Growth-dependent modulation of capacitative calcium entry in normal rat kidney fibroblasts

M.M. Dernison a, W.H.M.A. Almirza a, J.M.A.M. Kusters b, W.P.M. van Meerwijk a, C.C.A.M. Gielen b, E.J.J. van Zoelen a, A.P.R. Theuvenet a,⁎

a Department of Cell Biology, Radboud University Nijmegen, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands
b Department of Biophysics, Radboud University Nijmegen, Geert Grooteplein 21, 6525 EZ Nijmegen, The Netherlands

A B S T R A C T

Normal rat kidney (NRK) fibroblasts have electrophysiological properties and intracellular calcium dynamics that are dependent upon their growth stage. In the present study we show that this differential behavior coincides with a differential calcium entry that can be either capacitative or non-capacitative. Confluent cells made quiescent by serum deprivation, which have a stable membrane potential near −70 mV and do not show spontaneous intracellular calcium oscillations, primarily exhibit the capacitative mechanism for calcium entry, also called store-operated calcium entry (SOCE). When the quiescent cells are grown to density-arrest in the presence of EGF as the sole polypeptide growth factor, these cells characteristically fire spontaneously repetitive calcium action potentials, which propagate throughout the whole monolayer and are accompanied by intracellular calcium transients. These density-arrested cells appear to exhibit in addition to SOCE also receptor-operated calcium entry (ROCE) as a mechanism for calcium entry. Furthermore we show that, in contrast to earlier studies, the employed SOCs and ROCs are permeable for both calcium and strontium ions. We examined the expression of the canonical transient receptor potential channels (Trpcs) that may be involved in SOCE and ROCE. We show that NRK fibroblasts express the genes encoding Trpc1, Trpc3 and Trpc6, and that the levels of their expression are dependent upon the growth stage of the cells. In addition we examined the growth stage dependent expression of the genes encoding Orai1 and Stim1, two proteins that have recently been shown to be involved in SOCE. Our results suggest that the differential expression of Trpc5, Trpc6, Orai1 and Stim1 in quiescent and density-arrested NRK fibroblasts is responsible for the difference in regulation of calcium entry between these cells. Finally, we show that inhibition or potentiation of SOCE and ROCE by pharmacological agents has profound effects on calcium dynamics in NRK fibroblasts.

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1. Introduction

There is currently a great interest in the cellular and molecular mechanisms that underlie store-operated calcium entry (SOCE). Upon release of intracellular calcium ions from stores in the endoplasmic reticulum (ER), calcium channels in the plasma membrane are opened, which results in an influx of calcium ions and a refilling of the intracellular stores. A breakthrough in the understanding of this process has come from the identification of the proteins Stim1 and Stim2 as sensors of Ca2+ within the ER. Stim proteins sense the depletion of Ca2+ from the ER, oligomerize, translocate to junctions adjacent to the plasma membrane, organize plasma membrane calcium channels into clusters and open these channels to bring about SOCE [1,2]. Recent studies have identified particular members of the Orai and Trpc family as the plasma membrane calcium channels that are activated by this calcium-store depletion mechanism [3]. Although the components that play a role in store-operated calcium entry may have been identified, relatively few studies have functionally characterized this process under physiological conditions. Calcium entry into cells can take place through voltage-dependent calcium channels, including L-type and N-type channels, or through voltage-independent calcium channels. The latter group can be subdivided into store-operated calcium channels (SOCs) and receptor-operated calcium channels (ROCs). SOCs are activated by depletion of calcium stores after calcium release, whereas ROCs are activated through PLC-coupled receptors involving second messengers such as dicylglycerol (DAG), inositol 1,4,5-triphosphate (IP3) and arachidonic acid (AA). Orai channels work according to a SOC mechanism, whereas certain members of the Trpc channel family operate as SOC, and others as ROC.

