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Inhibition by calyculin A and okadaic acid of the Ca^{2+} release-activated Ca^{2+} entry pathway in rat basophilic leukemia cells: Evidence for regulation by Type 1/2A serine/threonine phosphatase activity

Nicholas E. Evans [1], Mark K.L. Forth [1], Anna K. Simpson, Michael J. Mason *

Department of Physiology, University of Cambridge, Downing Street, Cambridge CB2 3EG, UK

Abstract

Using a combination of fluorescence measurements of intracellular Ca^{2+} ion concentration ($[Ca^{2+}]_i$) and membrane potential we have investigated the sensitivity to serine/threonine phosphatase inhibition of Ca^{2+} entry stimulated by activation of the Ca^{2+} release-activated Ca^{2+} (CRAC) entry pathway in rat basophilic leukemia cells. In both suspension and adherent cells, addition of the type1/2A phosphatase inhibitor calyculin A, during activation of CRAC uptake, resulted in a fall in $[Ca^{2+}]_i$ to near preactivation levels. Pre-treatment with calyculin A abolished the component of the Ca^{2+} rise associated with activation of CRAC uptake and inhibited Mn^{2+} entry, consistent with a requirement of phosphatase activity for activation of the pathway. Depletion of intracellular Ca^{2+} stores is accompanied by a large depolarisation which is absolutely dependent upon Ca^{2+} entry via the CRAC uptake pathway. Application of calyculin A or okadaic acid, a structurally unrelated phosphatase antagonist inhibits this depolarisation. Taken in concert, these data demonstrate a marked sensitivity of the CRAC entry pathway to inhibition by calyculin A and okadaic acid.

1. Introduction

Release of Ca^{2+} from intracellular storage compartments is a ubiquitous signal for the activation of a Ca^{2+} entry pathway in the plasma membrane of non-excitable cells, a process termed store-regulated Ca^{2+} uptake (SRCU) or capacitative Ca^{2+} entry [1]. To date, both the molecular origin of the entry pathway and the signal linking depleted stores to its activation remain controversial. Much effort has focused upon the role of the superfamily of transient receptor potential channels as the molecular correlate of the pathway while numerous mechanisms have been postulated to explain the signal linking store depletion to pathway activation. Mechanisms including generation of a diffusable message released during store depletion and configurational or secretion-like coupling, a process

whereby Ca^{2+} stores reversibly couple to the plasma membrane during store depletion have been proposed (reviewed in [2]).

In spite of the uncertainty surrounding the mechanism of communication between intracellular stores and the plasma membrane, a large body of evidence has accumulated implicating a serine/threonine phosphorylation event in the regulation of the pathway. Activation of protein kinase C (PKC) by phorbol ester has been shown to inhibit SRCU in a large number of cells including the T-cell line Jurkat [3], human neutrophils [4], peripheral T-lymphocytes [5], *Drosophila* photoreceptors [6], HL60 cells [7] and rat basophilic leukemia (RBL) cells [8], while modest activation of PKC has been reported to enhance SRCU in oocytes [9] and a pancreatic cell line [10]. In contrast, we have found no effect of PKC stimulation on SRCU in rat thymic lymphocytes [11], a finding supported by work in RBL cells [12].

Modulation of SRCU by serine/threonine phosphorylation is further supported by experimental evidence derived from inhibition of serine/threonine phosphatase activity. Inhibition of type 1/2A phosphatase activity by addition of okadaic acid

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or calyculin A has been reported to inhibit thapsigargin-mediated SRCU in HeLa cells [13], thyroid FRTL-5 cells [14], human neutrophils [4], salivary epithelial cells [15], rat thymic lymphocytes [11], DD$_1$T-2 transformed smooth muscle cells [16], A7r5 smooth muscle cells [16] and human platelets [17].

The CRAC entry pathway is the prototypical and best electrophysiologically characterised SRCU pathway present in the RBL and Jurkat cell lines [18,19]. Electrophysiological isolation of the current correlate of CRAC uptake has revealed a highly Ca^{2+} selective pathway with a well-defined permeability sequence, marked inward rectification and Ca^{2+}-dependent fast and slow inactivation to name but five hallmarks of this pathway (reviewed in [2]). Electrophysiological isolation of SRCU in other cell types invites immediate comparison with the CRAC uptake pathway's well-described electrophysiological finger print. Interestingly, while calyculin A-mediated inhibition of SRCU has been reported in numerous cell types, the CRAC pathway has been reported by Bakowski and collaborators to be insensitive to modulation by this reagent [20]. It would appear that the CRAC entry pathway in RBL cells is distinct from SRCU in other cell types in its apparent lack of sensitivity to phosphatase inhibition. Thus, modulation of phosphatase activity cannot be a ubiquitous signal underlying the activation and/or regulation of the SRCU family.

In an attempt to reconcile this apparent discrepancy, we have undertaken experiments to re-evaluate the role of calyculin A and okadaic acid, established inhibitors of type1/2A serine/threonine phosphatase activity [21] in the modulation of the CRAC entry pathway in intact RBL cells. In these experiments, we have monitored both changes in [Ca^{2+}]$_i$ and membrane potential. In contrast to previous findings [20], we report an absolute requirement for calyculin A- and okadaic acid-sensitive phosphatase activity for the activation and maintenance of Ca^{2+} entry via the CRAC uptake pathway in intact RBL cells. Based upon these data, the role of modulation of the CRAC pathway by phosphatase activity must be re-evaluated. Regulation of phosphatase activity may be a crucial signal controlling activation and/or modulation of the entire class of SRCU pathways including the CRAC pathway.

2. Materials and methods

2.1. Cell culture

RBL-1 cells were kindly provided by Dr. Steve Ikeda, (NIH, Bethesda MD., USA). RBL-1 cells were propagated in Minimum Essential Medium plus Earles salts (Gibco, Paisley, UK), supplemented with 1% non-essential amino acids (Sigma, Gillingham, UK or Gibco, Paisley, UK), 2 mM L-glutamine (Sigma, Gillingham, UK or Gibco, Paisley, UK), 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin (Sigma, Gillingham, Dorset, UK) and 10% fetal calf serum (FCS) (Gibco, Paisley, UK). In some experiments, amphotericin was omitted. The presence or absence of amphotericin had no detectable impact upon the outcome of the experiments. Cultures were incubated in a humidified 95/5% air/CO$_2$ atmosphere at 37 °C. Cultures were propagated by passaging nonadherent cells taken from dense cultures as previously reported [22,23] or by firmly tapping the flask on the side of the lab bench to release lightly adherent cells.

In some experiments, cells were allowed to adhere to sterile glass cover slips for 24 to 48 h prior to their use.

2.2. Reagents

The acetoxymethyl (AM) ester derivatives of fluo-3 and fura-2 were purchased from TeFlabs (Austin, TX, USA) and were made up as 1 mM stocks in dimethylsulfoxide (DMSO) containing 20% pluronic. bis-(1,3-diethylthio-barbituric acid) trimethine oxonol (bis-oxonol) was purchased from Molecular Probes (Leiden, ND) and was made up as a 50 μM stock in DMSO. HEPES, NaCl, KCl, HCl, NaOH, KOH, CaCl$_2$, MgCl$_2$, GdCl$_3$, NiCl$_2$, MnCl$_2$, D-glucose, N-methyl-D-glucamine (NMG), DMSO, 2-aminoethoxydiphenyl borate (2-APB) and EGTA were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). 2-APB was made up in DMSO as a 50 mM stock. Thapsigargin and ionomycin were purchased from Merck Biosciences (San Diego, CA, USA) and were made up as concentrated stocks in DMSO and ethyl alcohol respectively. Calyculin A and the Na$^+$ salt of okadaic acid were purchased from LC Laboratories (Woburn, MA, USA) and were made up as concentrated stocks in ethyl alcohol and DMSO respectively. Gramicidin-D was purchased from Sigma-Aldrich (Gillingham, Dorset, UK) and was made up as a concentrated stock in ethyl alcohol (Sigma-Aldrich, Gillingham, Dorset, UK). GdCl$_3$ and NiCl$_2$ were made up as concentrated stocks in distilled H$_2$O.

