Functional importance of the dihydropyridine-sensitive, yet voltage-insensitive store-operated Ca^{2+} influx of U937 cells

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Abstract The Ca^{2+} current activated by Ca^{2+} store depletion in non-excitable cells is classically regarded as being dihydropyridine-insensitive, suggesting that store-operated Ca^{2+} channels (SOCs) are dissimilar to voltage-gated Ca^{2+} channels (VGCs) of excitable-cells. Here, we demonstrate dihydropyridine-sensitivity for the store-operated Ca^{2+} influx induced by ATP and thapsigargin (Tg) in the non-excitable U937 cell-line. Ca^{2+} store depletion by prior treatment of cells with either Tg or ATP, stimulated a Ca^{2+} entry mechanism that was inhibited by nicardipine, nifedipine, and the specific L-type Ca^{2+} channel blocker, calciseptine. A functional requirement for this Ca^{2+} influx mechanism in agonist-induced mitogenesis seemed likely, since nicardipine, a particularly potent inhibitor of store-operated Ca^{2+} influx in these cells, suppressed ATP- and Tg-stimulated cell proliferation. Depolarisation of cells with KCl, or gramicidin failed to elicit an increase in cytosolic Ca^{2+}, suggesting that while the store-operated Ca^{2+} influx channel of U937 cells shares pharmacologic properties with the L-type Ca^{2+} channel, it is voltage-insensitive and therefore may resemble an L-type Ca^{2+} channel lacking a voltage sensor.

Key words: Capacitative calcium influx; Store-operated calcium influx; Dihydropyridine, U937 cell; Cell proliferation

1. Introduction

Many agonist-receptor interactions result in an increase in the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which is often functionally coupled to cell response [1]. Agonist-induced increase in $[Ca^{2+}]_i$ is thought to be dependent on both Ca^{2+} mobilization from intracellular stores and Ca^{2+} influx across the plasma membrane. Recently, it has become widely accepted that agonist-induced Ca^{2+} influx is somehow linked to the state of filling of intracellular Ca^{2+} stores [2–4], and in recognition of this, the particular influx mechanism triggered by stimuli which share a common property of releasing stored Ca^{2+} within the cell has been termed store-operated, or capacitative Ca^{2+} influx. Although this influx mechanism appears to be ubiquitous in non-excitable cells [4] and has been reported in excitable-cells [5,6], very little is known regarding the physical nature of store-operated Ca^{2+} influx channels (SOCs), or the mechanism that communicates information between Ca^{2+} stores and SOCs. Furthermore, the functional significance of store-operated Ca^{2+} influx and its contribution to Ca^{2+} signalling and cellular response is difficult to assess for many systems, mainly due to the lack of known drug modulators of SOC activity.

Several mechanisms for SOC activation have been proposed, involving the release of a diffusible cytoplasmic influx factor (CIF) from Ca^{2+} stores, or the direct coupling of Ca^{2+} stores and plasma membrane channels [4]. Small G proteins may be implicated in SOC channel modulation [4], while CIF and cGMP might be analogous, since this cyclic nucleotide appears to potentiate Ca^{2+} influx activated in response to Ca^{2+} store depletion in a variety of cells [6–9]. It is noteworthy that the first SOC to be identified, the Ca^{2+} release-activated Ca^{2+} (CRAC) channel, has been proposed to be a mammalian homologue of the transient receptor potential (*trp*) gene product of *Drosophila* photoreceptors [4], which shares significant amino acid sequence homology to voltage-gated Ca^{2+} channels (VGCs) [10]. This may indicate structural and functional similarities between SOCs and VGCs, whilst also pointing to the possibility that specific drug modulators of VGCs might also be effective against SOCs. However, to date, no modulatory ligands of SOCs have been reported, with these channels being classically regarded as dihydropyridine-insensitive, and dissimilar to VGCs [4,11,12]. The lack of effective agonist or antagonist ligands of SOCs not only hinders their purification and characterisation, but also makes any functional assessment of the role of store-operated Ca^{2+} influx in Ca^{2+} signalling and cellular response difficult.

