully activated, could be entirely and reversibly abolished by replacing external Ca^{2+} with Mg^{2+} (Fig. 4*A* and *B*), suggesting a conductance carried exclusively by Ca^{2+} and highly selective for Ca^{2+} over Mg^{2+}.

Following hyperpolarizing voltage steps (for 1·85 s) to potentials from −60 to −120 mV, applied after maximal

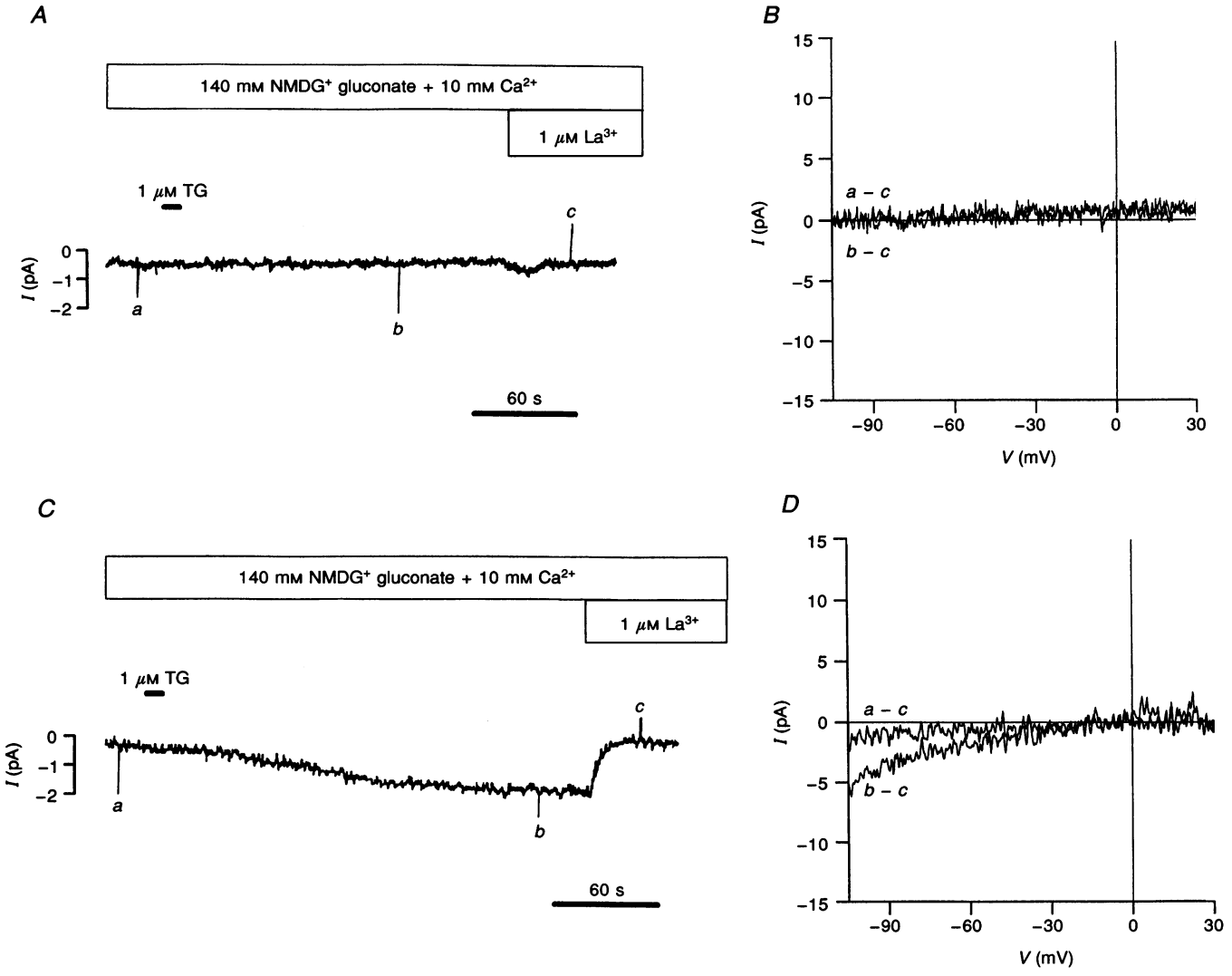

Figure 3. Detection of TG-evoked current in differentiated but not undifferentiated cells

The effect of 1 μM TG (10 s application) and the subsequent addition of 1 μM La^{3+} on whole-cell current measurements in undifferentiated (*A*) and differentiated cells (*C*) at a holding potential of −40 mV. Current–voltage relationships were obtained by applying 300 ms voltage ramps from −140 to +30 mV, before (*a*) and 2 min after TG application (*b*) and subtracting from ramps recorded after addition of La^{3+} (*c*) and are shown for undifferentiated cells (*B*), where the traces superimpose, and differentiated (*D*) cells. The intracellular solution contained 100 mM caesium gluconate and 10 mM Cs_4BAPTA while the external solution contained 140 mM $NMDG^+$ gluconate and 10 mM Ca^{2+}.

current activation, the current peaked and then inactivated to a new steady-state level with a single-exponential time constant (τ_s) of 230–270 ms at −120 mV (Fig. 4*C*). The amount of inactivation increased with the size of voltage step consistent with the phenomenon of Ca^{2+}-dependent slow inactivation. A small, rapid inactivation component (τ_f) of I_{CRAC} similar to that described in other cells (for example Zweifach & Lewis, 1993) was only observed (time constant, 20–60 ms at −120 mV) when currents, recorded during steps to −120 mV or greater in 10 mM Ca^{2+}, were subtracted from those obtained in nominally Ca^{2+}-free external solution (data not shown).