None of the reagents used adversely interacted with fluo-3 or bis-oxonol with the exception of GdCl$_3$, NiCl$_2$ and MnCl$_2$ which alter the fluorescence of fluo-3 and fura-2.

2.3. Solutions

Basic Na$^+$ containing solution had the following composition in mM; 145 NaCl, 5 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 Glucose, 10 HEPES, pH 7.4 with NaOH. When required, extra Ca^{2+} was added directly to this solution. Na$^+$-free solutions were made by equimolar replacement of Na$^+$ with NMG. Ca^{2+}-free solutions were made by omitting Ca^{2+} and in some cases adding EGTA at the indicated concentration. Solutions were stored long term at −20 °C and refrigerated short term.

2.4. Measurements of [Ca^{2+}]$_i$ in cell suspensions

[Ca^{2+}]$_i$ was measured fluorimetrically in cell suspensions using fluo-3. Culture flasks were gently tapped to release lightly adherent cells, the culture medium centrifuged and the cells resuspended in basic Na$^+$ solution. After determination of the cell density, cells were loaded with fluo-3 by incubation with the AM ester derivative of fluo-3. 25×10^6 cells/ml were loaded with 3 μM fluo-3-AM for 25 min at 37 °C. Cells were then centrifuged, resuspended in basic Na$^+$ solution and stored in the dark at room temperature until required. For intracellular Ca^{2+} measurements, cells were centrifuged and added directly to a cuvette to give a final concentration of between 1 and 2×10^6 cell/ml and fluo-3 fluorescence monitored under continual stirring in a cuvette-based fluorescence spectrophotometer (Cairn Research Ltd., Faversham, Kent, UK) (Ex 488 nm, 10 nm half bandwidth, Em 530 nm longpass, Comar Instruments, Cambridge, Cambridgeshire, UK). The photomultiplier tube output was externally filtered a 15 Hz using a lowpass Bessel filter (Frequency Devices Inc., Haverhill, MA, USA) and sampled at 30 Hz using a Digidata 1322A analogue to digital acquisition system (Axon Instruments, Union City, CA, USA) controlled by a Macintosh computer running Axograph Software (Axon Instruments, Union City, CA, USA). To calibrate the fluo-3 signal the maximum fluorescence in the presence of saturating Ca^{2+} (F_{max}) and the minimum fluorescence in the complete absence of Ca^{2+} (F_{min}) is required. At the end of each experiment maximum fluorescence was determined by the addition of ionomycin in the presence of 1 mM Ca^{2+}. This was followed by the addition of 3 to 5 mM Mn^{2+} and quenching of the fluo-3 signal (F_{Mn}). Experiments using free fluo-3 in solution revealed a consistent relationship between F_{max}, F_{min} and F_{Mn}. Fluorescence of fluo-3 in the presence of Mn^{2+} (F_{Mn}) was consistently greater than the fluorescence in the complete absence of Ca^{2+} (F_{min}). The decline in fluorescence from F_{max} observed by addition of Mn^{2+} is consistently 89% of the decline observed by complete removal of Ca^{2+} (F_{min}). This relationship was used to calculate the minimum fluorescence value

of fluo-3 for each experiment. $[Ca^{2+}]_i$ for a given fluorescence (F) was then calculated according to the following relationship:

$$[Ca^{2+}]_i = 390 \times (F - F_{min})/(F_{max} - F)$$

where F_{min} is the calculated minimum fluorescence in the absence of Ca^{2+}, F_{max} is the maximum fluorescence in the presence of ionomycin and extracellular Ca^{2+}, and 390 is the dissociation constant for fluo-3 in nM as taken from the Molecular Probes web page (http://probes.invitrogen.com/handbook/). Prior to applying the calibration, the data were corrected for fluo-3 leakage out of the cell. Leakage of fluo-3 out of the cell was evident as a slow linear increase in the fluorescence signal over the course of the experiment as fluo-3 leaked into the high Ca^{2+} environment of the external saline. The equation defining leakage was determined by fitting a linear regression to the initial baseline portion of the data. The regression was used to subtract the leakage component from each corresponding point in the data series.

All experiments were performed at room temperature.

2.5. Measurements of fluo-3 fluorescence in adherent cells

Fluo-3 fluorescence was recorded from adherent cells on a Zeiss Axiovert 100M inverted microscope equipped with a Zeiss LSM 510 confocal laser scanning module employing photo multiplier tubes for emission detection (Carl Zeiss Ltd., Welwyn Garden City, UK). Experiments were performed with the pinhole fully open thus ensuring the capture of fluorescence from the entire z plane of the cells. Small sections of coverslip were inserted into the experimental chamber and superfused with basic Na^+ containing solution to remove residual culture medium. For fluo-3 loading, half of the chamber volume was removed and replaced with an equivalent volume of solution containing fluo-3-AM (3 μM final loading concentration). Loading was confirmed after 10 to 15 min by capturing wide field images using the 488-nm excitation line of an argon ion laser and a long pass 505 emission filter. Images were captured at a resolution of 128×128 pixels using a Zeiss $40\times$ oil immersion objective (NA 1.3). Image fields contained from 6 to 35 adherent cells. Following confirmation of adequate fluo-3 loading the chamber was superfused with fluo-3-free basic Na^+ containing solution, image acquisition initiated and images captured every 3 s. Solution changes were made by gravity controlled superfusion with the exception of the application of calyculin A. For calyculin A application half the chamber volume was removed and mixed with calyculin A before careful reintroduction into the chamber (final concentration 200 nM).

Confocal control and image acquisition were achieved using proprietary software provided with the confocal module. Image fluorescence was analysed using LSM Toolbox (Dr. Christof Schwiening, Department of Physiology, University of Cambridge). Images were background subtracted and the average fluorescence from the entire field of view determined for each image in the experiment. Image fluorescence is expressed as f/f_o ratios to normalise the average image fluorescence (f) to the starting fluorescence immediately before addition of thapsigargin (f_o). This enables the comparison of image fields containing different cell numbers and thus average image fluorescence, and under conditions of different fluo-3 loading, laser intensity and photomultiplier tube sensitivity. We have chosen to use average fluorescence from entire image fields rather than regions of interest defining single cells as the calyculin A-dependent disruption of the cytoskeleton resulted in re-organisation of the cell morphology. This made the definition of individual cells in close proximity difficult. In control cells, where cytoskeletal disruption does not occur, a comparison of average image field versus average individual cell fluorescence yields identical dynamic changes in cell fluorescence thus validating our analysis.

2.6. Measurements of Mn^{2+} entry in cell suspensions

Mn^{2+} entry in cell suspensions was assessed using the quench of intracellular fura-2 at the Ca^{2+}-insensitive isosbestic wavelength [24]. Culture flasks were tapped to release lightly adherent cells, the culture medium centrifuged and the cells resuspended in basic Na^+ solution. After determination of the cell density, cells were loaded with fura-2 by incubation with the AM ester derivative of fura-2. 16 to 25×10^6 cells/ml were loaded with 5 μM fura-2-

AM for 25 min at 37 °C. Cells were then centrifuged, resuspended in basic Na^+ solution, aliquoted into individual microcentrifuge tubes and stored in the dark at room temperature until required. Cells were incubated with 200 nM calyculin A for 15 min at room temperature. Control cells were incubated with the appropriate ethanol concentration as a solvent control. After 15 min, the cells were centrifuged, added directly to a cuvette containing 200 nM calyculin A or appropriate ethanol volume as a solvent control and fura-2 fluorescence monitored under continual stirring at the isosbestic wavelength in the cuvette-based fluorescence spectrophotometer (Cairn Research Ltd., Faversham, Kent, UK). The isosbestic excitation wavelength was achieved using a Cairn Optoscan monochromator (Cairn Research Ltd., Faversham, Kent, UK). The accuracy of the isosbestic wavelength was assessed by monitoring the fluorescence change associated with application of high concentration of ionomycin to cells suspended in basic Na^+-containing solution containing 1 mM Ca^{2+} and 0.1 mM EGTA. Wavelengths between 360 and 363 nm (10 nm bandwidth) were used and emission was collected using a 494 nm longpass filter (Comar Instruments, Cambridge, Cambridgeshire, UK). The photomultiplier tube output was filtered and acquired as described for intracellular Ca^{2+} measurements employing fluo-3. For presentation, the data were further digitally filtered at 0.5 Hz using Axograph software. The control and calyculin A-treated traces were scaled to give equivalent starting fluorescence immediately before application of thapsigargin thus controlling for differences in fluorescence associated with differences in cell number in the cuvette. Both control and calycuin A-treated cells were taken from the same batch of fura-2-loaded cells.