Abbreviations: SOCE, store-operated calcium entry; ROCE, receptor-operated calcium entry; NRK, normal rat kidney; PGF2α, prostaglandin E2; OAG, 1-Oleoyl-2-acetyl-sn-glycerol; BHQ, 2,5-Di-t-butyl-1,4-benzohydroquinone; ER, endoplasmic reticulum; 2-APB, 2-aminoethoxydiphenyl borate; IP3, inositol 1,4,5-trisphosphate; Q-cells, quiescent NRK cells; DA-cells, density-arrested NRK cells.

⁎ Corresponding author. Tel.: +31 24 3652013; fax: +31 24 3652999.
E-mail address: a.theuvenet@science.ru.nl (A.P.R. Theuvenet).

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In a number of studies we have made a detailed characterization of the calcium homeostasis in normal rat kidney (NRK) fibroblasts as a function of their growth status [4,5]. When cultured at high density in serum-free medium these cells become quiescent, which is characterized by a stable membrane potential near −70 mV. Addition of prostaglandin F2α (PGF2α) to such quiescent cells, which activates the C-protein-coupled PGF2α-receptor (Ptgfr), results in degradation of inositol lipids and the production of IP3, which releases calcium ions from the ER by activation of the IP3-receptor. This process results in calcium oscillations, which are uncorrelated between different cells, and is accompanied by depolarization of the cells to a stable membrane potential of −20 mV. Upon addition of epidermal growth factor (EGF) and insulin to quiescent NRK cells, they can undergo one additional round of duplication, after which they stop proliferating as a result of density-dependent growth arrest. These density-arrested cells maintain a membrane potential near −70 mV, but show in addition periodically propagating calcium action potentials during which cells temporarily depolarize to positive values as a result of the opening of L-type calcium channels.

In an integrated model of calcium fluxes in NRK cells, we have previously shown that constitutive activation of plasma membrane calcium channels is essential for long-term calcium oscillations in PGF2α-treated quiescent cells, as well as for periodic calcium action potentials in density-arrested cells [6]. In the present study we have characterized experimentally the contribution of store-operated and receptor-operated calcium channels in the calcium homeostasis of quiescent and density-arrested NRK cells. Our results show that changes in calcium dynamics upon growing quiescent NRK cells to density-arrest coincide particularly with regulation of expression and changes in calcium dynamics upon growing quiescent NRK cells to density-arrest.[7] We have performed as described elsewhere[7].

2. Materials and methods

2.1. Cell culturing

Normal rat kidney fibroblasts (NRK clone 49F) were seeded at a density of 1.25 × 10^4 cells/cm^2 in bicarbonate-buffered Dulbecco’s modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% newborn calf serum (HyClone Laboratories, Logan, UT). Confluency was reached after four days. Cells were then incubated for three days in serum-free DF medium (1:1 mixture of DMEM and Ham's F-12 medium (Invitrogen)) supplemented with 30 mM Na_2SO_4 and 10 µg/ml human transferrin, to obtain quiescent cells. Density-arrested monolayers were obtained by incubation of quiescent cells for 48 h with 5 ng/ml EGF (Collaborative Research Incorporated, Bedford, MA) in combination with 5 µg/ml insulin (Sigma-Aldrich, St. Louis, MO). For calcium imaging experiments 1.2 × 10^5 NRK cells were seeded on 0.1% gelatin-coated glass coverslips with a diameter of 25 mm in 9.6 cm^2 wells.

