Activation of ryanodine receptors induces calcium influx in a neuroblastoma cell line lacking calcium influx factor activity

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We have further characterized the Ca^{2+} signalling properties of the NG115-401L (or 401L) neuroblastoma cell line, which has served as an important cell line for investigating SOC (store-operated channel) influx pathways. These cells possess an unusual Ca^{2+} signalling phenotype characterized by the absence of Ca^{2+} influx when Ca^{2+} stores are depleted by inhibitors of SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase). Previous studies found that Ca^{2+} -store depletion does not produce a CIF (Ca^{2+} influx factor) activity in 401L cells. These observations have prompted the question whether 401L cells possess the signalling machinery that permits non-voltage-gated Ca^{2+} influx to occur. We tested the hypothesis that ryanodine-sensitive Ca^{2+} pools and activation of RyRs (ryanodine receptors) constitute a signalling pathway capable of inducing Ca^{2+} influx in 401L cells. We found

that 401L cells express mRNA for RyR1 and RyR2 and that RyR activators induced Ca^{2+} release. Activation of RyRs robustly couples with Ca^{2+} influx responses in 401L cells, in sharp contrast with absence of Ca^{2+} influx when cells are treated with SERCA inhibitors. Thus it is clear that 401L cells, despite lacking depletion-induced Ca^{2+} influx pathways, express the functional components of a Ca^{2+} influx pathway under the control of RyR function. These findings further support the importance of the 401L cell line as an important cell phenotype for deciphering Ca^{2+} influx regulation.

Key words: calcium influx factor, conformational coupling, cyclopiazonic acid, depletion-activated calcium influx, ryanodine receptor, store-operated calcium influx.

INTRODUCTION

Changes in intracellular Ca^{2+} concentration serve as major ubiquitous signals triggering a wide spectrum of biological events, including fast responses such as contraction and secretion in addition to slower long-lasting changes in the growth properties of cells [1]. A large number of cells mediate the increases in cytosolic Ca^{2+} through the activation of receptors that couple with the production of IP_3 (inositol 1,4,5-trisphosphate), which mobilizes Ca^{2+} from internal stores in the ER (endoplasmic reticulum). Release of Ca^{2+} from ER stores couples with the activation of Ca^{2+} influx from the extracellular space, a pathway that has been observed in a great many non-excitable and excitable cell types and is often denoted as capacitative or store-operated Ca^{2+} entry [2]. The mechanism that links Ca^{2+} influx to the release of ER Ca^{2+} continues to be a poorly understood process. It is widely accepted, however, that the signal that initiates the opening of the influx channels is the depletion of ER Ca^{2+} stores [2–4]. These channels are therefore referred to as SOCs (store-operated channels) to denote their regulation by the Ca^{2+} content of the ER stores. Mechanisms proposed to activate SOC channels include the production of diffusible messengers, direct physical contact between SOC proteins and either IP_3 Rs (IP_3 receptors) or RyRs (ryanodine receptors), and direct insertion of SOC channel proteins into the plasma membrane [4]. These three ideas have been referred to respectively as the diffusible messenger, conformational coupling and secretion models to explain how Ca^{2+} influx is regulated by events initiated in the ER. A potentially powerful tool to assist in deciphering among the different modes of Ca^{2+} influx would be a native cell line that unambiguously operates in a single mode to mediate Ca^{2+} influx and, therefore,

represents a more tractable cell model system for investigating the regulation of Ca^{2+} influx.