All experiments were performed at room temperature.

2.7. Measurements of membrane potential in cell suspensions

Membrane potential was measured fluorimetrically in cell suspensions using bis-oxonol as previously reported by our laboratory [11,23,25,26]. Cells at a concentration of 1×10^6 cells/ml were equilibrated in a cuvette with 150 nM bis-oxonol and fluorescence monitored under continual stirring using the Cairn spectrophotometer (Ex 532 nm, 10 nm half bandwidth, Em 580 nm, 10 nm half bandwidth, Comar Instruments, Cambridge, Cambridgeshire, UK). The photomultiplier tube output was externally filtered at 15 Hz and sampled at 30 Hz as described for fluo-3 measurements. In some experiments an external calibration of the photomultiplier tube output was made by adding gramicidin to cells suspended in media containing varying ratios of Na^+ and NMG^+ and constructing a calibration curve as previously reported [11,23,25,26]. In the absence of a complete calibration, the 0 mV value was determined by adding gramicidin at the end of each experiment. Incorporation of a large monovalent cation conductance by the addition of gramicidin takes the membrane potential to approximately 0 mV when applied to cells suspended in basic Na^+ solution as previously reported [11,23,25,26].

All experiments were performed at room temperature.

3. Results

3.1. 2-APB sensitivity of the thapsigargin-mediated elevation in $[Ca^{2+}]_i$ in cell suspensions

Changes in $[Ca^{2+}]_i$ associated with thapsigargin-mediated activation of CRAC entry were monitored in cell suspensions of fluo-3-loaded RBL-1 cells. Fig. 1A shows a representative response to application of 100 nM thapsigargin recorded at room temperature in cell suspensions ($n = 12$). The rise in $[Ca^{2+}]_i$ induced by thapsigargin was biphasic, with a rapid rise in $[Ca^{2+}]_i$ followed by a secondary rise which occurred with a variable time constant. The secondary rise was followed by a decline in $[Ca^{2+}]_i$ to a plateau phase. Application of 2-APB, a potent inhibitor of I_{CRAC} in RBL cells ([20,27,28] and M.J. Mason unpublished observations), resulted in a marked decline in $[Ca^{2+}]_i$ to a value approaching pre-thapsigargin exposure.
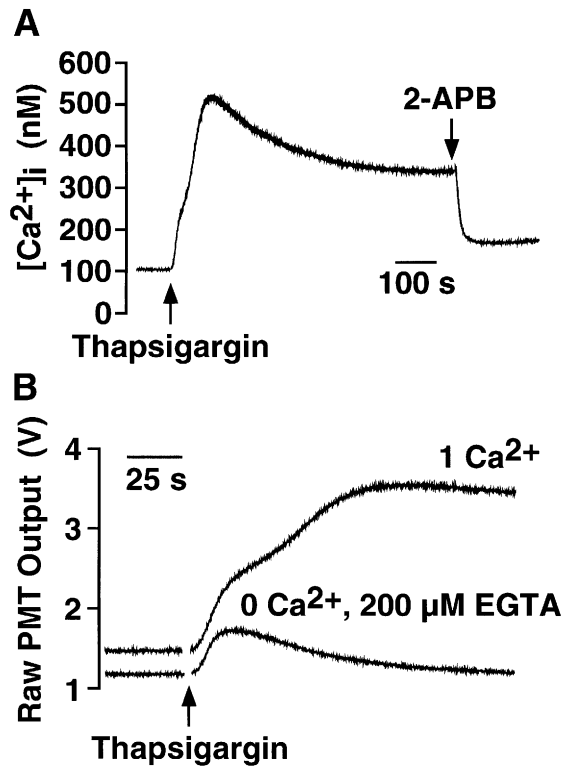
Fig. 1. Biphasic rise in $[Ca^{2+}]_i$ mediated by thapsigargin-induced activation of the CRAC entry pathway in RBL-1 cell suspensions. (A) Fluo-3-loaded cells were suspended in basic Na^+ solution containing 1 mM Ca^{2+}. Where indicated, 100 nM thapsigargin and 25 μM 2-APB were added. (B) Comparison of the $[Ca^{2+}]_i$ changes mediated by thapsigargin in the presence and absence of extracellular Ca^{2+}. Fluo-3-loaded cells were suspended in basic Na^+ solution in the presence or absence (200 μM EGTA added) of 1 mM extracellular Ca^{2+}. Where indicated, 100 nM thapsigargin was added directly to the cuvette.

These results are consistent with the sustained elevation in $[Ca^{2+}]_i$ being a result of CRAC uptake from the extracellular solution.

The initial biphasic elevation in $[Ca^{2+}]_i$ in response to thapsigargin most likely arises from release of Ca^{2+} from intracellular stores followed by influx of Ca^{2+} from the extracellular solution. This interpretation is supported by the observation that release of Ca^{2+} from intracellular stores in cells suspended in Ca^{2+} free solution shows a similar temporal rise in $[Ca^{2+}]_i$ devoid of the secondary Ca^{2+} rise seen in the presence of extracellular Ca^{2+} (Fig. 1B, $n=2$).

3.2. Calyculin A sensitivity of the CRAC uptake-mediated rise in $[Ca^{2+}]_i$

To investigate the role of type 1/2A phosphatase activity in the regulation of CRAC uptake, we have applied the phosphatase inhibitor calyculin A (200 nM) at the peak of the thapsigargin-mediated rise in $[Ca^{2+}]_i$. As shown in Fig. 2B ($n=7$), calyculin A application was accompanied, after a brief interval, by a dramatic fall in $[Ca^{2+}]_i$ to pre-thapsigargin levels and an abolition of the large 2-APB-sensitive component of the Ca^{2+} rise observed in control cells (Fig. 2A, $n=12$).

To address the sensitivity of the CRAC entry pathway activation process to phosphatase inhibition we have pre-

incubated cells at room temperature for 30 min with 200 nM calyculin A prior to application of thapsigargin. Application of thapsigargin under these conditions resulted in a single monophasic rise in $[Ca^{2+}]_i$ that was followed by a fall in Ca^{2+} to pre-thapsigargin levels (Fig. 3B, $n=3$). Application of 2-APB had no effect on $[Ca^{2+}]_i$ consistent with inhibition of the 2-APB-sensitive component of the Ca^{2+} rise (i.e., CRAC entry). Control experiments in which cells were treated with solvent under identical conditions showed marked activation of CRAC uptake as evidenced by the secondary rise in $[Ca^{2+}]_i$ in response to Ca^{2+} store depletion and a large 2-APB component of the sustained Ca^{2+} rise (Fig. 3A, $n=3$). Superimposing the experimental and control traces clearly shows that calyculin A pre-exposure inhibits the secondary rise in Ca^{2+} attributed to activation of the CRAC entry pathway while leaving the initial rise (attributable to Ca^{2+} release from intracellular stores) unaffected.