Here, and in contrast to previous studies, we report dihydropyridine-sensitivity for the store-operated Ca^{2+} influx induced by ATP and thapsigargin in non-excitable U937 cells, an immature human monocytic cell-line. This particular influx mechanism was also sensitive to the specific L-type Ca^{2+} channel blocker calciseptine, but appeared to be voltage-insensitive, suggesting that the SOC of U937 cells might resemble an L-type channel lacking a voltage sensor, similar to the *trp* of *Drosophilla melanogaster* photoreceptors [10]. As a result of identifying inhibitors of SOCs in U937 cells, we were able to assess the role of store-operated Ca^{2+} influx in agonist-stimulated mitogenesis. Using nicardipine, which was found to be a particularly potent inhibitor of SOC activity, results suggest a functional requirement for store-operated Ca^{2+} influx in ATP- and Tg-stimulated mitogenesis in U937 cells.

2. Materials and methods

2.1. Preparation of cells

Undifferentiated U937 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) and 1 μg/ml penicillin, 1 μg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 95% air, 5% CO_2, and were maintained in culture prior to use.

2.2. Analysis of free intracellular Ca^{2+}

For intracellular Ca^{2+} measurements, cells were loaded with 2 μM fura-2 AM in 2 ml Hanks' medium (pH 7.2), in the presence of 0.1% pluronic for 60 min at room temperature. Cells were washed in 2×2 ml aliquots of Hanks' medium to remove any residual dye, and were

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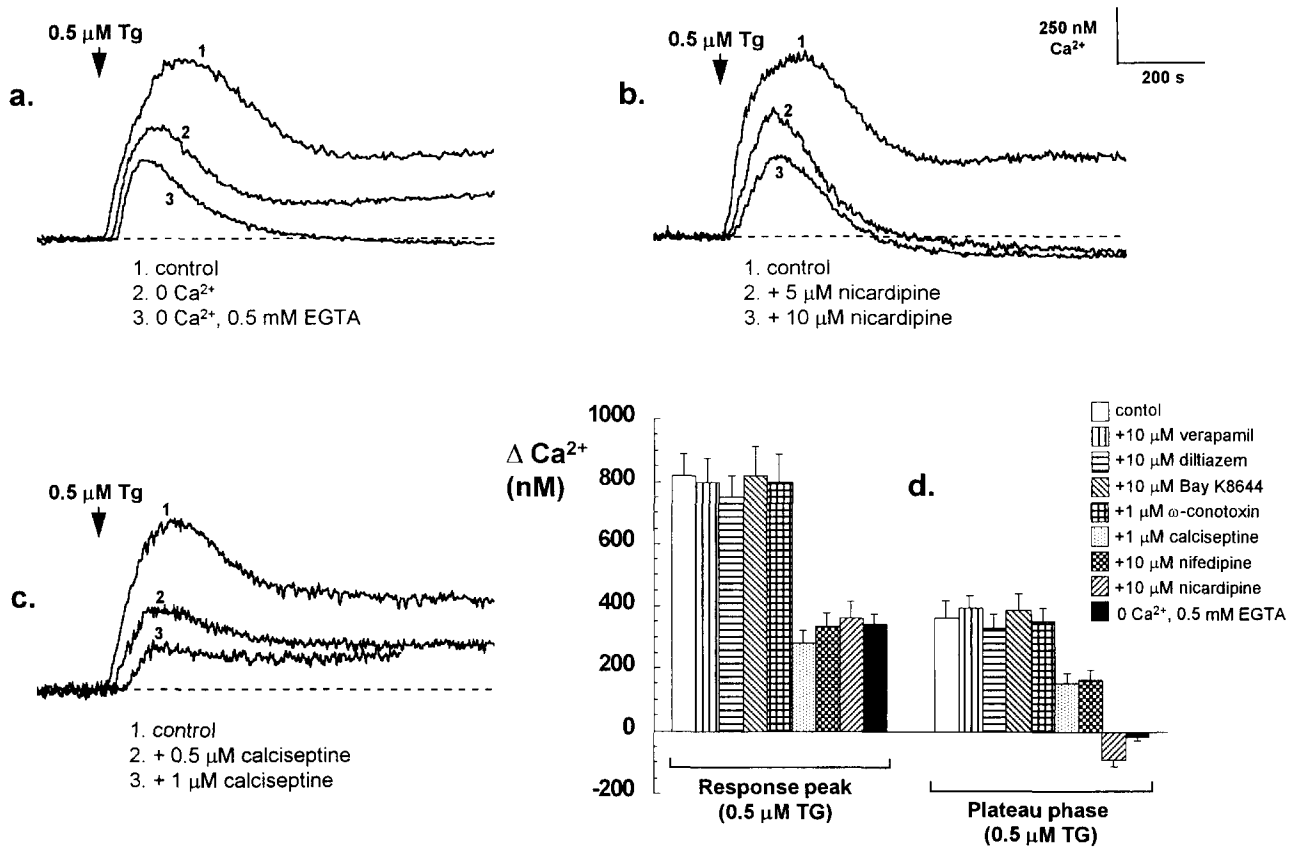