The NG115-401L (or 401L) neuroblastoma cell line has been a valuable cell line model for studies investigating the mechanisms of store-operated Ca^{2+} influx [5,6]. Previous studies have identified 401L cells as having an unusual phenotype with respect to responses to thapsigargin, the most potent inhibitor of the family of intracellular Ca^{2+} pump proteins known as the SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase) enzymes [5,6]. Indeed, the most widely accepted pharmacological paradigm for activating Ca^{2+} influx pathways employs thapsigargin treatment to deplete Ca^{2+} stores and activate SOC channels. However, in the 401L cell line, thapsigargin treatment fails to induce Ca^{2+} influx, in contrast with most cell types tested for this response [7]. Moreover, it has been shown that these cells fail to produce a small molecule CIF (calcium influx factor) activity when treated with thapsigargin, unlike T lymphocytes and other cells that exhibit pronounced SOC channel activation when treated with SERCA blockers [6,8]. The absence of a thapsigargin-induced Ca^{2+} influx messenger and response prompts the question whether 401L cells possess non-voltage-regulated Ca^{2+} entry pathways at all. These observations suggest that 401L cells may possess Ca^{2+} influx pathways dependent solely on conformational coupling by intracellular Ca^{2+} release channels, given the absence of a store-depletion-induced diffusible messenger. To address this question, we have examined the hypothesis that the 401L cell requires activation of RyRs to induce Ca^{2+} influx, a role described for RyRs in other neuronal cell types [9,10]. In the present study we report, using RT (reverse transcriptase)–PCR, the expression of mRNAs encoding two RyR isoforms (RyR1 and RyR2) in the 401L cell line. Moreover, we also report that Ca^{2+} influx can be

Abbreviations used: /AM, acetoxymethyl ester; CMC, 4-chloro-*m*-cresol; CPA, cyclopiazonic acid; ER, endoplasmic reticulum; HBSS, Hanks balanced salt solution; IP_3 , inositol 1,4,5-trisphosphate; IP_3 R, IP_3 receptor; PCB, pentachlorobiphenyl; PCB95, 2,2',3,5',6-pentachlorobiphenyl; RT, reverse transcriptase; RyR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase; SOC, store-operated channel.

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robustly induced in 401L cells using a spectrum of RyR pharmacological activators. We also find that Ca^{2+} influx can be induced in 401L cells by pro-inflammatory mediators that signal by activation of IP_3Rs . Thus the 401L cell line appears to express non-voltage-regulated Ca^{2+} influx pathways solely dependent on activation of intracellular Ca^{2+} release channels, although lacking influx pathways coupled with the depletion of intracellular Ca^{2+} stores. These findings further highlight the 401L cell line as an important model cell line for investigating the regulation of Ca^{2+} influx pathways.

EXPERIMENTAL

Materials

Ryanodine and thapsigargin were obtained from LC Laboratories (Woburn, MA, U.S.A.). CMC (4-chloro-*m*-cresol), ionomycin and CPA (cyclopiazonic acid) were from Calbiochem (La Jolla, CA, U.S.A.). Caffeine, ATP and poly-L-lysine were from Sigma (St. Louis, MO, U.S.A.). PCB95 (2,2',3,5',6-pentachlorobiphenyl) was purchased from Ultra Scientific (North Kingstown, RI, U.S.A.). Fura 2/AM (fura 2 acetoxymethyl ester) and the dispersing reagent Pluronic F-127 were from Molecular Probes (Eugene, OR, U.S.A.). Glass coverslips (9 mm $\times$ 22 mm) used for Ca^{2+} measurements on adherent cells were purchased from Bellco Glass (Vineland, NJ, U.S.A.). Cell culture reagents were purchased from Cambrex Bio Science (Walkersville, MD, U.S.A.). Omniscript RT, RNase inhibitor, MgCl_2 , dNTP, 10 vol. of PCR buffer, HotstarTaq™ DNA polymerase and RNeasy mini kit™ were purchased from Qiagen (Valencia, CA, U.S.A.). Random primers were obtained from Invitrogen (Carlsbad, CA, U.S.A.), rRNA (18 S) and RNase inhibitor from Ambion (Austin, TX, U.S.A.).

Cell culture

NG115-401L neuroblastoma cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 $\mu\text{g}/\text{ml}$ streptomycin and 100 units/ml penicillin. Cells were grown in 75 cm^2 (T75) tissue culture flasks (Phenix Research Products, Hayward, CA, U.S.A.) and passaged every 3 days at the ratio of 1:10. For Ca^{2+} measurements, NG115-401L cells were seeded on to poly-L-lysine-coated coverslips at a cell density of $1.5\text{--}2 \times 10^6$ cells/3 ml.