Experiments were undertaken to determine the influence of cell adherence on the calyculin A sensitivity of the rise in intracellular Ca^{2+} mediated via the CRAC pathway. Fig. 4A shows the average fluorescence of a field containing 35 adherent cells during application of 100 nM thapsigargin. The biphasic rise in fluo-3 fluorescence detected in adherent
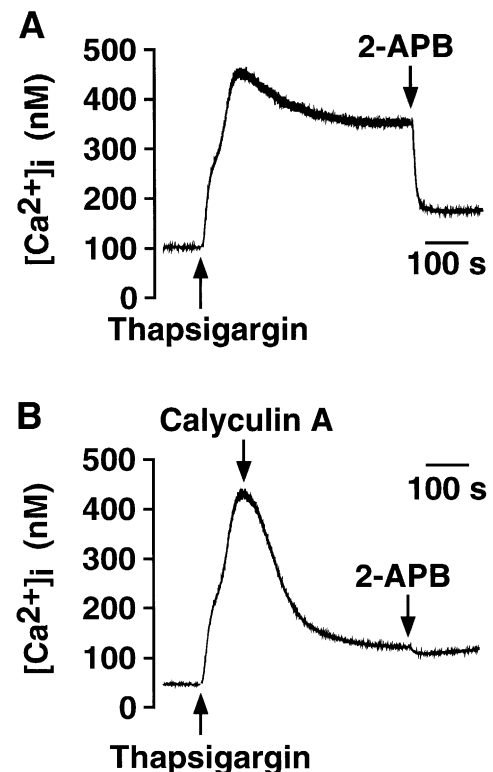

Fig. 2. Calyculin A sensitivity of the rise in $[Ca^{2+}]_i$ mediated by activation of the CRAC entry pathway in cell suspensions. (A) Control response to depletion of intracellular Ca^{2+} stores and the activation of the CRAC entry pathway. Fluo-3-loaded cells were suspended in basic Na^+ solution containing 1 mM Ca^{2+}. Where indicated, 100 nM thapsigargin and 25 μM 2-APB were added. (B) Effect of application of 200 nM calyculin A on the thapsigargin-mediated rise in $[Ca^{2+}]_i$. Fluo-3-loaded cells were suspended in basic Na^+ solution containing 1 mM Ca^{2+}. 100 nM thapsigargin, 200 nM calyculin A and 25 μM 2-APM were added to the cuvette where indicated.
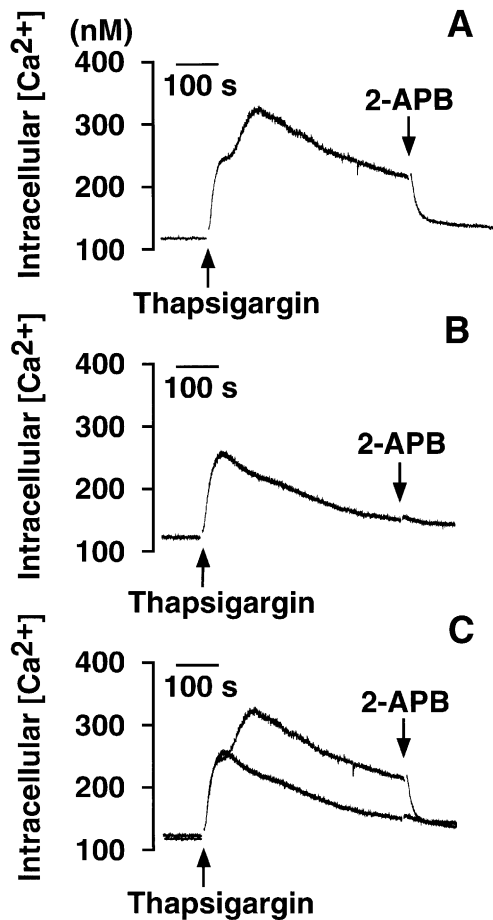
Fig. 3. Pre-incubation with calyculin A inhibits thapsigargin-mediated activation of the CRAC entry pathway in cell suspensions. (A) Fluo-3-loaded cells were incubated at room temperature for 30 min with solvent control (ethanol), spun and resuspended in the cuvette in basic Na^+ solution containing 1 mM Ca^{2+}. Where indicated, 100 nM thapsigargin and 25 μM 2-APB were added. (B) Fluo-3-loaded cells were incubated at room temperature for 30 min in the presence of 200 nM calyculin A, spun and resuspended in the cuvette containing basic Na^+ solution containing 1 mM Ca^{2+} and supplemented with 200 nM calyculin A. Where indicated 100 nM thapsigargin and 25 μM 2-APB were added to the cuvette. (C) The traces in panels A and B are superimposed for comparison of the $[Ca^{2+}]_i$ response to depletion of intracellular Ca^{2+} stores stimulated by addition of thapsigargin.

cells was indistinguishable from that observed in cell suspensions (Figs. 1 and 2) and was followed by a decline in cell fluorescence to a sustained plateau value. This sustained rise in fluo-3 fluorescence is dependent upon Ca^{2+} entry from the extracellular medium since removal of extracellular Ca^{2+} results in a marked decline in fluo-3 fluorescence. Re-addition of extracellular Ca^{2+} is accompanied by a rapid increase in fluorescence consistent with rapid Ca^{2+} entry. The thapsigargin-mediated rise in fluo-3 fluorescence presented in Fig. 4A is representative of the response observed in 7 of 7 control cover slips. The sensitivity of the thapsigargin-mediated rise in fluorescence to calyculin A in adherent cells was tested by applying the phosphatase inhibitor at the peak of the thapsigargin response. Fig. 4B shows the effect of addition of 200 nM calyculin A on the average fluorescence recorded from a field containing 14 cells. Addition of calyculin A is

accompanied by a marked decline in fluorescence with kinetics similar to those recorded in cell suspensions (Fig. 2B). In the presence of calyculin A, subsequent removal and reintroduction of extracellular Ca^{2+} resulted in only small changes in fluo-3 fluorescence consistent with potent inhibition of the CRAC entry pathway. The dramatic fall in fluo-3 fluorescence in the presence of calyculin A was observed in 3 of 3 coverslips tested.

On the basis of these data, we conclude that adherent cells respond to calyculin A in a manner indistinguishable from suspension cells.
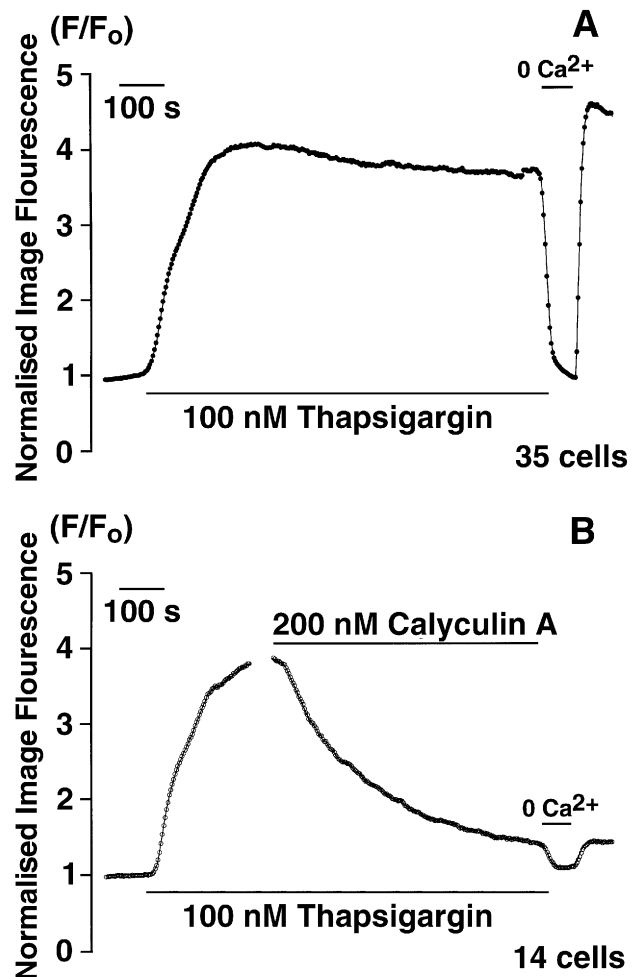