Fig. 1. Effect of extracellular Ca^{2+} chelation and drug modulators of L- and N-type Ca^{2+} channels on the Tg-induced increase in $[Ca^{2+}]_i$ of U937 cells. (a) 0.5 μM Tg was added to cells maintained in normal Hanks' medium, or to cells transferred to Ca^{2+} depleted Hanks' media (0 Ca^{2+}, or 0 Ca^{2+} +0.5 mM EGTA). (b) Pre-incubation of cells with 5 and 10 μM nicardipine for 10 min prior to addition of 0.5 μM Tg. (c) Pre-incubation of cells with 0.5 and 1 μM calciseptine for 10 min prior to addition of 0.5 μM Tg. (d) Effect of various Ca^{2+} channel modulators on the peak amplitude and elevated plateau phase of the Tg-induced Ca^{2+} response of U937 cells. Cells were pre-incubated with the indicated drugs for 10 min prior to addition of 0.5 μM Tg. Results are expressed as the mean change in $[Ca^{2+}]_i$ from the pre-stimulatory basal level ± S.D., derived from 4 separate experiments.

allowed to stand for 60 min at 22°C to ensure complete ester hydrolysis, prior to Ca^{2+} analysis.

Fluorimetric analysis of $[Ca^{2+}]_i$ was performed using a Perkin-Elmer LS 50 fluorimeter. Cells were loaded with the acetoxymethyl (AM) ester of Fura-2 (see above) and equivalent aliquots of the resulting cell suspension (containing approx. 5 million cells) were added to a stirred fluorimetry cuvette in a final volume of 3 ml Hanks' medium. Free cytosolic Ca^{2+} concentration was determined by calculating the ratio of fluorescence intensities at excitation wavelengths 340 and 380 nm, using an emission wavelength of 510 nm. Pairs of 340 and 380 nm fluorescence intensities were sampled every 0.8 s. Experiments were performed at 22°C. A low temperature was employed for Ca^{2+} measurements with fura-2, since this dye readily compartmentalises into cellular organelles at 37°C. Standard $CaCl_2$ solutions were used to calibrate the system, and viscosity corrections were made [13].

2.3. Assessment of Ca^{2+} influx by the manganese quench of cytosolic fura-2

Cells were loaded with the acetoxymethyl (AM) ester of fura-2 (see above) and equivalent aliquots of the resulting cell suspension (containing approx. 5 million cells) were added to a stirred fluorimetry cuvette in a final volume of 3 ml Hanks' medium. Mn^{2+} (0.2 mM) was added to the cuvette, together with drugs that stimulate or inhibit store-operated Ca^{2+} influx, and the resulting quench in fluorescence intensity was monitored at 510 nm using an excitation wavelength of 360 nm, at 22°C.

2.4. Cell proliferation assay

U937 cells were seeded into 6-well plates (approx. 200000 cells/well), and were incubated in 3 ml DMEM without FCS for 24 h in a humidified atmosphere of 95% air, 5% CO_2, at 37°C. For control and drug-treated cells, cell number was assessed following this time period using a Coulter Multisizer II cell counter. In experiments investigating the requirement of store-operated Ca^{2+} influx in ATP- and Tg-stimulated cell proliferation, cells were pre-treated with nicardipine or verapamil for 15 min, prior to addition of ATP or Tg. All results were compared for statistical significance using Student's *t*-test for unpaired observations. Statistical significance was taken at *P*-values less than or equal to 0.05.

2.5. Drugs and solutions

Experiments were performed in Hanks' medium (pH 7.2), containing 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.83 mM $MgSO_4$, 0.42 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$ and 5 mM glucose. For experiments carried out in low Ca^{2+} medium, $CaCl_2$ was omitted from the above, and 0.5 mM EGTA was added, yielding a free Ca^{2+} concentration of ~10 nM. DMEM, FCS, penicillin and streptomycin were from Gibco. Fura-2 AM was from Molecular Probes Inc. Bay K 8644 was from Calbiochem. Calciseptine, ω-conotoxin GVIA and D609 were from Procyon Pharmaceuticals Inc. Diltiazem, verapamil and all other drugs were from Sigma.