Calcium measurements

Monolayer cultures of NG115-401L cells growing on coverslips were loaded with 1.5 μM fura 2/AM for 30 min at room temperature (25 °C). The cells were gently washed with HBSS (Hanks balanced salt solution) and placed in a coverslip holder (PTI, Lawrenceville, NJ, U.S.A.) for insertion into cuvettes containing HBSS (2 ml). Depending on the experiment, the cells were suspended either in HBSS containing 1.8 mM Ca^{2+} or in a Ca^{2+} -free HBSS buffer. Changes in cytosolic Ca^{2+} were measured in cell population experiments using a fluorescence spectrophotometer (PTI) equipped with a thermostatically controlled sample compartment, permitting continuous stirring of samples. For cells loaded with fura 2/AM, excitation of the dye was achieved by rapidly alternating monochromator settings between 340 and 380 nm, with fluorescence emission measured at 510 nm. Changes in cytosolic Ca^{2+} concentrations are reported as the fluorescence ratio values for fura 2 measured at 340 and 380 nm. All ratios were corrected for autofluorescence by measuring fluorescence changes at 340 and 380 nm in 401L cells not loaded with fura 2

and subtracting these values from the experimental data obtained from fura 2-loaded cells.

Semi-quantitative RT-PCR

Total cellular RNA from 401L cells was extracted using an RNeasy mini kit™ according to the manufacturer's instructions. RNA was quantified by measuring absorbance spectrophotometrically at 260 nm and its integrity was assessed after electrophoresis in non-denaturing 1% agarose gels stained with ethidium bromide. Reverse transcription of 2 μg of total RNA was performed in 20 μl reaction volumes containing 4 units of Omniscript reverse transcriptase (Qiagen), 10 units of RNase inhibitor, 1 $\times$ buffer RT and 1 μM random primers (Invitrogen) for 60 min at 37 °C. For each 100 μl of PCR mixture, 5 μl of the RT product was used. The PCR mixture contained 250 μM dNTP, 2 mM MgCl_2 , 1 vol. of Qiagen buffer, 0.5 unit of HotstarTaq polymerase (Qiagen) and 1 μl each of forward and reverse primers (100 ng). The temperature programme for the amplification was 34 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. The final extension was completed at 72 °C for 7 min. PCR products were then analysed by electrophoresis on 1.6% (w/v) agarose gels stained with ethidium bromide and gels were photographed under UV light. As an internal control, 18 S rRNA expression (324 bp; Ambion) was used. The primer sequences used for RyR detection were based on published reports [11] and are as follows: for RyR1, 5'-GAAGGTTCTGGACAAACACGGG-3' (sense) and 5'-TCGCTCTTGTTGTAGAATTTGCGG-3' (antisense); for RyR2, 5'-GAATCAGTGAGTTACTGGGCATGG-3' (sense) and 5'-TTGGTCTCTCTGAGTTCTCCAAAAGC-3' (antisense); and for RyR3, 5'-CCTTCGCTATCAACTTCATCCTGC-3' (sense) and 5'-TCTTCTACTGGGCTAAAGTCAAGG-3' (antisense). The predicted amplicon sizes with these primer sets were 435 bp for RyR1, 635 bp for RyR2 and 505 bp for RyR3.

RESULTS

For the following results, Ca^{2+} responses are presented as changes in fluorescence ratio values measured at 340/380 nm for fura 2. The results are reported either as peak amplitude changes in fluorescence ratio values or as initial rates of fluorescence ratio changes and are expressed as means $\pm$ S.E.M., with the number of experiments indicated in parentheses [12].

RyR activation, but not SERCA inhibition, stimulates Ca^{2+} influx in 401L cells

NG115-401L neuroblastoma cells treated with the SERCA inhibitor CPA undergo a transient Ca^{2+} response, with no detectable Ca^{2+} influx inducible when extracellular Ca^{2+} levels are increased to 10 mM (Figure 1A). CPA (50 μM) induced a peak F_{340}/F_{380} fluorescence ratio increase of 0.52 ± 0.18 ($n = 4$) in 401L cells that rapidly returned to basal levels in the presence of extracellular Ca^{2+} (1.8 mM).