Fig. 4. Calyculin A sensitivity of the rise in fluo-3 fluorescence induced by activation of the CRAC entry pathway in adherent cells. Confocal measurement of fluo-3 fluorescence in adherent cells. (A) Cells were superfused with basic Na^+ solution containing 1 mM Ca^{2+} and wide field images from a field containing 35 adherent cells were collected every 3 s with the pin hole wide open to ensure capture of the fluorescence from the entire cell. Where indicated, 100 nM thapsigargin or 0 Ca^{2+} solution was superfused into the chamber. Average image fluorescence (*f*) was calculated for each image in the time series and normalised to the initial image fluorescence (f_o) immediately prior to thapsigargin application. (B) Cells were superfused with basic Na^+ solution containing 1 mM Ca^{2+} and wide field images from a field containing 14 adherent cells were collected every 3 s and analysed as in panel A. Where indicated 100 nM thapsigargin or 0 Ca^{2+} solution was superfused into the chamber. 200 nM calyculin A was added directly to the chamber where indicated as detailed in Materials and methods.
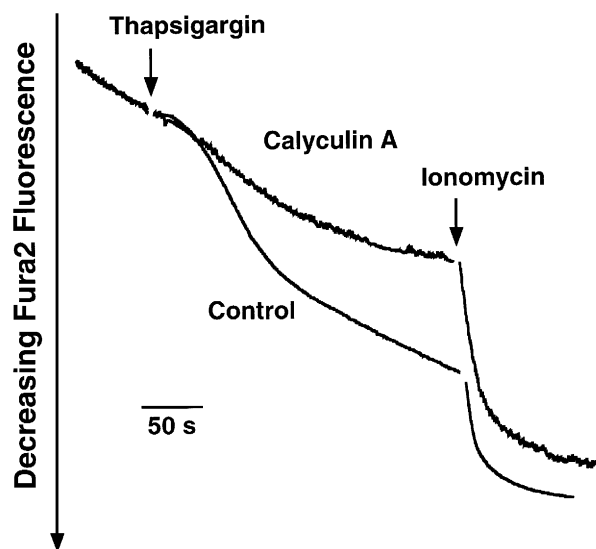
Fig. 5. Calyculin A sensitivity of thapsigargin-mediated Mn^{2+} entry in cell suspensions. Fura-2-loaded cells were incubated for 15 min in basic Na^+ solution augmented with 200 nM calyculin A or ethanol as a solvent control for calyculin A. The cells were spun down and added directly to the cuvette containing 200 nM calyculin A or appropriate ethanol concentrations as control and fura-2 fluorescence at the isobestic wavelength monitored. 25 μM $MnCl_2$ was added to the cuvette approximately 60 s prior to addition of 100 nM thapsigargin. 4 μM ionomycin was added to transport Mn^{2+} into the cell and fully quench fura-2 fluorescence.

3.3. Calyculin A inhibits thapsigargin-mediated Mn^{2+} entry in cell suspensions

The data above are consistent with calyculin A inhibition of the CRAC pathway or its activation process. However, these data may also be explained by upregulation of Ca^{2+} extrusion processes secondary to phosphatase inhibition. Mn^{2+} is poorly transported on Ca^{2+} extrusion pathways, therefore, Mn^{2+} entry is functionally unidirectional. We have previously reported that a small but measurable Mn^{2+} current is carried by the CRAC pathway in RBL-1 cells [22] while Mn^{2+} entry has been used to probe the CRAC entry pathway in RBL-2H3 cells [29]. Experiments were undertaken to define the influence of calyculin A on thapsigargin-mediated Mn^{2+} entry. A representative experiment is shown in Fig. 5. In control cells, addition of 100 nM thapsigargin results in pronounced augmentation of the basal Mn^{2+} entry rate consistent with activation of the CRAC entry pathway. Subsequent addition of 4 μM ionomycin results in ionophoretic Mn^{2+} transport into the cell and complete quench of fura-2 fluorescence. Pretreatment of cells with 200 nM calyculin A for 15 consistently resulted in a dramatic inhibition of the rate of thapsigargin-stimulated Mn^{2+} entry ($n=7$). Given the unidirectional nature of Mn^{2+} entry, these data are consistent with calyculin A inhibition of the CRAC entry pathway or its activation mechanism.

3.4. Depolarisation mediated by activation of the CRAC uptake pathway

Membrane depolarisation has a dramatic inhibitory influence on the CRAC entry pathway in RBL cells [23,30]. The influence of depolarisation arises both as a result of the loss of inwardly directed driving force and the inwardly rectifying nature of the current–voltage relationship for the CRAC entry pathway [18,23]. As a result, it can be proposed that the inhibitory influence of calyculin A arises not from modulation of CRAC uptake but rather from modulation of membrane potential resulting in depolarisation. It has been previously shown in RBL-2H3 cells that activation of the CRAC entry pathway by antigenic stimulation or inhibition of endosomal Ca^{2+}-ATPase activity with cyclopiazonic acid is accompanied by depolarisation [29,31,32]. We have previously reported in RBL-1 cells that thapsigargin-mediated activation of the CRAC entry pathway is accompanied by complex transitional changes in membrane potential between a hyperpolarised potential and a depolarised potential of approximately −15 mV when recorded at the single cell level under whole-cell current clamp [23]. When recorded in cell suspensions using *bis*-oxonol these membrane potential changes present as a marked sustained depolarisation, a finding attributed to the masking of the non-synchronised transitional changes in potential that occur at the single cell level [23]. A typical membrane potential response in RBL-1 cell suspensions in solution containing 5 mM extracellular Ca^{2+} is shown in Fig. 6A. Application of thapsigargin was
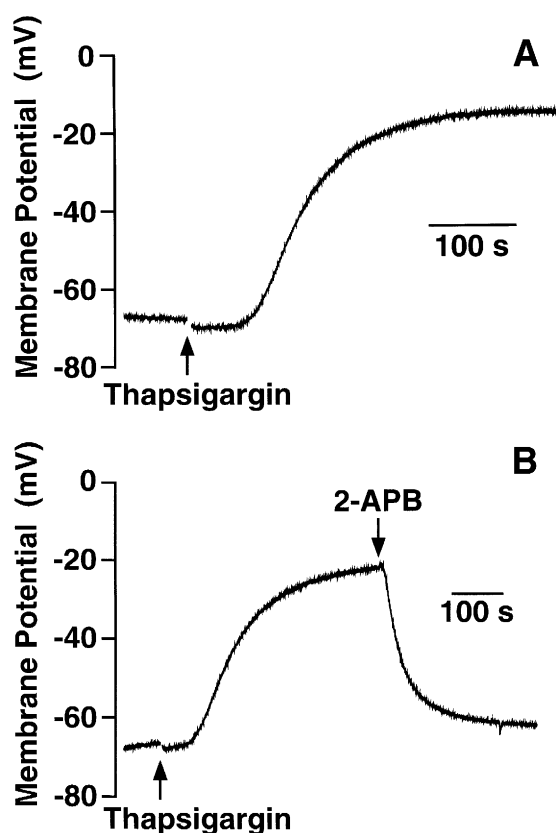

Fig. 6. Membrane depolarisation accompanying activation of the CRAC entry pathway is reversed by 2-APB addition. *Bis*-oxonol measurement of membrane potential in cells suspended in basic Na^+ solution containing 5 mM Ca^{2+}. (A) Following cell equilibration with *bis*-oxonol, 100 nM thapsigargin was added to the cuvette where indicated. (B) Following cell equilibration with *bis*-oxonol, 100 nM thapsigargin and 25 μM 2-APB were added to the cuvette where indicated.

accompanied by a small artifactual fall in *bis*-oxonol fluorescence attributable to solvent addition followed by a marked depolarisation to values between -20 mV and potentials exceeding 0 mV. The onset of the depolarisation displayed a variable time course with some cells showing a long latent period prior to depolarisation. Experiments were undertaken to define the role of activation of CRAC uptake in this marked depolarisation. Fig. 6B ($n=7$) shows that inhibition of CRAC entry by 2-APB addition reversed the depolarisation consistent with the ability of 2-APB to inhibit the rise in $[Ca^{2+}]_i$ mediated by thapsigargin addition (Fig. 1A).

