The lysosomal Ca^{2+} pool in MDCK cells can be released by Ins(1,4,5)P_3-dependent hormones or thapsigargin but does not activate store-operated Ca^{2+} entry

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In several cell types, Ca^{2+} release from intracellular Ca^{2+} stores by Ins(1,4,5)P_3 elicits Ca^{2+} influx from the extracellular space into the cytoplasm, termed store-operated Ca^{2+} entry (SOCE). In MDCK cells, the Ins(1,4,5)P_3-sensitive Ca^{2+} store giving rise to SOCE essentially overlaps with the thapsigargin (TG)-sensitive store. Recent evidence suggests that in MDCK cells lysosomes form a Ca^{2+} pool that is functionally coupled with the Ins(1,4,5)P_3-sensitive Ca^{2+} store: Ca^{2+} can be selectively released from lysosomes by glycyl-L-phenylalanine naphthylamide, an agent inducing lysosomal swelling with subsequent and reversible permeabilization of the vesicular membranes. This compartment is also depleted by Ins(1,4,5)P_3-dependent agonists or TG, indicating that it is part of a larger, Ins(1,4,5)P_3-sensitive Ca^{2+} pool. Here we show that whereas SOCE is triggered by Ca^{2+} release from the entire Ins(1,4,5)P_3-sensitive Ca^{2+} pool, selective Ca^{2+} release from lysosomes alone is unable to trigger SOCE. This finding is consistent with measurements of the store-operated cation current, a direct parameter for store-operated Ca^{2+} and Na^+ entry into MDCK cells. Hence it is proposed that the Ins(1,4,5)P_3-sensitive Ca^{2+} pool is composed of different intracellular compartments that do not uniformly stimulate Ca^{2+} entry into the cell.

INTRODUCTION

Capacitative Ca^{2+} entry or store-operated Ca^{2+} entry (SOCE) plays an important role to sustain Ca^{2+} signals evoked by various agonists [1–4]. In general the term is used to describe Ca^{2+} entry that follows the release of Ca^{2+} from the Ins(1,4,5)P_3-sensitive Ca^{2+} store. Beyond this functional definition, little is known about the organelles involved in SOCE and how the information for increased Ca^{2+} entry is conveyed from the stores to the plasma membrane. This lack of information is due to a considerable uncertainty about the nature and subcellular organization of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool itself. Although the endoplasmic reticulum (ER) is generally assumed to be the site of Ins(1,4,5)P_3-mediated Ca^{2+} release, final proof has never been given [5–7]. Rather, other specialized Ca^{2+} storage organelles independent of the ER have been proposed [8] as well as a 'functional coupling' between various Ins(1,4,5)P_3-insensitive and Ins(1,4,5)P_3-sensitive stores [9–11].

In this context we have recently described a lysosomal Ca^{2+} pool in MDCK cells that seems to be part of or functionally coupled with a larger, Ins(1,4,5)P_3-sensitive, Ca^{2+} pool, because Ca^{2+} is released out of the lysosomal compartment on treatment with ATP or the Ca^{2+}-ATPase blocker thapsigargin (TG) [12]. Furthermore, after Ca^{2+} depletion, a re-uptake of Ca^{2+} into lysosomes occurs, suggesting an involvement of this compartment in agonist-mediated Ca^{2+} signalling under physiological conditions. From these experiments we concluded that the Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} pool is compartmentalized and consists of at least two spatially different entities.

In this study we addressed the question of how these different pools are coupled with SOCE. We have recently characterized in MDCK cells an SOCE pathway that is highly Ca^{2+}-selective but permeable for both Ca^{2+} and Na^+ [13]. We therefore used two different approaches to investigate the above issue: (1) fluorimetric measurements of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) with fura 2, and (2) whole-cell patch-clamp measurements of the store-operated cation current (SOCC). We found that only the non-lysosomal, Ins(1,4,5)P_3- and TG-sensitive, Ca^{2+} pool is associated with SOCE, whereas the lysosomal Ca^{2+} pool is not. This is the first demonstration of two different intracellular Ca^{2+} pools, both of which are coupled with the action of Ins(1,4,5)P_3 or TG, but only one is able to stimulate SOCE.