2.2. Intracellular calcium measurements

Glass coverslips grown with quiescent monolayers of NRK fibroblasts were placed in a cell chamber and loaded for 30 min with 4 µM Fura-2/AM (Molecular Probes, Eugene, OR) in serum-free DF medium at room temperature. Medium was replaced by Ca^2+ -free HEPES-buffered saline (Ca^2+ -free HBS, containing 143 mM NaCl, 5 mM KCl, 1 mM MgCl_2, 10 mM glucose, 10 mM HEPES-KOH, and pH 7.4). Ca^{2+} or Sr^{2+}-containing HBS (128 mM NaCl, 10 mM CaCl_2 or SrCl_2, 5 mM KCl, 1 mM MgCl_2, 10 mM glucose, 10 mM HEPES-KOH, and pH 7.4) was mixed with an equal amount of Ca^{2+} -free medium to obtain a 5 mM Ca^{2+} or Sr^{2+}-containing medium in the chamber. Dynamic calcium video imaging was performed as described elsewhere [7]. Excitation wavelengths of 340 nm and 380 nm (bandwidth 8–15 nm) were provided by a 150 W Xenon lamp (Ushio UXL S150 M0, Ushio, Tokio, Japan), while fluorescence emission was monitored above 440 nm, using a 440 nm DCLP dichroic mirror and a 510 nm emission filter (40 nm bandwidth) in front of the camera. Image acquisition, using a camera pixel binning of 4 and computation of ratio images (F340/F380), was every 4 sec and operated through Metavue v.6.2 (Universal Imaging Corporation, Downingtown, PA). Camera acquisition time was 100 ms per excitation wavelength. The agents U73122, SKF96365, G06976 and OAG were purchased from Sigma-Aldrich (St. Louis, MO), BAPK was purchased from Calbiochem (Darmstadt, Germany) and 2-APB from Tocris (Avonmouth, UK).

2.3. Data analysis

At each measurement variations in intracellular calcium concentration as a function of time were measured simultaneously in 50 to 70 cells. The Mann–Whitney U ranking test was applied for comparing the frequency of calcium oscillations/transients in different cell groups. The increase of the F340/F380 ratio due to calcium influx through membrane channels or by calcium release from intracellular stores was determined by subtracting the mean ratio before (basal level) and after (peak) the calcium influx or release. Numerical data are represented as mean ± S.E.M throughout this article, with n representing the number of replicates in each experiment. Significance levels (denoted p) have been determined by double-sided student's T-test unless otherwise stated.

2.4. PCR primers and total RNA isolation

PCR primers for rat Trpc1-7, Orai1 and Stim1 were designed based on published sequences in GenBank (see Supplementary Table S1) using Oligo Perfect designed tool (Invitrogen). Total RNA was isolated from NRK cells using Trizol (Invitrogen) according to manufacturer's protocol.

2.5. RT-PCR

First strand cDNA was prepared from 1 µg of total RNA using SuperScript™ II RNase H− reverse transcriptase (Invitrogen) and 0.25 µg of hexamer primer. Thereto RNA samples were denaturated at 65 °C for 5 min and reverse transcription was performed for 50 min at 42 °C and stopped by heating the samples for 15 min at 70 °C. The cDNA was amplified by PCR using the specific primers for individual Trpc genes (see list in Supplementary Table S1) and Taq polymerase™ (Invitrogen). PCR amplification was performed using a PERKIN ELMER Gene Amp PCR System 2400 (Norwalk, CT) using 1 µl of first stranded cDNA reaction, 150 pmol of each degenerate primer, 50 µM of dNTPs, 2 units of Taq polymerase and 2.5 mM MgCl_2 in total volume of 50 µl. PCR conditions were as follows: 3 min at 94 °C, 40 cycles consisting of 30 s at 94 °C followed by 1 min at 72 °C. After completion of the 30 cycles, samples were incubated at for 10 min 72 °C. The PCR products were visualized on an ethidium bromide-stained agarose gel.