The thapsigargin-induced depolarisation was also dependent upon extracellular Ca^{2+}. Thapsigargin addition to cells suspended in nominally Ca^{2+}-free solution was associated with only a marginal depolarisation that reversed in a time-dependent fashion (Fig. 7, $n=4$). However, addition of 5 mM Ca^{2+} to the cell suspension is associated with a large depolarisation sensitive to 2-APB application. Since this experiment was performed in basic Na^+ solution containing 5 mM K^+, addition of gramicidin, a monovalent cation channel forming antibiotic, results in the suspension depolarising to approximately 0 mV (see Materials and methods). Application of gramicidin in non-calibrated experiments was used to indicate the 0-mV value and the relative magnitude of the changes in potential observed.

The thapsigargin-induced depolarisation was also reversed by application of 1 μM Gd^{3+} (Fig. 8A, $n=5$) or 5 mM Ni^{2+} (Fig. 8B, $n=3$), both established inhibitors of CRAC entry in RBL cells ([22,33] and M.J. Mason unpublished observations).

Further support for the conclusion that the thapsigargin-mediated depolarisation is dependent upon activation of the CRAC entry pathway is derived from a comparison of the latency of the depolarisation following thapsigargin addition with the biphasic rise in $[Ca^{2+}]_i$ observed in fluo-3-loaded cells. Both the depolarisation latency and the time to the onset of the secondary rise in $[Ca^{2+}]_i$ showed a variability. However, when
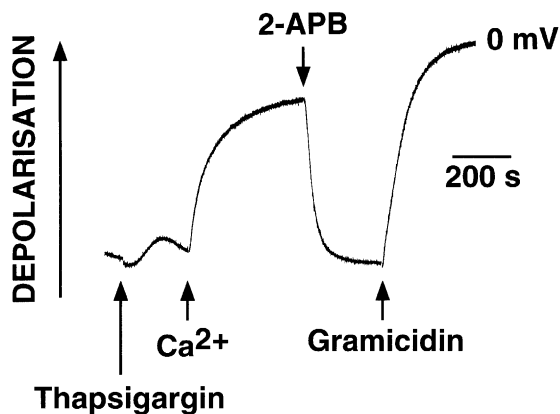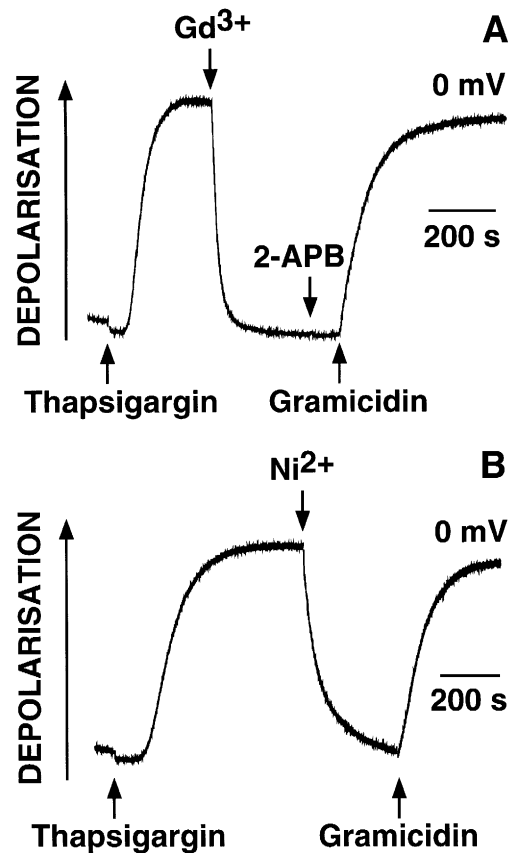
Fig. 8. Membrane depolarisation accompanying activation of the CRAC entry pathway is reversed by extracellular Gd^{3+} and Ni^{2+}. (A) Following cell equilibration with *bis*-oxonol in basic Na^+ solution containing 5 mM Ca^{2+}, 100 nM thapsigargin was added to the cuvette where indicated. At the peak of the depolarising response 1 μM GdCl$_3$ was added followed by 25 μM 2-APB. 200 nM gramicidin was added to depolarise the cell suspension to 0 mV. (B) Following cell equilibration with *bis*-oxonol in basic Na^+ solution containing 5 mM Ca^{2+}, 100 nM thapsigargin was added to the cuvette where indicated. At the peak of the depolarising response 5 mM NiCl$_2$ was added followed by 200 nM gramicidin.

compared on the same day both relationships showed a similar temporal relationship. Fig. 9 shows the temporal relationship between the changes in potential and the changes in $[Ca^{2+}]_i$ in

Fig. 7. Membrane depolarisation accompanying activation of the CRAC entry pathway is dependent upon extracellular Ca^{2+}. Following cell equilibration with *bis*-oxonol in nominally Ca^{2+}-free basic Na^+ solution, 100 nM thapsigargin was added where indicated. Following the small transient depolarisation, 5 mM Ca^{2+} was added directly to the cuvette, followed by addition of 25 μM 2-APB. 200 nM gramicidin was added to depolarise the cell suspension to 0 mV in accordance with the incorporation of a large monovalent cation conductance into the plasma membrane (see Materials and methods).
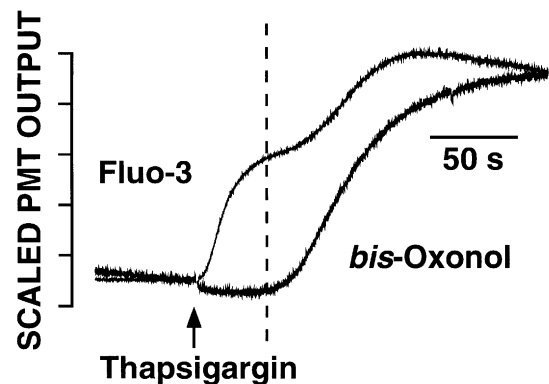
Fig. 9. A comparison of the temporal relationship between the $[Ca^{2+}]_i$ and the membrane potential responses to depletion of intracellular Ca^{2+} stores induced by thapsigargin. For comparison purposes, the photomultiplier tube output of the *bis*-oxonol record has been scaled. The dashed line indicates the point of best estimate of the first indication of the onset of depolarisation in the presence of 5 mM extracellular Ca^{2+}.

cells taken from the same cell culture. In the three experiments in which this comparison was performed, an identical relationship was observed, with the onset of the depolarisation correlating with the onset of the secondary increase in $[Ca^{2+}]_i$ attributed to activation of the CRAC entry pathway.

3.5. Sensitivity of CRAC uptake-mediated depolarisation to calyculin A and okadaic acid

The Ca^{2+} dependence of the depolarisation, its sensitivity to reversal by 2-APB, Gd^{3+} and Ni^{2+} and the close temporal relationship between the onset of the depolarisation and the secondary phase of the Ca^{2+} rise attributable to activation of CRAC entry all support the contention that activation of the CRAC entry pathway leading to Ca^{2+} influx is an absolute requirement for membrane depolarisation. On the basis of this conclusion, if calyculin A is an inhibitor of the CRAC entry pathway one would predict that its addition should reverse the depolarisation mediated by thapsigargin. Fig. 10A shows a

representative result of application of 200 nM calyculin A during the CRAC uptake pathway-dependent depolarisation. Addition of calyculin A brings about complete repolarisation of the cell suspension ($n = 12$). Clearly calyculin A is not altering the ability of *bis*-oxonol to report the membrane potential as addition of gramicidin results in a significant increase in fluorescence in accordance with depolarisation of the suspension to 0 mV. Pre-treatment of suspensions with 200 nM calyculin A also inhibits the thapsigargin-mediated depolarisation (Fig. 10B, $n = 3$) consistent with the effect of pre-treatment on the thapsigargin-mediated changes in $[Ca^{2+}]_i$ reported in Fig. 3.