These results are consistent with earlier findings demonstrating that SERCA inhibition due to thapsigargin treatment also fails to induce Ca^{2+} influx in 401L cells [5,6].

Previous studies have shown that the RyR family of intracellular Ca^{2+} release channels can participate in the regulation of Ca^{2+} influx pathways [13,14]. We hypothesized that 401L cells represent a cell phenotype possessing Ca^{2+} influx pathways controlled by RyR function but lacking influx pathways coupled directly with Ca^{2+} -store depletion. We sought to determine whether 401L cells contain RyR-releasable Ca^{2+} pools and whether RyR-activated Ca^{2+} release could, in contrast with CPA, induce measurable

Figure 1 Ca^{2+} influx is induced by ryanodine but not by CPA in NG115-401L cells

(A) The addition of CPA ($50 \mu\text{M}$) in the presence of extracellular Ca^{2+} (1.8 mM) increased the F_{340}/F_{380} fluorescence ratio, but failed to activate Ca^{2+} influx when extracellular Ca^{2+} levels were increased to 10 mM . (B) 401L cells were incubated in a Ca^{2+} -free medium (0 Ca^{2+} HBSS) and stimulated with ryanodine ($1 \mu\text{M}$). After the decay of the ryanodine response, Ca^{2+} (1 mM) was added back to the cells and responses were tested for sensitivity to Ni^{2+} (1 mM). (C) Cells stimulated as in (B) were tested for sensitivity to EGTA (5 mM) to determine their dependence on extracellular Ca^{2+} . (D) Ba^{2+} (1 mM) was added to 401L cells stimulated with ryanodine ($1 \mu\text{M}$) in Ca^{2+} -free HBSS. Arrows indicate the approximate time points of addition of the various agents. Bars indicate different treatment methods for bivalent ion exposure.

Ca^{2+} influx. Using semi-quantitative RT-PCR with RyR-specific primers (see the Experimental section), we observed expression of the RyR1 and RyR2 but not the RyR3 isoform in 401L cells (results not shown). 401L cells treated with ryanodine ($1 \mu\text{M}$) in Ca^{2+} -free media produced robust Ca^{2+} release responses (0.92 ± 0.22 peak fluorescence ratio units, $n = 8$), as shown in Figure 1. For these experiments, we used a Ca^{2+} add-back assay to test for the opening of influx channels after RyR activation. As shown in Figure 1(B), addition of Ca^{2+} (1 mM) after the decay of the ryanodine-activated discharge response elicited a rapid initial increase in the F_{340}/F_{380} fluorescence ratio (0.8 ± 0.37 fluorescence ratio units/min, $n = 8$), indicating that RyR activation resulted in opening of the Ca^{2+} influx channels. The ryanodine-induced Ca^{2+} influx response was also sensitive to Ni^{2+} treatment (Figure 1A). Indeed, addition of Ni^{2+} (1 mM) resulted in the complete reversal of the influx response at an initial decay rate of 0.33 ± 0.07 fluorescence ratio units/min ($n = 8$). Chelation of extracellular Ca^{2+} with EGTA (5 mM) resulted in a rapid and complete reversal of the ryanodine-induced Ca^{2+} influx response with an initial decay rate of 0.80 ± 0.12 fluorescence ratio units/min ($n = 4$; Figure 1C). In addition, Figure 1(D) shows that ryanodine-induced Ca^{2+} release stimulated a robust Ba^{2+} influx pathway in 401L cells (1.06 ± 0.18 fluorescence ratio units/min, $n = 4$), also consistent with the activation of a Ca^{2+} influx pathway [15]. The fluorescence signal induced by Ba^{2+} influx shows a continuing gradual increase, probably as a result of the inability of the cell to extrude the ion.