EXPERIMENTAL

Whole-cell patch clamp

This was performed as described in detail previously [13]. In brief, MDCK cells [cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 26 mM $NaHCO_3$ at 37 °C in humidified air containing 5% CO_2] were seeded at low density on a plastic Petri dish and single cells were patched 1–3 days later. Measurements of the whole-cell currents (I) were performed under constant perfusion with a control bath solution containing (in mM): 129 NaCl, 11 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 Hepes and 10 glucose, pH 7.4 (adjusted with NaOH). In fluorimetric experiments, Ca^{2+}-free solutions included 1 mM EGTA. In whole-cell patch-clamp experiments, Ca^{2+}-free solutions were nominally Ca^{2+}-free (i.e. without added Ca^{2+}; $[Ca^{2+}]$ 3 μM), because this dramatically increased the stability of the seal. The control pipette solution contained (in mM): 137 potassium gluconate, 9 KCl, 1 $MgCl_2$, 0.1 EGTA and 10 Hepes, pH 7.4. Na^+ (7 mM) was added by titration with NaOH. During a whole-cell patch-clamp experiment, repetitive voltage ramps (20–60/min) were performed from a holding potential (V_H) of −65 to +65 mV, followed by voltage steps back to 0 and −65 mV, with a pCLAMP 5.5 routine. Under these conditions, as described in detail previously [13], inward currents (downward) at −65 mV represent SOCC, whereas outward currents (upward) at +65 mV represent Ca^{2+}-activated K^+ current [$I_{K(Ca)}$].

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; ER, endoplasmic reticulum; GPN, glycyl-L-phenylalanine naphthylamide; $I_{K(Ca)}$, Ca^{2+}-activated K^+ current; SOCE, store-operated Ca^{2+} entry; SOCC, store-operated cation current; TG, thapsigargin.

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Fluorescence measurements

$[Ca^{2+}]_i$ was determined as described in detail previously [12]. In brief, coverslips with subconfluent layers of MDCK cells were loaded with 1.2 μM fura 2 acetoxymethyl ester in Dulbecco's modified Eagle's medium for 15 min and then mounted in a perfusion chamber. The cells were exposed to control solution (see above) for 5 min before experimental solutions were applied. Measurements were made under an inverted microscope (Axiovert 35; Zeiss, Oberkochen, Germany) equipped for epifluorescence and photometry. The cells were exposed to consecutive light flashes of 20 ms at each wavelength (340, 360 and 380 nm) at intervals of 1 s. Excitation light was further attenuated by a grey filter of 3 % transmission before being deflected by a 420 nm dichroic mirror into the objective (Zeiss Fluar, $\times$40 oil). The emitted fluorescence was directed through a 420 nm cut-off filter to a photomultiplier tube (R928; Hamamatsu Herrsching, Germany). To collect fluorescence from a single cell, a pinhole was placed in the image plane of the phototube. Data acquisition was performed with Pulse software (Heka, Lambrecht, Germany), and data analysis with Datgraf software (Cyclobios, Innsbruck, Austria). $[Ca^{2+}]_i$ was calculated by the method of Grynkiewicz et al. [14]. For each cell, the minimal fluorescence ratio (R_{min}) was determined by the addition of ionomycin (20 μM) to a Ca^{2+}-free (5 mM EGTA) perfusate, and the maximal fluorescence ratio (R_{max}) by addition of ionomycin to the control solution containing 5 mM Ca^{2+}. Background fluorescence was subtracted by quenching fura 2 fluorescence with Mn^{2+} (5 mM) and ionomycin at the end of each experiment.

Materials

Fura 2 acetoxymethyl ester was purchased from Molecular Probes (Eugene, Oregon, U.S.A.). All other chemicals were obtained from Sigma (Munich, Germany).