It was necessary to ensure that the ability of calyculin A to inhibit the CRAC entry pathway arises from its reported inhibition of type 1/2A phosphatase activity and not as a result of a direct inhibitory influence at the level of the pathway. To investigate this, experiments were undertaken with okadaic acid, a type 1/2A phosphatase inhibitor structurally unrelated to calyculin A [21] but previously reported by our laboratory to inhibit store-regulated Ca^{2+} uptake in rat thymic lymphocytes [11]. Cell suspensions were incubated with 4 µM okadaic acid at 37 °C for 15 min prior to addition to the cuvette, equilibration with *bis*-oxonol and the application of thapsigargin. Addition of thapsigargin under these conditions resulted in a very small and much delayed, 2-APB-sensitive depolarisation (Fig. 11B, $n = 3$). In contrast, cells incubated with solvent as a control showed a robust depolarisation in response to thapsigargin (Fig. 11A, $n = 3$).

4. Discussion

4.1. Sensitivity of CRAC uptake in RBL cells to type 1/2A serine/threonine phosphatase inhibitors

The present results demonstrate the marked sensitivity of the CRAC entry pathway in intact RBL-1 cells to inhibition by type 1/2A phosphatase inhibitors. Calyculin A- and okadaic acid-sensitive phosphatase activity is an absolute requirement for the activation and sustained Ca^{2+} transport via the CRAC uptake pathway. This conclusion is not based solely upon measurements of $[Ca^{2+}]_i$ but also unidirectional measurements of Mn^{2+} entry. Furthermore, we have exploited the large CRAC entry-dependent membrane depolarisation as a bioassay for activation of the CRAC entry pathway. These membrane potential measurements have also ensured that depolarisation does not underlie the inhibition detected in the Ca^{2+} measurements. This is particularly important given the inward rectification of the CRAC current in RBL cells. Interestingly, application of calyculin A is accompanied by a dramatic repolarisation which would favour Ca^{2+} entry rather than stimulate the significant decline in $[Ca^{2+}]_i$ that is observed. In this regard, it is interesting that under control conditions the initial biphasic rise in $[Ca^{2+}]_i$ is followed by a decline in $[Ca^{2+}]_i$ to a sustained plateau value. This fall in $[Ca^{2+}]_i$ mirrors the continued depolarisation phase of the cell suspension evident in Fig. 9, consistent with this depolarisation underlying the fall in $[Ca^{2+}]_i$ in accordance with the influences of membrane potential on the CRAC entry pathway.
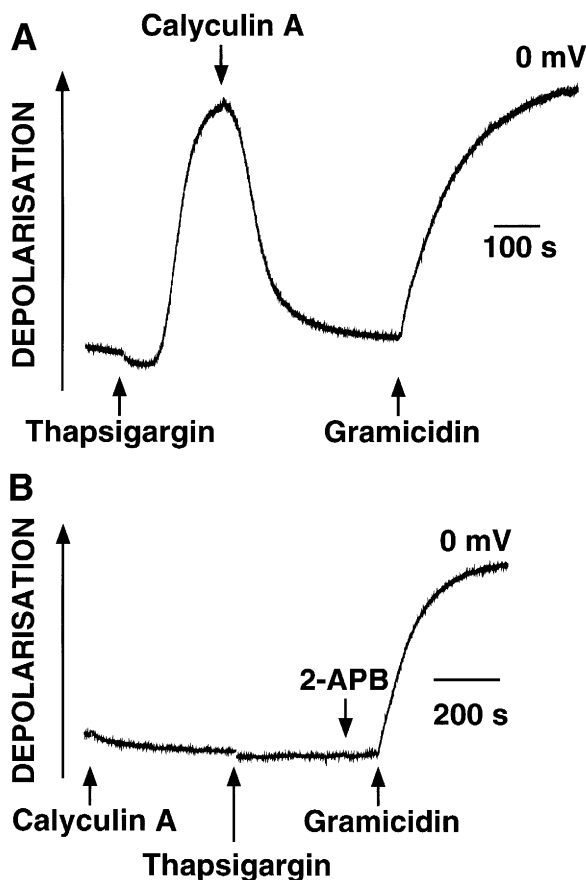

Fig. 10. Inhibition by calyculin A of the membrane potential depolarisation stimulated by activation of the CRAC entry pathway. (A) Following cell equilibration with *bis*-oxonol in basic Na⁺ solution containing 5 mM Ca²⁺, 100 nM thapsigargin was added to the cuvette where indicated. At the peak of the depolarising response, 200 nM calyculin A was added followed by 25 µM 2-APB. 200 nM gramicidin was added to depolarise the cell suspension to 0 mV. (B) Following cell equilibration with *bis*-oxonol in basic Na⁺ solution containing 5 mM Ca²⁺ calyculin A was added and the cells incubated for an additional 7 min prior to the addition of 100 nM thapsigargin. Where indicated, 25 µM 2-APB and 200 nM gramicidin were added.
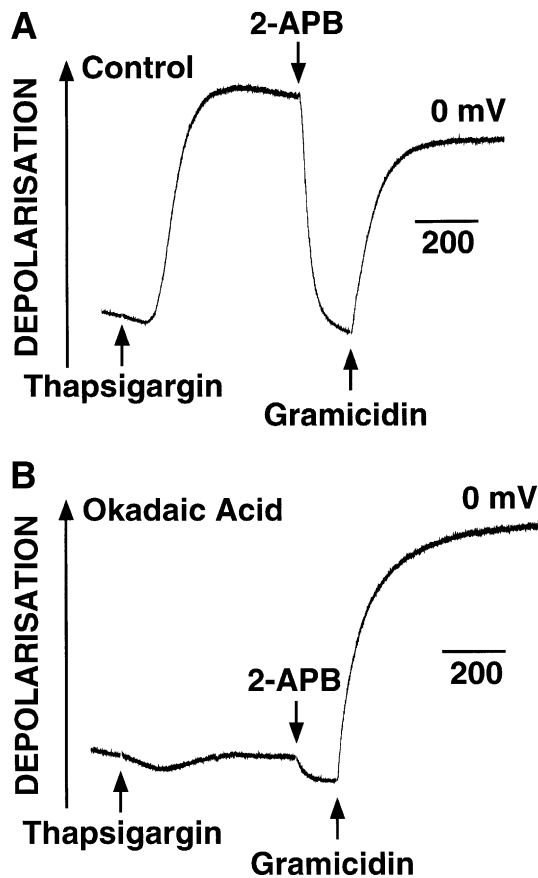
Fig. 11. Inhibition by okadaic acid of the membrane potential depolarisation stimulated by activation of the CRAC entry pathway. (A) Control membrane potential response accompanying activation of the CRAC entry pathway. Following cell equilibration with *bis*-oxonol in basic Na^+ solution containing 5 mM Ca^{2+}, 100 nM thapsigargin was added to the cuvette where indicated. At the peak of the depolarising response 25 μM 2-APB was added followed by 200 nM gramicidin to depolarise the suspension to 0 mV. (B) Cells were incubated for 15 min with 4 μM okadaic acid at 37 °C in basic Na^+ solution containing 1 mM Ca^{2+}. Cells were then centrifuged and resuspended in basic Na^+ solution containing 5 mM Ca^{2+} and supplemented with 4 μM okadaic acid. Following equilibration of the cells with *bis*-oxonol 100 nM thapsigargin, 25 μM 2-APB and 200 nM gramicidin were added where indicated.