The La^{3+} sensitivity of this inwardly rectifying conductance, the absence of current noise, its high selectivity for Ca^{2+} over Mg^{2+} and its inactivation kinetics are all consistant with properties of I_{CRAC} as described in other cell types (Hoth & Penner, 1992; Zweifach & Lewis, 1995*a*, *b*).

DISCUSSION

We have investigated the effect of dbcAMP differentiation on store-mediated Ca^{2+} entry in the human monocytic cell line U937, using both single-cell fura-2 fluorescence and patch clamp measurements. In fluorescence studies, by first releasing intracellular Ca^{2+} stores in nominally Ca^{2+}-free external solution and then re-introducing external Ca^{2+}, we were able to demonstrate a differentiation-dependent increase in both the size of TG-sensitive Ca^{2+} stores and the

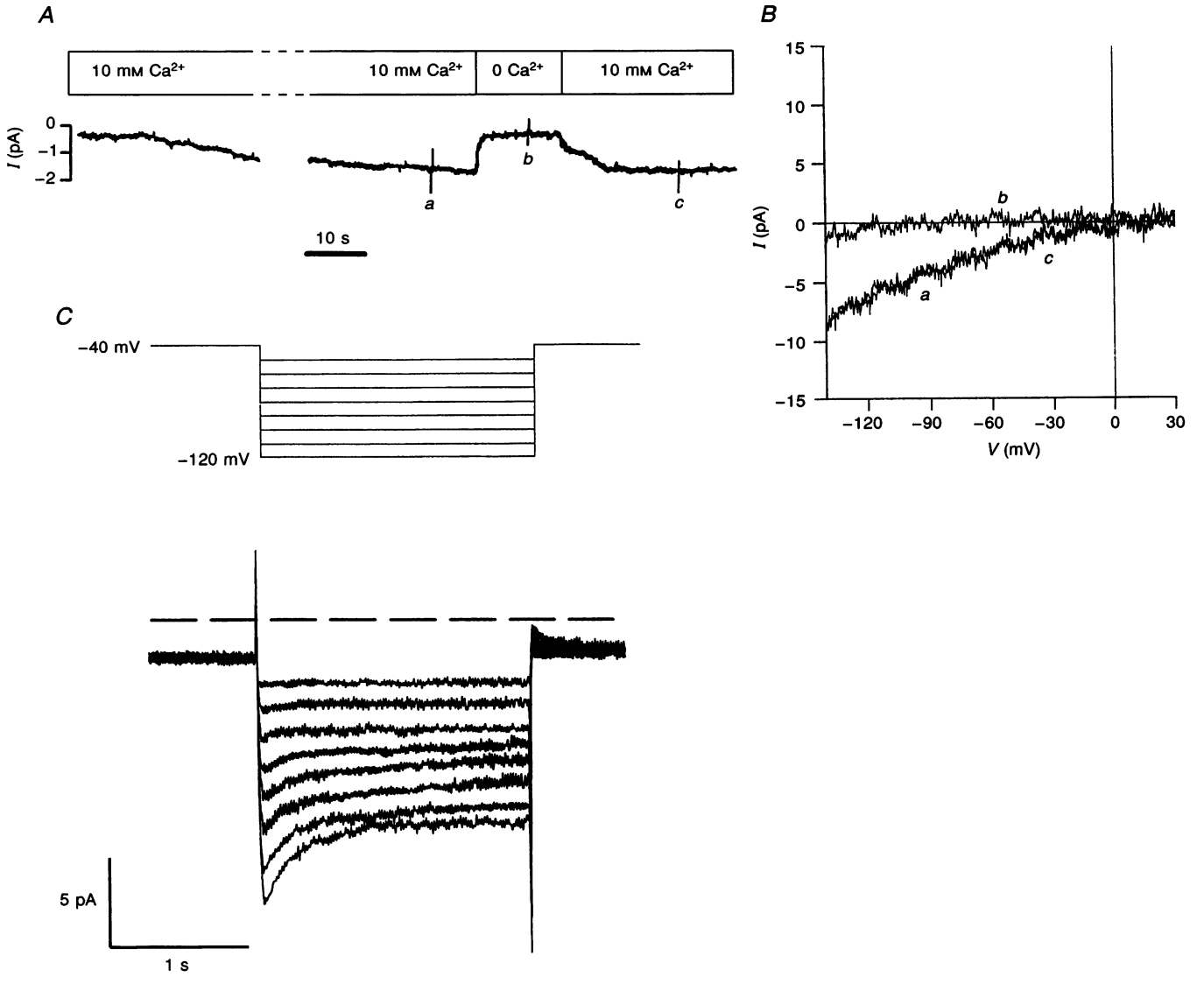

Figure 4. Ionic selectivity and inactivation properties of TG-evoked current

A, effect of changing from 10 mM Ca^{2+} + 1 mM Mg^{2+} to 11 mM Mg^{2+} (0 Ca^{2+}) external solution on the TG-evoked whole-cell current in a differentiated cell at −40 mV holding potential. *B* shows current–voltage relationships obtained by 300 ms voltage ramps, from −140 to +30 mV, applied before (*a*), during (*b*) and after (*c*) a 13·7 s perfusion of Ca^{2+}-free solution (traces *a* and *c* superimpose). *C*, typical current traces from a differentiated cell, held at −40 mV in 10 mM Ca^{2+}, responding to a series of 1·85 s hyperpolarizing voltage steps, given at 2 s intervals following maximal activation of TG-evoked current.

amount of capacitative Ca^{2+} entry. Since valinomycin maintained a constant electrical driving force for Ca^{2+} influx (Fig. 2), this enhanced Ca^{2+} influx must be due to a greater Ca^{2+} selective membrane permeability.

The cause of the cell-to-cell variability in the level of capacitative Ca^{2+} entry, seen in control cells (Fig. 1*A*) and markedly increased in differentiated cells (Fig. 1*C*), is unclear. There does not seem to be any correlation between the size of a cell's TG-sensitive Ca^{2+} store and the level of capacitative Ca^{2+} entry (Fig. 1*B* and *D*) implying that these two mechanisms are under independent control. The variability may reflect intrinsic heterogeneity of U937 cells and might be amplified by differing responses to dbcAMP treatment. Inhibition of capacitative Ca^{2+} entry during mitosis (Preston, Sha'afi & Berlin, 1991) could explain the variations in control cells but not in dbcAMP-treated cells where cell cycles are halted in the G_0/G_1 phase (Sheth *et al.* 1988).