3. Results

3.1. Inhibition of thapsigargin-induced Ca^{2+} influx in U937 cells by selective L-type Ca^{2+} channel antagonists

The endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor, thapsigargin (Tg), is well established as an agent that activates

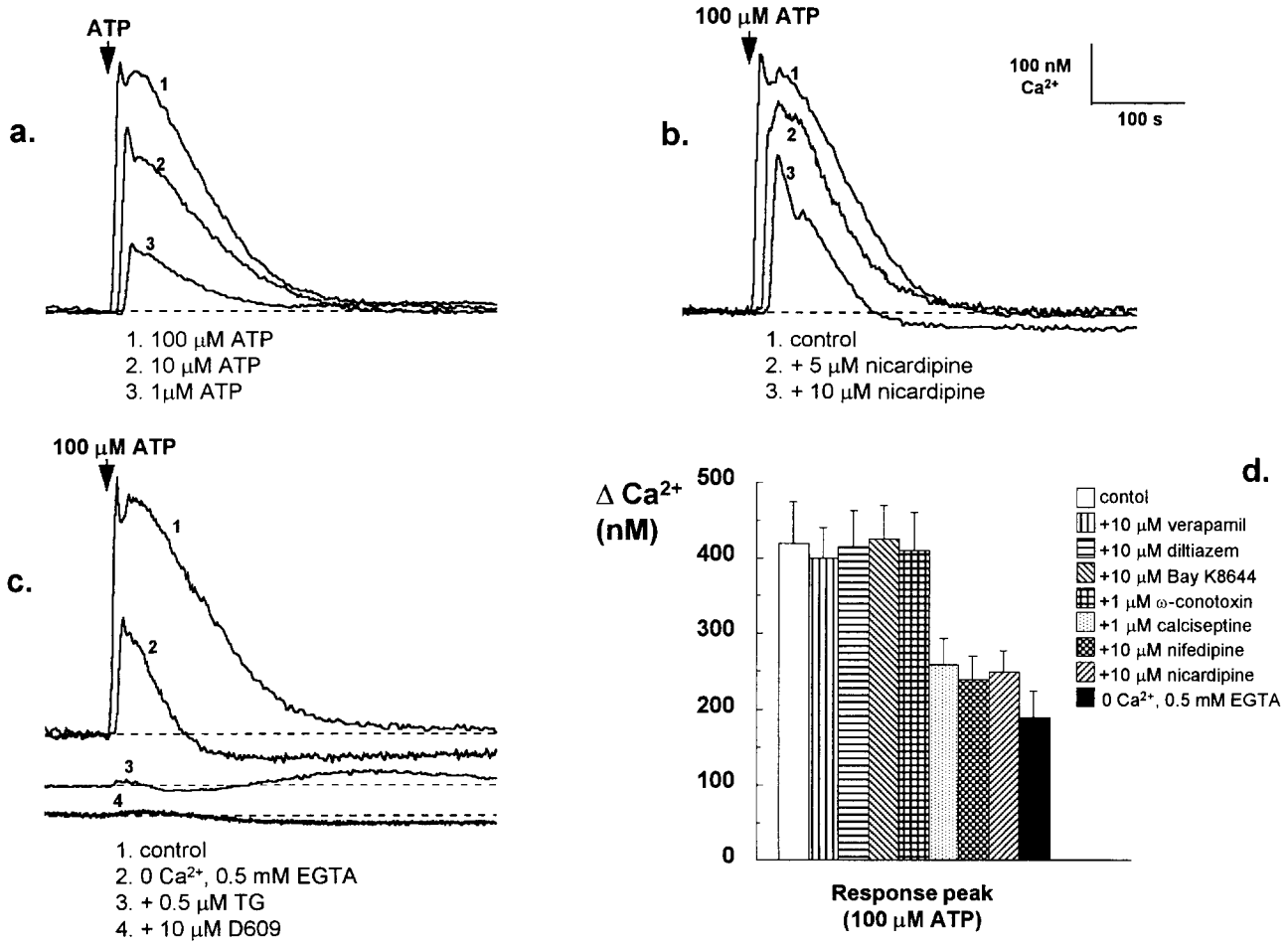

Fig. 2. Effect of extracellular Ca^{2+} chelation, and pre-treatment of cells with Tg, D609, and drug modulators of L- and N-type Ca^{2+} channels on the ATP-induced increase in $[Ca^{2+}]_i$ of U937 cells. (a) ATP-induced increase in $[Ca^{2+}]_i$ of U937 cells. (b) Pre-incubation of cells with 5 and 10 μM nicardipine for 10 min prior to addition of 100 μM ATP. (c) Transfer of cells to Ca^{2+} depleted Hanks' medium (0 Ca^{2+} +0.5 mM EGTA), or pre-treatment for 15 min with 0.5 μM Tg, or 10 min with 10 μM D609, prior to addition of ATP. (d) Effect of various Ca^{2+} channel modulators on the peak amplitude of the ATP-induced Ca^{2+} response of U937 cells. Cells were pre-incubated with the indicated drugs for 10 min prior to addition of 100 μM ATP. Results are expressed as the mean increase in $[Ca^{2+}]_i$ above the pre-stimulatory basal level ± S.D., derived from 4 separate experiments.

store-operated Ca^{2+} influx in cells by depleting intracellular Ca^{2+} stores [14–16]. Consequently, for U937 cells, the resulting Tg-induced increase in $[Ca^{2+}]_i$ was found to be dependent on extracellular Ca^{2+} (Fig. 1a). Removal of extracellular Ca^{2+} from the bathing medium resulted in a decrease in the peak amplitude, and abrogation of the raised plateau phase of the Tg-induced Ca^{2+} response (Fig. 1a). This suggests that Ca^{2+} mobilization and influx contribute to the overall magnitude of the response, while the sustained, elevated plateau phase is assumed to be due to store-operated Ca^{2+} influx. A similar effect was witnessed for cells maintained in normal Ca^{2+} (1.3 mM) medium and pre-treated