We proceeded to test whether the 401L neuroblastoma cell line would respond to other commonly employed pharmacological activators of RyRs. Recent studies have identified a subgroup of polychlorobiphenyls (PCBs) that serve as useful agents to investigate the properties of Ca^{2+} release from RyR-sensitive stores

Figure 2 Common pharmacological activators of RyRs induce Ca^{2+} influx in NG115-401L cells

(A) 401L cells were treated with PCB95 ($10 \mu\text{M}$) in Ca^{2+} -free HBSS, followed by challenge with Ca^{2+} (1 mM) and exposure to Ni^{2+} (1 mM). (B) In a Ca^{2+} -containing (1.8 mM) medium, CMC ($250 \mu\text{M}$; solid trace) induced $[\text{Ca}^{2+}]_i$ release and activated sustained Ca^{2+} entry responses. The addition of high Ca^{2+} concentrations (10 mM) further increased the Ca^{2+} influx. CMC-induced Ca^{2+} influx was inhibited by La^{3+} ($100 \mu\text{M}$). In a Ca^{2+} -free medium, CMC ($250 \mu\text{M}$, broken trace) induced a transient F_{340}/F_{380} fluorescence peak. Restoration of external Ca^{2+} (1.2 mM) induced a Ca^{2+} influx response that was completely inhibited by Ni^{2+} (1 mM) treatment. (C) The RyR activator caffeine (40 mM) induced Ca^{2+} release in 401L cells in the presence of extracellular Ca^{2+} (1.8 mM). Caffeine treatment resulted in Ca^{2+} influx responses when high Ca^{2+} (10 mM) was added to the cells. Caffeine-induced Ca^{2+} influx responses were inhibited by Zn^{2+} (1 mM). Arrows indicate the approximate time points of addition of the various agents. Bars indicate different treatment methods for bivalent ion exposure.

[16]. It has been shown that the PCB95 congener can specifically mobilize Ca^{2+} from RyR-sensitive stores in PC12 cells [17]. Figure 2(A) shows that the application of PCB95 ($10 \mu\text{M}$) to 401L cells in Ca^{2+} -free media resulted in the rapid discharge of intracellular Ca^{2+} stores, with a peak fluorescence change corresponding to 1.05 ± 0.23 fluorescence ratio units ($n = 6$). As with ryanodine, the PCB95-induced RyR activation was accompanied

by a rapid initial increase in the influx rate (0.84 ± 0.26 fluorescence ratio units/min, $n = 6$) when extracellular Ca^{2+} (1 mM) was added back to the cells. PCB95-induced Ca^{2+} influx was also sensitive to Ni^{2+} blockade, showing a rapid initial decrease (0.32 ± 0.08 fluorescence ratio units/min, $n = 6$) and complete reversal of the influx response (Figure 2A).

Figure 2(B) shows 401L cell responses to the RyR agonist CMC [18]. The application of 250 μM CMC to fura 2-loaded 401L cells in the presence of extracellular Ca^{2+} (1.8 mM) induced a sustained response (peak fluorescence ratio change of 0.44 ± 0.18 , $n = 7$), presumably reflecting both Ca^{2+} release and influx. The addition of high Ca^{2+} (10 mM) during the CMC-induced response stimulated a further increase in Ca^{2+} levels with an initial rate increase of 0.55 ± 0.14 fluorescence ratio units/min ($n = 7$; Figure 2B). Ca^{2+} influx responses induced by 250 μM CMC were rapidly inhibited by La^{3+} ions (0.35 ± 0.08 fluorescence ratio units/min, $n = 5$). It is apparent, however, that La^{3+} (100 μM), while rapidly inhibiting CMC-induced Ca^{2+} influx, failed to reverse completely the increased Ca^{2+} signals to pre-stimulus levels, suggesting the recruitment of influx pathways insensitive to La^{3+} blockade. Figure 2(B) also shows 401L cell responses to CMC (250 μM) in Ca^{2+} -free HBSS. In zero- Ca^{2+} buffers, CMC stimulation resulted in a transient Ca^{2+} response (peak fluorescence ratio change of 0.16 ± 0.08 , $n = 6$) that returned to pre-stimulus levels. Restoration of extracellular Ca^{2+} (1.2 mM) induced a rapid Ca^{2+} influx response that was completely inhibited by Ni^{2+} (1 mM) treatment (Figure 2B).