Statistics

Each experiment was repeated at least five times; the figures show representative experiments. Results are reported as means $\pm$ S.E.M. Student's t test was used for statistical analysis. Differences between means were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Previous experiments in MDCK cells have shown that glycyl-L-phenylalanine naphthylamide (GPN) preferentially accumulates in acidic compartments and specifically and reversibly permeabilizes lysosomes to some extent, allowing low-molecular-mass compounds to pass though the vesicular membrane [12]. The underlying mechanism is the substrate-specific cleavage of GPN by the dipeptidyl aminopeptidase I, an exopeptidase located exclusively in lysosomes [15–18]. The amino acids liberated by GPN cleavage accumulate within the vesicles and lead to

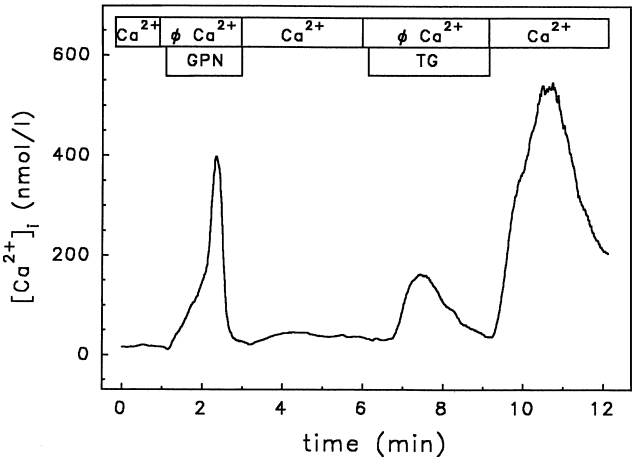

Figure 2 Effect of Ca^{2+} addition following intracellular Ca^{2+} release by GPN or TG

Ca^{2+} readdition to the bath after treatment with 200 μM GPN resulted in a small increase in $[Ca^{2+}]_i$. In contrast, prior treatment with 200 nM TG caused a marked increase in $[Ca^{2+}]_i$ on readdition of Ca^{2+}. ϕ Ca^{2+} = Ca^{2+}-free plus 1 mM EGTA.

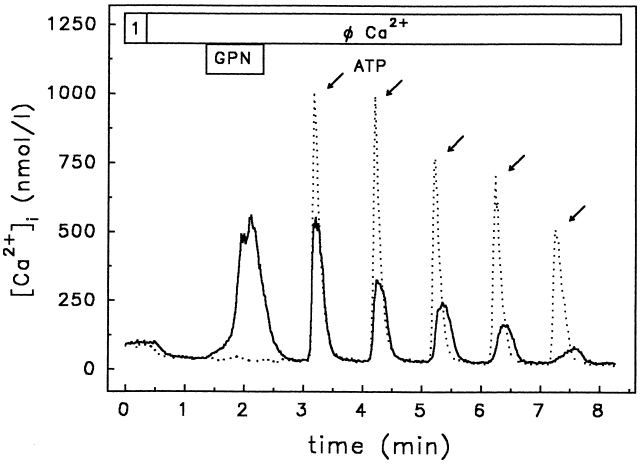

Figure 1 Decreased ATP-induced intracellular Ca^{2+} release following GPN treatment

Solid line, effect of 200 μM GPN and subsequent applications of ATP (10 μM, arrows) in solutions indicated above the tracing (1, $[Ca^{2+}]$ = 1 mM). Broken line, an identical experimental protocol, performed in the same cell after a 10 min reincubation in a Ca^{2+}-containing bath, but without GPN. ϕ Ca^{2+} = Ca^{2+}-free plus 1 mM EGTA.