for 10 min with either 5–10 μM nicardipine, 5–10 μM nifedipine, or 0.5–1 μM of the specific L-type Ca^{2+} channel blocker, calciseptine. As for extracellular Ca^{2+} chelation, all these drugs reduced the peak amplitude of the Tg-induced Ca^{2+} response (Fig. 1b,c,d) and either significantly reduced (nifedipine, calciseptine), or completely abrogated (nicardipine) the elevated plateau phase (Fig. 1b,c,d), suggesting that they were inhibiting store-operated Ca^{2+} influx. Other known inhibitors of L-type Ca^{2+} channels, including verapamil and diltiazem, the L-type Ca^{2+} channel agonist Bay K 8644, and the N-type Ca^{2+} channel blocker ω-conotoxin, did not affect the Tg-induced Ca^{2+} response (Fig. 1d) at the indicated concentrations.

Further evidence for dihydropyridine- and calciseptine-sensitivity for Tg-induced Ca^{2+} influx in U937 cells was derived from manganese quench experiments (Fig. 3a), and the re-introduction of Ca^{2+} to store-depleted cells bathed in low extracellular Ca^{2+} medium (Fig. 3b). Pre-treatment of cells for 3 min with 10 μM nicardipine resulted in a reduction in the Tg-stimulated manganese quench of cytosolic fura-2 (Fig. 3a), indicating that nicardipine was inhibiting Tg-induced Ca^{2+} influx. A similar effect was seen when cells were pre-treated for 3 min with 10 μM nifedipine, or 1 μM calciseptine (data not shown). Verapamil (10 μM), diltiazem (10 μM), Bay K 8644 (10 μM) and ω-conotoxin (1 μM) did not affect Tg-induced manganese quench of cytosolic fura-2 (data not shown), in line with their lack of effect on the Tg-induced Ca^{2+} response (Fig. 1d).

In cells pre-treated with Tg and bathed in low Ca^{2+} medium (0 Ca^{2+}, 0.5 mM EGTA), in order to deplete Ca^{2+} stores and stimulate SOC activity, the re-introduction of Ca^{2+} to the bathing medium resulted in an increase in $[Ca^{2+}]_i$, due to Ca^{2+} influx (Fig. 3b). For store-depleted cells