2.6. Quantitative real-time RT-PCR

The mRNA levels for genes of interest were analyzed by using quantitative RT-PCR (Detection System 5700 ABI Prism, Applied Biosystems, Foster City, CA). A total of 1 µg of cDNA, synthesized as described above using the primers shown in supplementary Table S2, was amplified using SYBR Green PCR Mastermix (Applied Biosystems) under the following conditions: initial denaturation for 10 min at 95 °C, followed by 40 cycles consisting of 15 s at 94 °C and 1 min at 60 °C. Expression values were calculated from threshold cycles at which an increase in reporter fluorescence above baseline signal could first be detected.
3. Results

3.1. Characterization of store-operated calcium entry in quiescent and density-arrested NRK fibroblasts

Store-operated calcium entry (SOCE) was studied in quiescent (Q) and density-arrested (DA) NRK fibroblasts by measuring calcium influx after release of calcium from intracellular stores. Emptying of these stores was induced by placing the cells in a nominal calcium-free medium in the additional presence of the sarco-endoplasmatic reticulum Ca²⁺-ATPase (SERCA) inhibitor BHQ. SOCE was subsequently measured by increasing the extracellular calcium concentration to 5 mM.

Fig. 1 shows on the basis of the F340/F380 ratio of Fura-2 fluorescence that the release of calcium from the stores results in an increase in cytoplasmic calcium ions, which are rapidly pumped out of the cells by the plasma membrane Ca²⁺-ATPase. Subsequent addition of calcium to the extracellular medium results in a strong, transient increase in cytoplasmic calcium concentration due to the activity of store-operated calcium channels. Fig. 1A shows that the release of calcium from intracellular stores in Q-cells resulted in an increase in F340/F380 ratio of 0.080±0.008 (mean±SEM, n=19), as measured from the basal level to the peak value after the addition of BHQ. Addition of extracellular calcium ions resulted in a rise of the F340/F380 ratio of 0.14±0.01 (mean±SEM, n=19), as measured from the basal level to the peak value after addition of Ca²⁺. Comparable experiments for DA-cells (see Fig. 1B) resulted in an increase in F340/F380 ratio of 0.10±0.02 (mean±SEM, n=6) for BHQ treatment and of 0.15±0.02 (mean±SEM, n=6) upon subsequent calcium addition. Fig. 1C shows that addition of extracellular calcium to Q-cells without prior depletion of calcium stores by BHQ treatment resulted in a small increase in the F340/F380 ratio of only 0.030±0.021 (mean±SEM, n=4). These data show that SOCE in density-arrested NRK cells is higher than in quiescent cells, although the difference was below the 95% confidence interval for statistical significance (p=0.08).

Since DA-cells can undergo spontaneous calcium action potentials, accompanied by calcium influx due to transient opening of the L-type calcium channel, the above experiments were carried out in the presence of the L-type channel inhibitor nifedipine. Control experiments showed that nifedipine had no effect on the intracellular calcium level of both Q- and DA-cells (data not shown).

3.2. Characterization of receptor-operated calcium entry in quiescent and density-arrested NRK fibroblasts

Receptor-operated calcium entry (ROCE) was studied in quiescent (Q) and density-arrested (DA) NRK fibroblasts by pre-incubating the cells in nominal calcium-free medium with the DAG-analogue OAG and measuring the increase in intracellular calcium concentration upon addition of 5 mM extracellular Ca²⁺. Nifedipine was added in the experiments to prevent calcium influx through voltage-dependent L-type calcium channels.

Fig. 2A shows that addition of extracellular calcium to Q-cells in the presence of OAG resulted in a F340/F380 increase of 0.045±0.013 (mean±SEM, n=10) above the basal fluorescence level. Fig. 2B shows that the addition of calcium ions to OAG-treated DA-cells resulted in an increase in F340/F380 of 0.11±0.01 (mean±SEM, n=10), which is 2.4 times higher (p<0.01) than the value observed in Q-cells. When these experiments were carried out in DA-cells without prior incubation with OAG (Fig. 2C), a value of 0.11±0.01 (mean±SEM, n=10) was found, which is not significantly different from the increase found in the presence of OAG. This suggests that density-arrested NRK cells may already contain sufficient DAG to activate receptor-operated calcium channels. Fig. 2D shows that pretreatment of DA-cells with PLC-inhibitor U73122 in order to prevent PIP₂ degradation and concomitant DAG production, reduced the increase in F340/F380 to 0.061±0.007 (mean±SEM, n=7), which is indeed
45% lower than the value observed for these cells in the absence of this inhibitor, either with or without additional OAG.