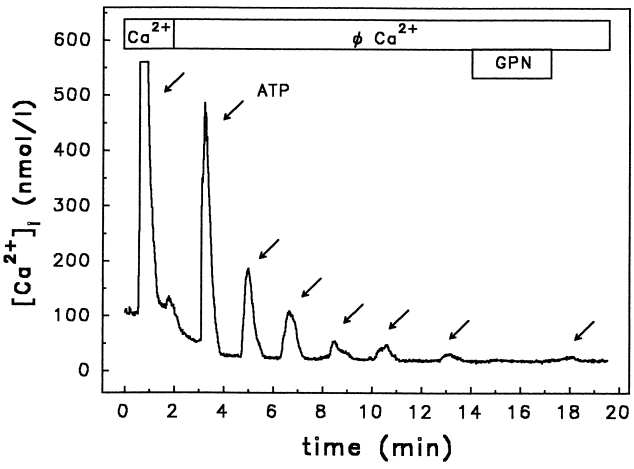

Figure 3 Inhibition of GPN (200 μM)-induced increase in $[Ca^{2+}]_i$ after repeated addition of ATP (10 μM, arrows) to Ca^{2+}-free perfusate in a single MDCK cell

ϕ Ca^{2+} = Ca^{2+}-free plus 1 mM EGTA.

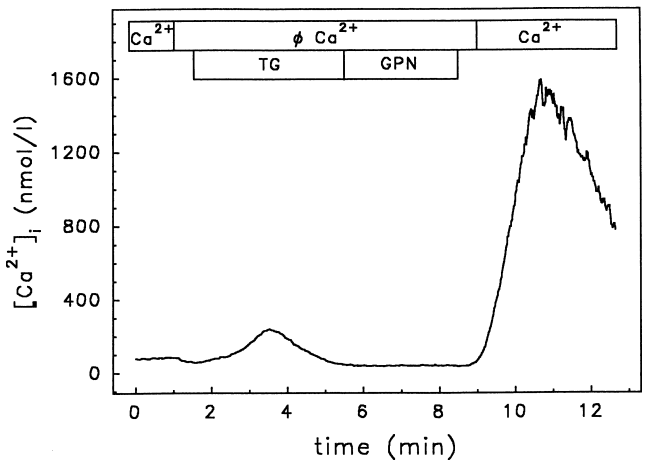

Figure 4 TG-induced increase in $[Ca^{2+}]_i$ on readdition of Ca^{2+} preceded by application of 200 μM GPN

osmotically induced permeabilization. During this process, considerable quantities of Ca^{2+} are released into the cytosol, as evidenced by a large, transient and non-recurrent rise of $[Ca^{2+}]_i$ in a Ca^{2+}-free solution (Figures 1 and 2). Thus GPN was used in our experiments as a tool to specifically discharge Ca^{2+} stored in lysosomes. Importantly, GPN-induced Ca^{2+} release clearly originated from a site distinct from, but functionally coupled with, the Ins(1,4,5)P_3- or TG-sensitive Ca^{2+} pool as shown previously [12] and summarized in Figures 1 and 3: Ca^{2+} release from the Ins(1,4,5)P_3- or TG- sensitive Ca^{2+} pool was largely unaffected following Ca^{2+} release from lysosomes (Figure 1). When the Ins(1,4,5)P_3- or TG-sensitive pool was depleted of Ca^{2+}, however, a further Ca^{2+} release from the lysosomal pool by GPN was no longer feasible (Figures 3 and 4), indicating that this pool had also lost its Ca^{2+}. We explained these findings by intracellular Ca^{2+} transfer from the lysosomal pool to the ER [the putative Ins(1,4,5)P_3-sensitive pool] or less probably by the endowment of lysosomes with membranes containing Ins(1,4,5)P_3 receptors and sarcoplasmic/endoplasmic-reticulum-type Ca^{2+} ATPase [12].