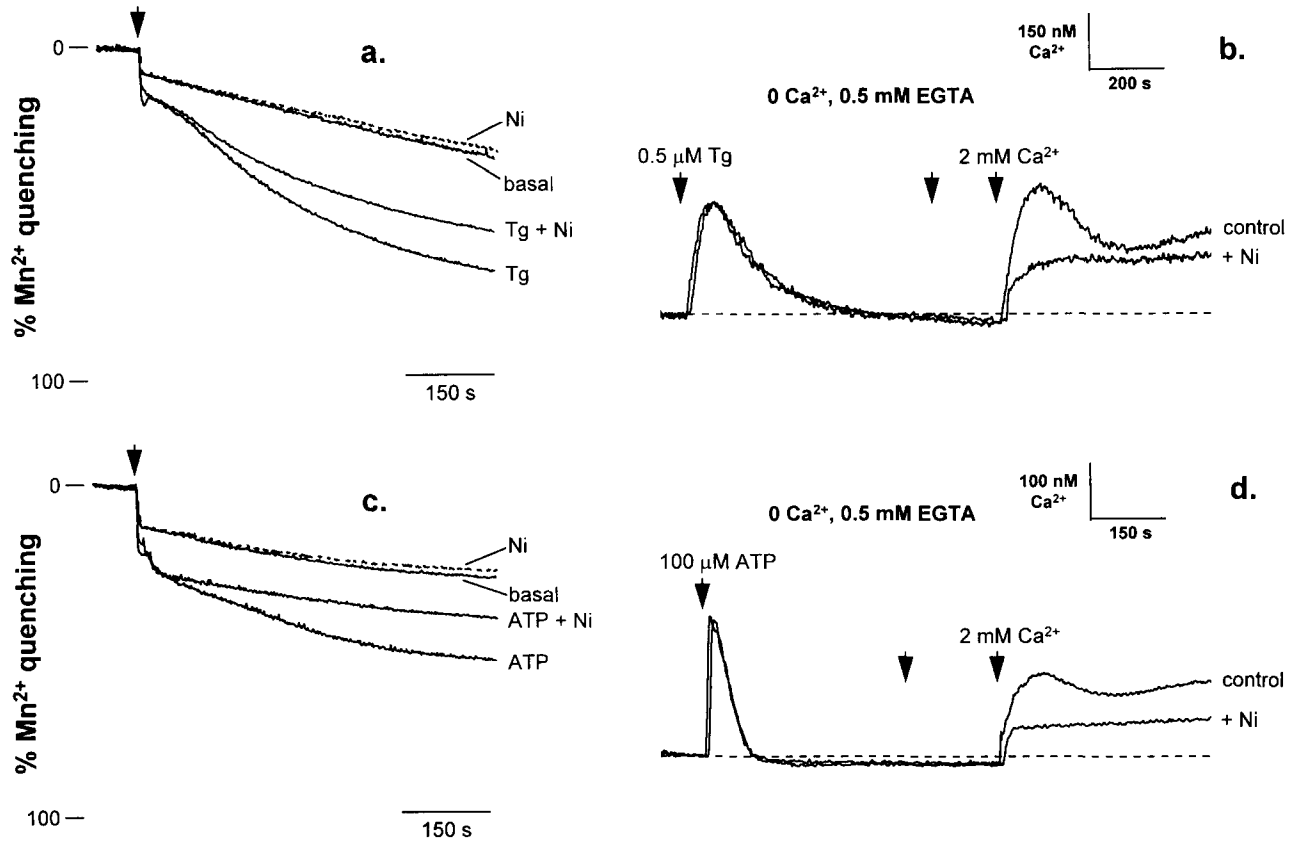

Fig. 3. Effect of nicardipine on Tg- and ATP-induced Ca^{2+} influx in U937 cells. Inhibition of Tg- (a) and ATP-stimulated (c) Mn^{2+} quench of cytosolic fura-2 by nicardipine. Basal Mn^{2+} quench (basal) was determined by the addition of 0.2 mM Mn^{2+} (arrowed) to cells. Pre-treating cells with 10 μM nicardipine for 3 min (Ni) prior to addition of Mn^{2+} (arrowed) did not alter basal quench. Store-operated Ca^{2+} influx was stimulated and monitored by the co-addition of 0.5 μM Tg (a), or 100 μM ATP (c) and 0.2 mM Mn^{2+} (arrowed) to cells (Tg, ATP). Pre-incubating cells with 10 μM nicardipine for 3 min inhibited Tg- (a), and ATP-stimulated (c) Mn^{2+} quench (Tg + Ni, ATP + Ni). (b and d) Ca^{2+} influx inhibition by nicardipine in cells maintained in low Ca^{2+} medium (0 Ca^{2+}, 0.5 mM EGTA) and whose Ca^{2+}-stores were depleted by the prior addition of 0.5 μM Tg (b), or 100 μM ATP (d). For nicardipine-treated cells (+ Ni), 10 μM nicardipine was added (unlabelled arrow) 3 min prior to the re-introduction of 2 mM Ca^{2+} to the bathing medium, in (b) and (d). For control cells (control), nicardipine was omitted. $[Ca^{2+}]_i$ was monitored in the same way as above. All traces of Fig. 3 are representative of at least 3 separate experiments.