We also tested 401L cells for responses to the RyR activator caffeine (Figure 2C). Caffeine (40 mM) induced a varying peak Ca^{2+} response in the presence of extracellular Ca^{2+} (0.22 ± 0.12 fluorescence ratio units, $n = 8$). The addition of high Ca^{2+} (10 mM) to caffeine-stimulated cells resulted in a rapid Ca^{2+} influx response (0.37 ± 0.11 fluorescence ratio units/min, $n = 8$) that was sensitive to Zn^{2+} ions (Figure 2C). Indeed, Zn^{2+} (1 mM) treatment led to rapid (0.43 ± 0.11 fluorescence ratio units/min, $n = 4$) and complete reversal of the influx response to basal Ca^{2+} levels. Thus four structurally distinct RyR activators were capable of inducing Ca^{2+} discharge in 401L cells. Moreover, in contrast with CPA-induced Ca^{2+} release, RyR-activated Ca^{2+} release coupled efficiently with robust Ca^{2+} influx responses, as determined by the sensitivity of these responses to external Ca^{2+} and to a spectrum of commonly applied blockers of Ca^{2+} influx pathways.

Characterization of Ca^{2+} pools in the 401L cell line

In view of the possibility that Ca^{2+} influx may be linked to Ca^{2+} mobilization from discrete agonist-specific Ca^{2+} pools, we sought to clarify the relationship between stores discharged by SERCA blockers and stores sensitive to RyR agonists. We observed that prior treatment of 401L cells with thapsigargin (1 μM), in the absence of extracellular Ca^{2+} , effectively abolished responses to the subsequent addition of both CMC (500 μM) and ryanodine (1 μM ; Figures 3A and 3B). To determine whether RyR-mediated Ca^{2+} release represents all or part of the total Ca^{2+} -releasable pools in 401L cells, the order of ryanodine and thapsigargin addition was reversed. Figure 3(C) shows that prior treatment with ryanodine (1 μM) in the absence of extracellular Ca^{2+} abolishes the subsequent thapsigargin (1 μM) response, suggesting that the thapsigargin- and RyR-releasable storage compartments are the same. Furthermore, the addition of a Ca^{2+} ionophore (2 μM ionomycin) in the absence of extracellular Ca^{2+} after thapsigargin (1 μM) and CMC (500 μM) application elicited a response indicating additional Ca^{2+} stores not sensitive to SERCA blockers or RyR agonists (Figure 3D).

Figure 3 Relationship of the RyR-gated and thapsigargin-sensitive Ca^{2+} pools in NG115-401L cells

(A) In a Ca^{2+} -free medium (0 Ca^{2+} HBSS), addition of thapsigargin (TG, 1 μM) induced a $[\text{Ca}^{2+}]_i$ transient that rapidly decayed to pre-stimulus levels. Subsequent addition of CMC (500 μM) failed to stimulate an increase in $[\text{Ca}^{2+}]_i$. (B) Response of 401L cells to ryanodine (1 μM) after stimulation with TG (1 μM) in Ca^{2+} -free media. (C) The addition of ryanodine (1 μM) to 401L cells in a Ca^{2+} -free medium induced a $[\text{Ca}^{2+}]_i$ transient reflecting release from intracellular Ca^{2+} stores. Subsequent addition of TG (1 μM) failed to cause an increase in $[\text{Ca}^{2+}]_i$. (D) As in (A, B), addition of TG (1 μM) in a Ca^{2+} -free medium elicited a $[\text{Ca}^{2+}]_i$ transient that returned to pre-stimulus levels. Subsequent addition of CMC (500 μM) failed to induce a response. The subsequent addition of ionomycin (2 μM) induced an increase of $[\text{Ca}^{2+}]_i$ in 401L cells. Arrows indicate the approximate time points of addition of the various agents.