We tested whether, in addition to intracellular Ca^{2+} release, this pool might also be involved in SOCE. As shown in Figure 2, Ca^{2+} readdition to the bath after a 3 min application of 200 μM GPN (which is sufficient to deplete the lysosomal compartments of Ca^{2+} entirely) caused a small transient elevation of $[Ca^{2+}]_i$ from 99 ± 22 nM ($n = 8$) to 165 ± 40 nM ($n = 8$), which was the same when GPN was still present during Ca^{2+} readdition ($n = 7$). This increase, although significant, is not caused by GPN because incubation in a Ca^{2+}-free bath for 3 min had the same effect [13]. This is in good agreement with the findings of Jacob [19] that partial depletion of the internal pool results in partial activation of SOCE. However, GPN treatment clearly had no additional effect on this elevation of $[Ca^{2+}]_i$, excluding the possibility that depletion of the GPN-sensitive Ca^{2+} store stimulated Ca^{2+} entry. In contrast, a 3 min pretreatment with 200 nM TG caused a marked increase in $[Ca^{2+}]_i$ on addition of Ca^{2+}, up to 655 ± 72 nM ($n = 8$). Importantly, this TG-induced $[Ca^{2+}]_i$ peak was unaffected by a concomitant or prior application of GPN (Figure 4), excluding the possibility that GPN or one of its degradation products directly inhibited SOCE. We therefore conclude that Ca^{2+} release from the lysosomal pool alone is not sufficient to trigger SOCE, whereas a combined release from the non-lysosomal plus the lysosomal pool by TG is a strong stimulus for SOCE.

Whole-cell patch-clamp experiments were performed to relate

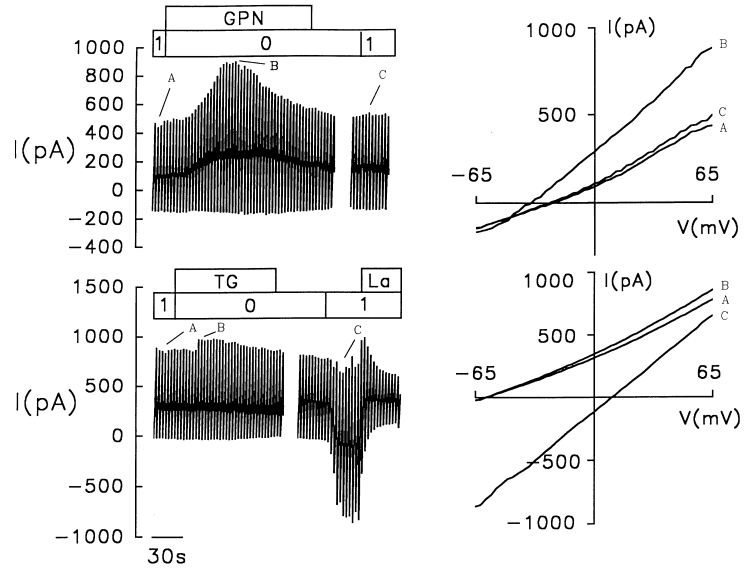

Figure 5 Original current (*I*) traces (left panels) with corresponding current–voltage relationships depicted at times A, B and C (right panels) of two whole-cell patch-clamp experiments (top and bottom) in single MDCK cells

Repetitive voltage ramps from −65 to +65 mV were performed, followed by a voltage step to 0 mV and to −65 mV. The thickening of the current trace at 0 mV arises from uncompensated capacitive current transients. Capacitive current transients at −65 mV were not recorded. As described in the Experimental section and in [13], SOCC is recorded as a stimulation of the inward current (i.e. downward deflection) at −65 mV and $I_{K(Ca)}$ is recorded as a stimulation of the outward current (i.e. upward deflection) at +65 mV. Addition of drugs is indicated above the tracings: [GPN] = 200 μM, [TG] = 200 nM, [La] = 10 μM. The bath Ca^{2+} concentration is indicated as 1 (1 mM) and 0 (Ca^{2+}-free) above the tracings.