pre-treated with 10 μM nicardipine for 5 min, the increase in $[Ca^{2+}]_i$ on re-introduction of 2 mM Ca^{2+} to the bathing medium was reduced compared to untreated controls, again suggesting that nicardipine inhibits store-operated Ca^{2+} influx in U937 cells. As for the previous experiments, 10 μM nifedipine and 1 μM calciseptine mimicked the action of 10 μM nicardipine, while no Ca^{2+} influx inhibition was observed for cells treated with 10 μM verapamil, 10 μM diltiazem, 10 μM Bay K 8644, or 1 μM ω-conotoxin (data not shown).

3.2. *Inhibition of ATP-induced Ca^{2+} influx in U937 cells by selective L-type Ca^{2+} channel antagonists*

Addition of 1–100 μM ATP to U937 cells, resulted in a transient increase in $[Ca^{2+}]_i$ (Fig. 2a), via purinergic receptor activation. This ATP-induced Ca^{2+} response was abolished by prior treatment of cells with Tg, or the phospholipase C inhibitor, D609, and was substantially reduced when cells were transferred to low Ca^{2+} medium (Fig. 2c). This suggests that the response is comprised of an inositol-1,4,5-trisphosphate (IP_3) -mediated Ca^{2+} mobilization component and a Ca^{2+} influx component, with the influx component being dependent on the mobilization component, and therefore presumably store-operated. As for extracellular Ca^{2+} chelation, pre-treating cells for 10 min with 5–10 μM nicardipine resulted in a reduction in the ATP-induced Ca^{2+} response (Fig. 2b), with both nifedipine and calciseptine mimicking the effect of nicardipine (Fig. 2d). Verapamil, diltiazem, Bay K 8644 and ω-conotoxin did not affect the ATP-induced Ca^{2+} response at the concentrations indicated (Fig. 2d), in line with their lack of effect on the Tg-induced Ca^{2+} response, and suggesting the possible delineation of ATP-induced store-operated Ca^{2+} influx by nicardipine, nifedipine and calciseptine. This was confirmed from manganese quench experiments (Fig. 3c), and the re-introduction of Ca^{2+} to store-depleted cells bathed in low extracellular Ca^{2+} medium (Fig. 3d), where broadly similar effects of the above drugs were witnessed for ATP-stimulated Ca^{2+} influx as for Tg-stimulated Ca^{2+} influx (Fig. 3). These results strongly suggest that nicardipine, nifedipine and calciseptine inhibit ATP- as well as Tg-induced store-operated Ca^{2+} influx in U937 cells.

3.3. *Depolarisation of U937 cells*

Depolarising U937 cells by addition of 50 mM KCl or 1 μM gramicidin to the bathing medium, did not result in an increase in $[Ca^{2+}]_i$. This suggests that U937 cells do not possess voltage-sensitive Ca^{2+} channels, consistent with their non-ex-

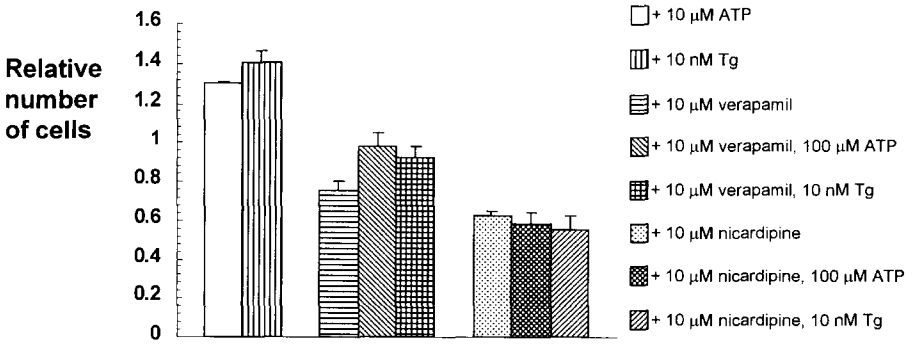

Fig. 4. Suppression of ATP- and Tg-stimulated U937 cell proliferation by nicardipine. Untreated control and drug-treated cells were incubated in DMEM for 24 h, prior to counting. For experiments investigating the requirement of store-operated Ca^{2+} influx in ATP- and Tg-stimulated cell proliferation, cells were pre-treated with nicardipine (10 μM), or verapamil (10 μM) for 15 min, prior to addition of ATP (100 μM) or Tg (10 nM) and 24 h incubation. Results are expressed as the mean change in cell number ± S.D. relative to untreated control cells (control = 1), derived from 3 estimations.

citable status. Hence, although the store-operated Ca^{2+} influx of these cells is dihydropyridine-sensitive, it is unlikely to be voltage-sensitive.