Activation of IP_3Rs induces Ca^{2+} influx

401L cells serve as an important sensory neuron model cell line, having several features in common with primary afferent sensory neurons [5,19,20]. Indeed, they represent an important cell line for elucidating the signalling pathways involved in neural inflammatory responses [5,21,22]. As such, 401L cells express receptors for the key inflammatory mediators bradykinin and ATP, which release Ca^{2+} through the phosphatidylinositol pathway and IP_3R activation. Thus we wished to determine whether activation of these pro-inflammatory IP_3R -coupled pathways constituted a sufficient signal to induce Ca^{2+} influx in 401L cells. The addition of ATP (100 μM) to 401L cells in the presence of extracellular Ca^{2+} (1.8 mM) induced responses similar to those observed for RyR agonists, with an initial release of Ca^{2+} from internal stores (a peak value of 0.75 ± 0.16 fluorescence ratio units, $n = 6$) followed by a further increase in cytosolic Ca^{2+} when the external Ca^{2+} concentration was increased to 5 mM (Figure 4A). The initial rate of the Ca^{2+} influx stimulated by ATP treatment was similar to responses induced by RyR agonists (0.52 ± 0.17 fluorescence ratio units/min, $n = 6$). As described above for RyR agonists, the Ca^{2+} influx response induced by ATP was completely inhibited by Ni^{2+} (1 mM) at a decay rate of 0.31 ± 0.08 fluorescence ratio units/min ($n = 6$; Figure 4A). Prior exposure of 401L cells to thapsigargin (1 μM) in the absence of extracellular Ca^{2+} eliminated subsequent ATP (100 μM)-induced responses (Figure 4B), suggesting that the ATP-releasable pools overlap or are a sub-compartment of the thapsigargin-releasable Ca^{2+} pools. Moreover, when the order of addition of these stimulants is reversed, prior ATP (100 μM) treatment abolished thapsigargin-induced

Figure 4 ATP induces Ca^{2+} influx in NG 115-401L cells

(A) In a Ca^{2+} -containing (1.8 mM) medium ATP (100 μM) mobilized Ca^{2+} from intracellular stores. Further addition of high Ca^{2+} concentrations (5 mM) to the medium increased the $[\text{Ca}^{2+}]_i$ responses. The ATP-induced Ca^{2+} influx responses were inhibited by Ni^{2+} (1 mM). (B) In a Ca^{2+} -free medium, thapsigargin (TG, 1 μM) increased $[\text{Ca}^{2+}]_i$. Subsequent addition of ATP (100 μM) failed to increase $[\text{Ca}^{2+}]_i$. Restoration of Ca^{2+} (1.2 mM) to the cells induced Ca^{2+} influx responses. (C) Similar results were obtained by reversing the order of addition in (B); TG (1 μM) failed to increase $[\text{Ca}^{2+}]_i$ following Ca^{2+} release induced by ATP (100 μM). Adding back Ca^{2+} (1.2 mM) stimulated Ca^{2+} influx in the 401L cells. Arrows indicate the approximate time points of addition of the various agents.

Ca^{2+} release, suggesting that the two pools may completely overlap (Figure 4C), as was observed for the thapsigargin- and RyR-sensitive pools. Interestingly, the addition of Ca^{2+} (1.2 mM) after thapsigargin treatment induced Ca^{2+} influx in 401L cells also exposed to ATP (either before or after thapsigargin addition), suggesting that, as was observed for RyRs, intracellular Ca^{2+} channel/receptor activation is required and that mere store depletion (through thapsigargin-induced SERCA inhibition) is an insufficient stimulus to activate Ca^{2+} influx (Figures 4B and 4C; compare Figure 1A). Indeed, initial rates of Ca^{2+} influx were 0.56 ± 0.15 ($n = 4$) and 0.73 ± 0.11 ($n = 5$) fluorescence ratio units/min when cells were treated with ATP either after or before

treatment with thapsigargin respectively (Figures 4B and 4C). We observed a response similar to that of ATP when 401L cells were treated with the inflammatory mediator bradykinin (1 μM ; results not shown). Thus it appears that physiological stimuli of inflammatory responses can induce a stringently regulated Ca^{2+} influx pathway in the 401L cell line that requires activation of IP_3Rs and/or RyRs , while displaying a refractory sensitivity to general Ca^{2+} -store depletion.