The differentiation-dependent increase in TG-evoked Ca^{2+} conductance was confirmed by patch clamp recording of differentiated U937 cells, which identified a highly Ca^{2+}-selective TG-triggered current, blocked by La^{3+} and showing a similar current–voltage relationship to the I_{CRAC} of mast cells (Hoth & Penner, 1992) and other cell types. Although control U937 cells showed a small level of Ca^{2+} influx in fluorescence experiments, no I_{CRAC} was observed, illustrating the higher threshold for detection of capacitative Ca^{2+} entry using patch clamp techniques. In addition, because the whole-cell patch clamp configuration results in cytoplasmic dialysis, these electrophysiological recordings confirm that the observed increase in capacitative Ca^{2+} influx is a differentiation-dependent event and unlikely to be caused by acute effects of cAMP-dependent channel phosphorylation.

This I_{CRAC} conductance showed a slow inactivation (τ_s of 230–270 ms at 120 mV) to steady state following hyperpolarizing steps similar to the slow Ca^{2+}-dependent inactivation ($\tau = 100$ ms, −120 mV) observed in Jurkat cells by Zweifach & Lewis (1995*b*), although the longer time constants seen in U937 cells may reflect lower Ca^{2+} binding affinity or slower kinetics of the inactivation mechanism. This slow I_{CRAC} inactivation may contribute, at least in part, to the relaxation from peak to plateau $[Ca^{2+}]_i$ seen following re-addition of Ca^{2+} to store-depleted U937 cells (Fig. 1*A* and *B*) and other cell types (Fasolato *et al.* 1994)

The molecular identity of the CRAC channel still remains unknown, although the development of Jurkat cell mutants with markedly reduced capacitative Ca^{2+} entry (Serafini *et al.* 1995; Fanger, Hoth, Crabtree & Lewis, 1995) as well as the identification of T cells from patients with a primary immunodeficiency showing defects in CRAC channel activity (Partiseti, Le Diest, Hivroz, Fischer, Korn & Choquet, 1994) may facilitate cloning attempts. If the differentiation-dependent increase in capacitative Ca^{2+} entry in U937 cells is due to upregulated CRAC channel expression and not merely due to an increased proportion of active channels, then the U937 cell system may prove an equally useful and more widely available target for subtraction cloning strategies. In addition, our findings raise the possibility that the control of CRAC channel expression (at least in U937 cells) is via a cAMP-dependent pathway.

The functional consequence of altering levels of capacitative Ca^{2+} entry will be to modify the pattern, amplitude and duration of agonist-induced Ca^{2+} signals (for example, the duration and amplitude of Fcγ receptor-activated Ca^{2+} oscillations in U937 cells is critically dependent on Ca^{2+} influx through I_{CRAC} (Floto, Somasundaram & Mahaut-Smith, 1994) and there will be subsequent effects on the types of Ca^{2+}-dependent signalling events triggered and their levels of activation. At least potentially, the modulation by cells of CRAC channel activity may provide a powerful method of altering functional responses to stimuli. It is not yet known, however, whether dbcAMP treatment of U937 cells correlates to a pathophysiological differentiation of native monocytes/macrophages and if modulation of CRAC channel activity plays a role in the immunological function of these cells.

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