3.4. *Inhibition of ATP- and Tg-stimulated cell proliferation by nicardipine*

For cells treated with ATP (100 μM) or a low concentration of Tg (10 nM) for 24 h, cell number was significantly increased by 30–40% compared to untreated controls (Fig. 4). For cells treated with nicardipine (10 μM), or verapamil (10 μM) for 24 h, cell number was reduced compared to untreated controls; however, this was not due to increased cell death in the former. Pre-treatment of cells for 15 min with nicardipine (10 μM), prior to addition of ATP (100 μM) or Tg (10 nM), prevented stimulation of cell growth by the latter (Fig. 4). Conversely, verapamil (10 μM), which did not inhibit SOC activity in U937 cells, did not prevent stimulation of cell growth by ATP and Tg (Fig. 4).

4. Discussion

Results from this study support the existence of a dihydropyridine-sensitive, yet voltage-insensitive store-operated Ca^{2+} influx mechanism in U937 cells. We therefore propose that the SOC of U937 cells might resemble an L-type Ca^{2+} channel lacking a voltage sensor. Several models have been described in the literature which appear strikingly similar to the SOC of U937 cells, and may therefore be structural and functional homologues of this channel. (i) The *Drosophila melanogaster trp* gene product has a similar sequence to the α_1 subunit of the L-type Ca^{2+} channel, but lacks charged residues in the S_4 segment [10], the part of the α_1 subunit that is thought to sense a change in voltage [17]. Transfection of mammalian *trp* gene homologues in a mammalian cell line (COS-M6) enhanced store-operated Ca^{2+} entry beyond that found in non-transfected cells, while transfection of mouse fibroblast L-cells with antisense cDNA fragments of murine *trp* homologues resulted in the abolition of store-operated Ca^{2+} influx [18]. This indicates that mammalian *trp* gene homologues encode proteins that can participate in store-operated Ca^{2+} entry in non-excitable mammalian cells. (ii) A murine erythroleukemia cell line expresses a truncated form of the L-type Ca^{2+} channel α_1 subunit, in which the first four transmembrane segments are absent [19]. While this would render the channel voltage-insensitive, the dihdropyridine binding site, residing between the S_5 and S_6 segments of the α_1 subunit, is intact, thus conferring dihydropyridine-sensitivity. (iii) The anti-Ig-induced Ca^{2+} influx of rat B-lymphocytes is mediated by cGMP, and is both dihydropyridine- and calciseptine-sensitive, and is also inhibited by an anti-peptide antibody raised against the α_1 subunit of the L-type Ca^{2+} channel, yet is voltage-insensitive [20]. (iv) A cGMP modulated, store-operated Ca^{2+} influx channel of rat pituitary GH_3 cells is nicardipine-sensitive [6].

Taken as a whole, a picture is emerging which suggests that SOCs may constitute a new class of Ca^{2+} channel with significant structural commonality to L-type Ca^{2+} channels, and perhaps do not consist of a disparate group of unrelated conductances, as has previously been suggested [21]. Furthermore, since there is also mounting evidence that cGMP can modulate a number of SOCs, it is possible that these channels may also share structural similarity to cyclic nucleotide gated channels (CNGs), especially when considering that the S_5-S_6 linkers of VGCs and CNGs show significant resemblance [22,23].

Although the store-operated Ca^{2+} influx of U937 cells was sensitive to dihydropyridines and calciseptine, it was insensitive to the L-type Ca^{2+} channel blockers verapamil, and diltiazem. This may imply different binding domains for the above drug classes. It is also noteworthy that verapamil failed to prevent ATP- or Tg-induced stimulation of cell growth, in line with its lack of effect on store-operated Ca^{2+} influx, while nicardipine was inhibitory. Taken together, these observations underscore a functional requirement for store-operated Ca^{2+} influx in agonist-stimulated U937 cell proliferation. Since nicardipine and verapamil alone reduced cell growth following 24 h incubation compared to untreated controls, these drugs might also inhibit other Ca^{2+} channels which help maintain the resting state $[Ca^{2+}]_i$ within close limits.

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