These results show that density-arrested NRK cells display significantly higher levels of ROCE than quiescent cells. Furthermore they suggest that at least in density-arrested cells activation of receptor-operated calcium entry takes place in a PLC-dependent manner.

3.3. Mechanism of strontium uptake in NRK cells

We have previously shown that PGF2α-mediated calcium oscillations in quiescent, as well as spontaneous calcium action potentials in density-arrested NRK cells, also occur in the presence of externally added strontium ions [8]. These studies provided evidence that during an action potential L-type calcium channels can mediate the uptake of strontium ions into NRK cells. However, the ion channels involved in strontium uptake in the absence of an action potential have not been characterized yet. Fig. 3A, B compares the uptake of calcium and strontium ions, respectively, by store-operated ion channels in DA-cells. In BHQ-treated cells 5 mM Ca2+ induced a F340/F380 increase of 0.14 ± 0.01 (mean ± SEM, n = 19), while 5 mM Sr2+ induced a fluorescence increase of 0.034 ± 0.008 (mean ± SEM, n = 11). Fig. 3C, D shows the rise in fluorescence ratio by the uptake of calcium or strontium ions, respectively, by receptor-operated channels in DA-cells. Addition of calcium ions resulted in a F340/F380 increase of 0.11 ± 0.01 (mean ± SEM, n = 12), while strontium ions induce an increase in fluorescence ratio of 0.075 ± 0.019 (mean ± SEM, n = 6). These data indicate that strontium ions can be taken up by NRK cells through both store-operated and receptor-operated ion channels. All the measured increases of the fluorescence ratio in these experiments were significant compared to the baseline values.

When interpreting these data it should be taken into account that calcium and strontium ions both change the fluorescence properties of Fura-2, but do so with a different affinity (Ca2+:Kd = 224 nM; Sr2+:Kd = 9.2 µM [9]). The relatively small change in fluorescence upon addition of strontium ions therefore corresponds to a relatively high permeability of the plasma membrane for Sr2+, when compared to calcium ions. Calibration of the Fura-2 ratio signal by the method of Grynkiewic [10] was not conclusive due to the small increase in the fluorescence ratio when strontium was added. Based on the above Kd-values and the known Hill coefficient of Fura-2, the observed increase in fluorescence ratio from 0.52 to 0.65 upon the addition of extracellular calcium (Fig. 3A) corresponds to an increase in intracellular calcium concentration of 0.44 µM. In comparison, the relatively small increase from 0.49 to 0.52 upon the addition of extracellular strontium (Fig. 3B) corresponds to an intracellular strontium concentration up to 10 µM. This indicates that strontium ions can permeate through store-operated ion channels at least as good as calcium ions. Moreover, the relatively high fluorescence increase for strontium versus calcium ions during receptor-operated ion uptake indicates that strontium ions are well taken up by NRK cells by both SOCE and ROCE mechanisms.

Fig. 2. Non-store-operated calcium entry is larger in density-arrested than in quiescent cells. Increase in intracellular calcium upon re-addition of calcium in the presence of 100 µM OAG to quiescent cells (A) and density-arrested cells (B) (significance: **, p < 0.001, compared to A, n = 10). Increase in intracellular calcium upon re-addition of calcium to density-arrested cells in the absence of OAG (N.S., compared to B, n = 10) (C). Calcium entry upon re-addition of calcium to density-arrested cells in the presence of the PLC-inhibitor U73122 (D) (significance: *, p < 0.05, compared to C, n = 7). All recordings in density-arrested cells were performed in the presence of nifedipine to prevent entry of calcium through L-type calcium channels. The gray band around the traces represents the SEM-error bars for every datapoint.
3.4. Endogenous expression of Trpc family members, Orai1 and Stim1 in NRK fibroblasts