DISCUSSION

Previous studies have identified the NG115-401L cell line as an important signalling cell line model for sensory neuron function [19,21–23]. As such, there is an interest in the characterization of the signalling pathways that mediate responses to key inflammatory regulators such as ATP and bradykinin. Further studies characterizing hormone-sensitive Ca^{2+} pathways in 401L cells have revealed an unusual phenotype with respect to intracellular Ca^{2+} -store regulation. A key observation in these studies is the failure of thapsigargin treatment to induce Ca^{2+} influx in 401L cells [5,6]. This result suggests that the 401L cell line lacks the machinery required to relay depleted Ca^{2+} stores to the activation of Ca^{2+} influx. Thus the 401L cells represents an important cell line for investigating the signalling mechanism that couples Ca^{2+} stores with Ca^{2+} entry through plasma-membrane-resident SOC channels, motivating the need for further investigation into which components of this signalling pathway are present. It may be possible, therefore, to identify putative Ca^{2+} influx pathway regulators by determining which components, when added exogenously or selectively expressed in the 401L cell, restore thapsigargin-induced Ca^{2+} influx.

The lack of a classical capacitative Ca^{2+} entry pathway in the 401L cell line prompted us to investigate whether non-voltage-gated Ca^{2+} influx responses could be induced at all in these cells. Specifically, we have tested the hypothesis that 401L cells possess RyR-activated pathways that couple with Ca^{2+} influx, a pathway that has been observed in sensory neurons and other excitable cell types [9,10,24]. We show in the present study that the atypical thapsigargin response in 401L cells is most probably not due to an absence of ion channel components, since we observed expression of mRNA for RyR1 and RyR2 channel isoforms as well as robust Ca^{2+} influx responses inducible by four structurally distinct RyR activators: ryanodine, PCB95, caffeine and CMC. Moreover, we observed that Ca^{2+} influx could be stimulated in 401L cells treated with agents that release Ca^{2+} through the activation of IP_3Rs as well. Thus 401L cells require the activation of intracellular Ca^{2+} release channels to couple effectively with Ca^{2+} influx pathways. Indeed, depletion of intracellular Ca^{2+} stores is not an efficient primary stimulus to trigger the opening of SOC channels in these cells. This mode of Ca^{2+} influx regulation in the 401L cell is consistent with the conformational coupling hypothesis whereby RyR or IP_3R activation produces conformational changes permitting either direct or indirect gating of Ca^{2+} permeability on the plasma-membrane SOC channels [1–4]. Since the proposal of the conformational coupling hypothesis as a mechanism explaining capacitative Ca^{2+} entry, several studies have reported evidence that both IP_3Rs and RyRs can interact with surface SOC channels, serving as the gating device for controlling the Ca^{2+} permeability of these channels [13,14,25–27].

The Ca^{2+} signalling features of the 401L cell suggest the use of particular Ca^{2+} influx pathways to suit the specialized needs of the cell. Therefore it may be that some cells, such as sensory neurons of the peripheral nervous system, require greater control over stimuli that trigger Ca^{2+} influx. To produce a Ca^{2+} influx response, Ca^{2+} release through activation of the intracellular Ca^{2+}

release channels must first occur, linking regulation of the RyR or IP₃R to Ca²⁺ entry. Linking the activation of Ca²⁺ influx to regulation of the RyR or IP₃R offers the cell more stringent control on influx responses than that attainable by a system activated more generally by depletion of Ca²⁺ stores. Indeed, ER Ca²⁺-store levels can be altered potentially by a variety of physiological processes, such as SERCA pump modulation (e.g. phospholamban) or expression of the apoptosis regulators of the bcl-2 family of proteins [28–30]. Perhaps, sensory neurons, and neurons in general, are less vulnerable to undergoing inappropriate apoptosis responses as a result of uncoupling Ca²⁺-store depletion from Ca²⁺ influx, given that these are post-mitotic cells that cannot be replaced once cell-death pathways are activated.

We thank Dr I. Pessah (Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, CA, U.S.A.) for the polychlorobiphenyl compounds and many helpful discussions. D. W. T. was supported by a New Investigator grant from the American Association of Colleges of Pharmacy.

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Received 27 May 2004/8 October 2004; accepted 14 October 2004

Published as BJ Immediate Publication 14 October 2004, DOI 10.1042/BJ20040900