these findings with SOCC, a Ca^{2+} and cation current recently described in MDCK cells [13]. As outlined in [13], SOCC is recorded as an inward current at -65 mV, whereas a stimulation of the outward current at $+65$ mV is predominantly $I_{K(Ca)}$, an indicator of near-membrane $[Ca^{2+}]_i$. As shown in Figure 5, GPN caused a slow transient activation of $I_{K(Ca)}$ in a Ca^{2+}-free solution ($n = 10$), consistent with intracellular Ca^{2+} release. Subsequent addition of Ca^{2+} to the bath (either with or without GPN) did not elicit SOCC, consistent with the lack of SOCE activation described above. When GPN was applied in the continuous presence of Ca^{2+} ($n = 6$), the same observation was made, i.e. a transient activation of $I_{K(Ca)}$ without activation of SOCC. GPN-induced $I_{K(Ca)}$ was confirmed by a complete inhibition with 5 mM Ba^{2+} ($n = 3$). This is in contrast with the significant activation of SOCC after treatment with TG (Figures 2 and 4) [13].

This is the first demonstration of an intracellular Ca^{2+} pool that is not coupled with SOCE. It is also an additional evidence that GPN, unlike ionomycin or other ionophores, selectively permeabilizes a Ca^{2+} store that is clearly distinct from, but functionally coupled with, the TG-sensitive Ca^{2+} store. Our findings, in line with previous observations [12], suggest that the Ins(1,4,5)P_3-sensitive pool might be a collection of independent compartments. We also conclude from our results that SOCE is not simply triggered by intracellular store depletion but activated after Ca^{2+} depletion of a particular subcompartment of the overall Ins(1,4,5)P_3-sensitive store.

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REFERENCES

1 Putney, Jr., J. W. and Bird, G.St.J. (1993) Cell **75**, 199—201
2 Penner, R., Fasolato, C. and Hoth, M. (1993) Curr. Opin. Neurobiol. **3**, 368–374
3 Fasolato, C., Innocenti, B. and Pozzan, T. (1994) Trends Pharmacol. Sci. **15**, 77-83
4 Berridge, M. J. (1995) Biochem. J. **312**, 1–11
5 Pozzan, T., Rizzuto, R., Volpe, P. and Meldolesi, J. (1994) Physiol. Rev. **74**, 595–636
6 Rossier, M. F. and Putney, Jr., J. W. (1991) Trends Neurosci. **14**, 310–314
7 Rossier, M. F., Bird, G.St.J. and Putney, Jr., J. W. (1991) Biochem. J. **274**, 643–650
8 Volpe, P., Krause, K. H., Hashimoto, S., Zorzato, F., Pozzan, T., Meldolesi, J. and Lew, D. P. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 1091–1095
9 Ghosh, T. K., Mullaney, J. M., Tarazi, F. I. and Gill, D. L. (1989) Nature (London) **340**, 236–239
10 Hajnóczky, G., Lin, C. and Thomas, A. P. (1994) J. Biol. Chem. **269**, 10280–10287
11 Hampe, W., Zimmermann, P. and Schulz, I. (1990) FEBS Lett. **271**, 62–66
12 Haller, T., Dietl, P., Deetjen, P. and Völkl, H. (1996) Cell Calcium **19**, 157–165
13 Delles, C., Haller, T. and Dietl, P. (1995) J. Physiol. (London) **486**, 557–569
14 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
15 Jadot, M., Colmant, C., Wattiaux-de Coninck, S. and Wattiaux, R. (1984) Biochem. J. **219**, 965–970
16 Jadot, M. and Wattiaux, R. (1985) Biochem. J. **225**, 645–648
17 Berg, T. O., Strømhaug, P. E., Løvdal, T., Seglen, P. O. and Berg, T. (1994) Biochem. J. **300**, 229–236
18 Berg, T. O., Strømhaug, P. E., Berg, T. and Seglen, P. O. (1994) Eur. J. Biochem. **221**, 595–602
19 Jacob, R. (1990) J. Physiol. (London) **421**, 55–77