In order to test which calcium channels may be involved in the observed SOCE and ROCE, we tested NRK cells for expression of channel encoding genes by RT-PCR analysis. Fig. 4A shows that of the various Trpc channels, NRK cells expressed particularly the genes encoding Trpc1, Trpc5 and Trpc6. As a comparison, rat brain tissue expressed at least the genes encoding Trpc1, Trpc3, Trpc4, Trpc5, and Trpc6, but possibly also those encoding Trpc2 and Trpc7. Trpc1 is generally considered as a channel involved in SOCE\[11\], while Trpc6 is known to be DAG dependent\[12\]. Trpc5 is less well characterized in this respect. The observation that NRK cells express these three genes, confirms our functional studies that NRK cells contain both SOCs and ROCs. As shown in Fig. 4B, quantitative RT-PCR analysis indicated that particularly Trpc5 (10-fold) and Trpc6 (6-fold) are strongly up-regulated when quiescent NRK cells are grown to density-arrest. In contrast, the high expression level of Trpc1 is not enhanced upon density-arrest of the cells.

Under similar experimental conditions, transcripts of both Stim1 and Orai1 were detected in NRK fibroblasts by RT-PCR. Fig. 5A, C shows the PCR products obtained for Stim1 and Orai1, respectively. Gel electrophoresis confirmed that the PCR product sizes corresponded to rat Stim1 (293 bp) and rat Orai1 (375 bp). Sequencing of the PCR products showed agreement with the original GenBank sequences. Upon density-arrest, the expression of Stim1 and Orai1 was up-regulated 1.6 and 5.5 fold, respectively (Fig. 5B, D).

Although these data do not necessarily reflect changes in channel densities, the results indicate that the genes for most calcium channels tested and for the calcium sensor Stim1 are strongly up-regulated, when NRK cells are grown from quiescence to density-arrest. These data agree with our functional studies showing that both SOCE and ROCE are clearly enhanced upon growing NRK cells to density-arrest (see Sections 3.1 and 3.2).

3.5. Calcium influx limits calcium oscillations

We have previously shown that calcium influx is required for the persistent calcium oscillations that are induced by PGF2α in quiescent NRK cells [5]. Several pharmaceutical agents are known to inhibit or enhance SOCE and ROCE. We have determined the inhibitory or enhancing effect of three of these compounds. In Fig. 6 we show that 2-APB and SKF96365 had profound inhibitory effects on SOCE in quiescent (Fig. 6A, C, respectively) and ROCE in density-arrested cells (Fig. 6B, D, respectively) (see also Supplementary Table S3). In the remainder of Fig. 6 we show that pre-incubation with the protein kinase C inhibitor Gö6976 potentiated SOCE in quiescent cells (Fig. 6A C, respectively) and ROCE in density-arrested cells (Fig. 6B, D, respectively) (see also Supplementary Table S3). The present observations show that calcium entry into NRK cells can be either inhibited or enhanced by pharmacological treatments. Because of the inhibitory effects of 2-APB on IP3 receptors [13], we choose SKF96365 and Gö6976 to test their inhibitory and potentiating effects, respectively, on PGF2α-induced calcium oscillations.
that in Q-cells potentiation by Gö6976 as well as inhibition by SKF96365 of calcium entry both significantly reduced the frequency of the PGF$_{2α}$-induced calcium oscillations, while in DA-cells only inhibition of calcium entry caused a significant reduction in the frequency of the action potential-induced calcium transients.

4. Discussion

In this study we show a differential role for SOCs and ROCs in the calcium dynamics of quiescent and density-arrested NRK fibroblasts. For the first time the occurrence of both types of calcium entry has been demonstrated experimentally in quiescent and density-arrested NRK cells. In an earlier study we already predicted the necessity of a calcium store-dependent calcium entry in quiescent and density-arrested fibroblasts.