The finding of an absolute requirement for phosphatase activity for the activation and maintenance of Ca^{2+} uptake via the CRAC entry pathway in RBL-1 cells is in agreement with the previous report of inhibition of the pathway by PKC stimulation [8]. However, further experiments performed in RBL-1 cells by members of the same research group revealed no sensitivity of the CRAC entry pathway to calyculin A [20], a result difficult to reconcile with the present results. On first look, it is tempting to ascribe this difference to the use by these investigators of the whole-cell patch clamp technique. Dialysis of the cytoplasm with the constituents of the patch pipette could result in removal of soluble phosphatases and/or their regulatory subunits or target proteins resulting in a loss of sensitivity of the signalling process to calyculin A. However, a similar lack of calyculin A-sensitivity was reported in a small sample of single-cell measurements of $[Ca^{2+}]_i$ in intact cells. Therefore, dialysis of key regulatory elements does not appear to account for the difference in

calyculin A sensitivity reported by Bakowski and collaborators [20]. An additional point worth considering is that the experiments of Bakowski et al. [20] and Parekh and Penner [8] were performed on cells adherent to glass coverslips. Adherence has been reported to modulate PKC activity [12] with adherent RBL-2H3 cells displaying constitutively active PKC activity while cells in suspension did not. Furthermore, RBL cells grown on coverslips show a very distinct morphology compared to those grown in suspension, with adherent cells displaying thin, tail-like projections on the surface of the coverslip [20] rather than the spherical shape of RBL cells in suspension. The cytoskeleton has been proposed to play a critical role in the activation/regulation of SRCU in the DD_1T-2 transformed smooth muscle cell line [16], the A7r5 smooth muscle cell line [16] and human platelets [17]. Cell adherence does not reconcile the current differences, however, since we have demonstrated that the modulation of the Ca^{2+} response by calyculin A following activation of the CRAC pathway is indistinguishable in adherent and suspension cells (Fig. 4).

4.2. Origin of the thapsigargin-induced depolarisation

The origin of the depolarisation observed in response to activation of the CRAC uptake pathway in RBL-1 cells is worthy of consideration. Elevations in $[Ca^{2+}]_i$ which accompany activation of the pathway may be responsible for modulation of endogenous channel conductances. Activation of a Ca^{2+}-activated Na^+ channel would be expected to result in depolarisation. However, such a channel cannot explain the present results given that an identical calyculin A-sensitive depolarisation is observed when extracellular Na^+ is replaced with the membrane impermeant monovalent cation NMG (M.J. Mason, unpublished observations). Activation of a Ca^{2+}-gated Cl^- channel could also account for the depolarisation. However, the existence of such a channel in RBL cells is unclear. Ca^{2+}-mediated inhibition of the inwardly rectifying K^+ channel has been reported [34,35]. The inward rectifier represents the dominant conductance setting membrane potential in RBL cells and inhibition of this channel would be expected to depolarise RBL cells. The sensitivity of this channel in RBL cells to inhibition by elevations in $[Ca^{2+}]_i$ is unclear with reports of both Ca^{2+}-dependent inhibition [36] and reports of no significant modulation by intracellular Ca^{2+} [37]. While a detailed investigation of the sensitivity of the inward rectifier has not been undertaken, our preliminary experiments suggest that Ca^{2+}-dependent inhibition of this channel does not underlie the depolarisation in the present experiments.

In the presence of extracellular divalents the CRAC entry pathway is a low conductance pathway [19] which is highly Ca^{2+} selective [33,38]. We have previously proposed that the depolarisation associated with activation of the CRAC uptake pathway in RBL-1 cells arises from the interaction between the very small inward CRAC current and the low membrane conductance region of the inwardly rectifying K^+ channel current–voltage relationship. We have previously shown that

generation of an inward current of less that 6 pA was sufficient to transitionally depolarise RBL cells under whole-cell current clamp from a potential of approximately −70 mV to values in excess of 0 mV [23]. While it is unclear what the magnitude of the CRAC current is under the present experimental conditions, a current of this magnitude is observed in RBL cells under whole-cell patch clamp conditions with ionic conditions designed to isolate Ca^{2+} currents [8,18,20,22,33,39]. To increase the magnitude of the inward CRAC current we have used 5 mM extracellular Ca^{2+} in the *bis*-oxonol experiments. While marked depolarisations can be observed with 1 mM extracellular Ca^{2+}, 5 mM Ca^{2+} resulted in consistently large depolarisations of the cell suspension mediated by the interaction between the CRAC current and the inwardly rectifying K^+ current.

Knowledge of the exact mechanism underlying the depolarisation is not central to the interpretation of the membrane potential results. The data presented clearly demonstrate that the depolarisation is dependent upon activation of the CRAC pathway and as such, membrane potential measurements provide a useful bioassay for studying activation and regulation of the CRAC entry pathway. This is important in that such measurements are not dependent upon invasive techniques such as whole-cell patch clamp or loading cells with fluorescent Ca^{2+} indicators which can significantly alter the true Ca^{2+} dynamics of the cell as a result of alterations in intracellular Ca^{2+} buffering.

4.3. Site of action of the phosphorylation/dephosphorylation cycle

While it is well established that serine/threonine phosphorylation/dephosphorylation reactions modulate SRCU, [3–10,13–16] the site or sites of phosphorylation are unclear. Given the reported inhibitory influence of protein kinase C activity on the CRAC uptake pathway [8] modulation of phosphatase activity becomes a critical regulatory process particularly during activation of the CRAC pathway mediated via physiological agonists linked to activation of phospholipase C. Under these conditions, the generation of inositol 1,4,5 trisphosphate is accompanied by the production of diacylglycerol and an increase in PKC activity. CRAC activation can only occur if phosphatase activity can effectively counter the PKC influences on the phosphorylation state of the target proteins, an observation previously appreciated in the light of the inhibitory influence of PKC stimulation [8].

The finding that phosphatase activity is absolutely required for CRAC entry in our hands is not confined to the RBL-1 clone. We have also found that the depolarisation induced by thapsigargin is inhibited by calyculin A and okadaic acid in RBL-2H3 cell suspensions (M.J. Mason unpublished observations). Additionally, incubation with nor-okadaone, a structurally related okadaic acid analogue lacking inhibitory phosphatase activity had no influence on the thapsigargin-mediated depolarisation, consistent with phosphatase inhibition underlying repolarisation of the cell suspension (M.J. Mason,

unpublished observations). Further experiments in RBL-2H3 cells have demonstrated that thapsigargin-induced Mn^{2+} entry measured as quench of intracellular indo-1 fluorescence at its isosbestic wavelength was also completely inhibited by calyculin A (M.J. Mason, unpublished observations). Taken in concert, these data indicate that the CRAC uptake pathway in RBL cells, independent of their clonal origin, displays a requirement for type 1/2A phosphatase activity, a finding consistent with previous observations of SRCU in a variety of cell types [4,11,13–17].

While the present experiments were undertaken at room temperature in an attempt to mimic the experimental conditions of Bakowski et al. [20], preliminary experiments were undertaken at 37 °C. We have observed identical calyculin A sensitivity of the membrane potential changes induced by thapsigargin at 37 °C. Additionally, thapsigargin-stimulated Mn^{2+} entry in RBL-2H3 cells is also inhibited at this temperature. These results are consistent with a requirement for phosphatase activity across a broad range of temperatures.

The present results demonstrate that inhibition of phosphatase activity effectively inhibits CRAC entry both when applied before depletion of Ca^{2+} stores or after full activation of the pathway, indicating that prior activation of the pathway is not required for the inhibitory influence of calyculin A to be manifested. Modulation of phosphatase and/or kinase activity by the Ca^{2+} status of the intracellular stores provides a mechanism that could explain the link between intracellular Ca^{2+} stores and the regulation of the CRAC uptake pathway. In this model net dephosphorylation of target protein(s) would lead to activation of the CRAC entry pathway. Net dephosphorylation could occur directly at the level of the CRAC entry pathway or more indirectly at the level of control of more complex signalling cascades proposed to underlie activation following Ca^{2+} store depletion such as diffusible messengers [40–45], conformational coupling [17,46] or secretion-like coupling [16,47,48]. Type 1/2A serine/threonine phosphatase activity is regulated by numerous regulatory subunits which interact with its catalytic subunit [49,50]. Phosphatase activity may be regulated by modulation of subunit availability controlled by the Ca^{2+} content of intracellular store. More work is required to understand the regulation of type1/2A phosphatase activity during Ca^{2+} signalling and to localise the site(s) at which phosphorylation regulates the CRAC entry pathway in RBL cells. However, it is clear from the present experiments that phosphatase activity plays a critical role in its activation and/ or regulation.

Acknowledgement

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