In contrast to previous studies [14–16] we have shown that both SOCs and ROCs have a significant permeability for Sr$^{2+}$-ions. Due to the low affinity of Fura-2 for Sr$^{2+}$, the influx of Sr$^{2+}$ into the cells resulted in only a rather small increase in the fluorescence ratio. This modest increase represents, however, a quite large Sr$^{2+}$ influx. Previously we have shown that density-arrested cells can repetitively fire action potentials in calcium-free medium supplemented with strontium for prolonged periods of time [8]. Our results suggest that...
strontium entry is facilitated by the same activation pathways as calcium entry and that those pathways are different from the L-type voltage-gated channels.

In quiescent cells, the addition of extracellular calcium after calcium deprivation without SERCA inhibition resulted in a small calcium influx (Fig. 1C). In density-arrested cells, however, calcium addition after calcium deprivation resulted in a significantly higher calcium influx (Fig. 2C). These results indicate that density-arrested cells have another ensemble of calcium influx pathways than quiescent cells. Besides store-operated calcium entry, the density-arrested cells also exhibit a

Fig. 6. Effects of pharmacological agents on calcium entry in NRK fibroblasts. A and B show the effect of 75 µM 2-APB on BHQ-induced store-operated calcium entry in quiescent cells (A) and receptor-operated calcium entry in density-arrested cells in the presence of OAG (B). C and D show the effect of SKF9365 (10 µM) on SOCE and ROCE in quiescent (C) and density-arrested cells (D), respectively. The inhibition of calcium entry by 2-APB and SKF9365 in quiescent cells is compared with control experiments (Fig. 1A) (significance: ****, p<10^{-8}). The inhibition of calcium entry by 2-APB and SKF9365 in density-arrested cells is compared with control experiments (Fig. 2B) (significance: ***, p<10^{-6}). E and F show the effect of pre-incubation (30 min) with 100 nM Gö6976 (assigned by (Gö6976) in the figure) on SOCE in quiescent (E) and ROCE in density-arrested cells (F). Potentiation of calcium entry in quiescent and density-arrested cells is compared with control experiments (Figs. 1A and 2B, respectively) (significance: **, p<10^{-4}; and *, p<0.01). All recordings in density-arrested cells were performed in the presence of the L-type calcium channel blocker nifedipine to prevent entry of calcium through this type of calcium channels.
receptor-operated calcium entry mechanism. The addition of OAG, a DAG-derivative, did not result in an additional increase of the calcium influx. However, incubation of the density-arrested cells with PLC-inhibitor U73122 inhibited the calcium entry in these cells (Fig. 2D). This indicates that in density-arrested NRK cells a PLC-dependent hydrolysis of PIP$_2$ into IP$_3$ and DAG plays a role in the stimulation of ROCs. This is supported by earlier findings by Harks et al. [4], who have shown that density-arrested cells produce and secrete low amounts of PGF$_{2\alpha}$. This low concentration of PGF$_{2\alpha}$ activates the G-protein coupled FP receptor and activates PLC. These results suggest that the low concentrations of PGF$_{2\alpha}$ present in the culture medium of the density-arrested cells can result in an increased intracellular DAG level activating ROCE. Earlier studies [17] have proposed an iPLA$_2$-dependent pathway as an activation mechanism for SOCE. However, inhibition of iPLA$_2$ did
not affect SOCE, showing that this pathway is not relevant in NRK fibroblasts (data not shown).

Our findings are further supported by the differential expression pattern of Trpc genes in quiescent and density-arrested cells. Trpc channels have been described earlier as candidate channels for mediating SOCE and ROCE [18]. While the expression level of Trpc1 was found to be independent of cell density, expression levels of Trpc5 and Trpc6 were clearly increased in density-arrested cells. Trpc1 is generally considered as the channel involved in SOC formation [11], while Trpc6 is known to be DAG dependent [12]. Although the exact activation pathway is not conclusive, Trpc5 seems to be activated by receptors coupled to PLC, suggesting a role together with TRPC in ROCE [19]. On the other hand, Zhu et al. [20] have shown that Trpc5 is desensitized by phosphorylated PKC. The binding of DAG to the C1 domain results in activation and translocation of PKC [21], whereby the production of DAG would inhibit the activity of Trpc5. The potentiating effect of PKC inhibitor Gö6976 suggests a role together with Trpc6 in ROCE [19]. On the other hand, we have shown that the PLC-inhibitor U73122, expected to suggest that a PKC-dependent calcium entry channel is present. The potentiating effect of PKC inhibitor Gö6976 clearly increased in density-arrested cells. Trpc1 is generally considered as responsible for calcium entry, whether receptor-operated or store-operated. Density-arrested cells, however, behave in a more complex manner. In a previous study [26] we have suggested a pacemaker–follower system to explain the spontaneous calcium action potentials in density-arrested cells. Under conditions of density-arrest inhomogeneity in the local production of PGF2α might give rise to localized islands of depolarized cells with increased intracellular IP3. In these ‘pacemaker’-islands IP3-induced intracellular calcium oscillations synchronize resulting in depolarizations at the border between polarized and depolarized cells, giving rise to propagating action potentials which depolarize the surrounding ‘follower’-cells. According to Harks et al. [4] density-arrested cells have a PGF2α concentration of only 1.5 nM in the extracellular medium. Therefore the cytosolic level of IP3 in density-arrested cells is presumably much lower than in quiescent cells stimulated with 1 μM PGF2α. Density-arrested cells, however, seem to have a larger calcium entry facilitated by SOCs and ROCs, so in pacemaking cells the concentration of IP3 might be in a regime near 0.2 μM in combination with a membrane calcium conductance of 0.04 nS (Fig. S1B). An increase/potentiation of calcium entry conductance (to 0.10 nS, Fig. S1C), while maintaining a constant level of [IP3]cyt, would not stop the calcium oscillations. This shows that the higher calcium influx in density-arrested cells results in a broader range of IP3 concentrations that can induce calcium oscillations. Apparently, density-arrested cells are more sensitive to IP3 with respect to the ability to induce calcium oscillations.

The effect of increasing calcium entry on the calcium oscillations in NRK fibroblasts suggests that proliferation of these cells from the quiescent to the density-arrested stage may not only result in an increased production of PGF2α, but also in an enhanced expression of both SOCs and ROCs. This differential mechanism of calcium entry provides NRK fibroblasts with an elegant pathway to meet their calcium requirements under the different growth conditions. Our previous study [6] has shown that a regulated calcium entry is required to couple membrane excitability with calcium dynamics, in order to maintain calcium homeostasis in NRK fibroblasts. Our results thereby suggest that Trpc channels, most likely in combination with Stim1 and Orai1, are able to provide this calcium entry pathway. We are currently testing this hypothesis by using a shRNA approach to selectively knockdown the genes for these proteins.

5. Conclusions

In this study we have shown that Trpc1, Trpc5 and Trpc6, Stim1 and Orai1 are expressed in NRK fibroblasts. Moreover, Trpc5, Trpc6 and Orai1 are differentially expressed in quiescent and density-arrested cells. The increased expression of these genes in density-arrested cells coincides with an increase of SOCE and ROCE at this growth stage. The involvement of Trpc6 in ROCE is supported by our observation that
ROCE is a PLC-dependent process. Earlier observations of sustained oscillations in Sr\(^{2+}\)-containing media are supported by our finding that in NRK fibroblasts SOC as well as ROC are not only permeable for Ca\(^{2+}\) but also for Sr\(^{2+}\). The earlier notion from the mathematical model of NRK fibroblasts that calcium entry limits the range in which IP\(_3\)-dependent calcium oscillations and action potentials can occur, is supported by the present experimental findings.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellsig.2010.02.007.

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