R-RAS ALTERS CA$^{2+}$ HOMEOSTASIS BY INCREASING THE CA$^{2+}$ LEAK ACROSS THE ENDOPLASMIC RETICULAR MEMBRANE

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**Abbreviations:** R-Ras: Ras-like Ras, DAG: 1,2-diacylglycerol, CCK₈: C-terminal octapeptide of cholecystokinin, Tg: thapsigargin.

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SUMMARY

Evidence in the literature implicating both R-Ras and intracellular Ca$^{2+}$ in programmed cell death and integrin-mediated adhesion prompted us to investigate the possibility that R-Ras alters cellular Ca$^{2+}$ handling. CHO cells expressing the cholecystokinin (CCK)-A receptor were loaded with indo-1 to study the effects of constitutively active V38R-Ras and dominant negative N43R-Ras on the kinetics of the thapsigargin (Tg)- and CCK$_8$-induced Ca$^{2+}$ rises using high-speed confocal microscopy. In the absence of extracellular Ca$^{2+}$, both Tg (1 µM), a potent and selective inhibitor of the Ca$^{2+}$ pump of the intracellular Ca$^{2+}$ store, and CCK$_8$ (100 nM) evoked a transient rise in Ca$^{2+}$, the size of which was significantly decreased following expression of V38R-Ras. At 0.1 nM, CCK$_8$ evoked periodic Ca$^{2+}$ rises. The frequency of these Ca$^{2+}$ oscillations was significantly reduced in V38R-Ras-expressing cells. In contrast to V38R-Ras, N43R-Ras did not alter the kinetics of the Tg- and CCK$_8$-induced Ca$^{2+}$ rises. The present findings are compatible with the idea that V38R-Ras expression increases the passive leak of Ca$^{2+}$ out of the store leading to a decrease in Ca$^{2+}$ content of this store, which, in turn, leads to a decrease in frequency of the CCK$_8$-induced cytosolic Ca$^{2+}$ oscillations. The effect of V38R-Ras on the Ca$^{2+}$ content of the intracellular Ca$^{2+}$ store closely resembles that of the anti-apoptotic protein Bcl-2 observed earlier. Together with reports on the role of dynamic Ca$^{2+}$ changes in integrin-mediated adhesion this leads us to propose that the reduction in ER Ca$^{2+}$ content may underlie the anti-apoptotic effect of R-Ras, whereas the decrease in frequency of stimulus-induced Ca$^{2+}$ oscillations may play a role in the inhibitory effect of R-Ras on stimulus-induced cell detachment and migration.
INTRODUCTION

The Ras-related G-protein, R-Ras, is a member of the Ras subfamily of small GTP-binding proteins (1,2). The R-Ras protein is localized at the inner leaflet of the outer membrane, shares 55% identity with the prototypic Ras, but is 26 amino acids longer at its N-terminus. In vitro, R-Ras interacts with several known Ras regulatory proteins including RasGRF1, Ras-GRP/CalDag-GEFII, Ras-GRP3/CalDAG-GEFIII, GAP1IP4BP (3) and the three downstream effector proteins Raf1, phosphatidyl inositol 3-kinase and RalGDS (4,5,6).

Several of the Ras exchange factors with which R-Ras interacts including RasGRF1, CalDAG-GEFII (Ras-GRP), CalDAG-GEFIII (RasGRP3) and CalDAG-GEFIII are sensitive to Ca$^{2+}$ and/or diacylglycerol (DAG), the endogenous activator of protein kinase C, whereas the GTPase-activating protein GAP1IP4BP with which R-Ras also interacts is activated by inositol 1,3,4,5-tetrakisphosphate (3).

R-Ras has been implicated in cell transformation, cell adhesion (7) and cell cycle control (6,8). These functions appear to be mediated by few, if any, of the signaling pathways taken by Ras (4,6,9,10,11). Several studies have shown that Ras and R-Ras have opposing effects on apoptosis, or programmed cell death, in that R-Ras stimulates this process under conditions where Ras is protective (1,12,13). Early studies employing the yeast two hybrid system suggested a physical interaction between R-Ras and the anti-apoptotic Bcl-2 (14). Thusfar, however, this interaction could not be demonstrated in a mammalian cell system (15). Other studies have shown that under certain experimental conditions activated mutants of R-Ras can act through the phosphatidylinositol 3-kinase pathway to inhibit cell death (16,17). Finally, constitutively active V38R-Ras has been shown to keep cellular integrins in an active state thus allowing attachment to surfaces coated with integrin ligands (7).

Recent studies have implicated the Ca$^{2+}$-dependent enzyme calpain in cell detachment during cell migration (18) and inhibition of integrin-induced stress fiber assembly and cell spreading.
These findings explain previously reported effects of alterations in intracellular Ca\(^{2+}\) concentration on integrin-mediated adhesion (20). A role for Ca\(^{2+}\) in apoptosis became apparent when it was shown that a modest reduction in endoplasmic reticulum (ER) Ca\(^{2+}\) content prevented cell death (21,22).

The involvement of R-Ras and intracellular Ca\(^{2+}\) in both programmed cell death and integrin-mediated adhesion prompted us to investigate the possibility that R-Ras might exert its actions through an effect on cellular Ca\(^{2+}\) handling. Here we show that constitutively active V38R-Ras decreases the ER Ca\(^{2+}\) content in a manner similar to the pro-apoptotic protein Bcl-2 and slows down the frequency of stimulus-induced periodic Ca\(^{2+}\) rises. We propose that the reduction in ER Ca\(^{2+}\) content may underly the anti-apoptotic effect of R-Ras described by Suzuki and co-workers (16,17). Furthermore, we propose that the decrease in frequency of the stimulus-induced cytosolic Ca\(^{2+}\) rises may inhibit stimulus-induced activation of calpain thus causing inhibition of cell detachment and migration and favoring integrin-mediated cell attachment and spreading.
EXPERIMENTAL PROCEDURES

Transient transfection of CHO cells with R-Ras mutants

The development of a CHO cell line stably expressing the CCK\(_A\) receptor (CHO-CCK\(_A\)) has been described in detail elsewhere (23). CHO-CCK\(_A\) cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere of 5% CO\(_2\) at 37°C. For transfection, cells were trypsinized (5x10\(^6\) cells/300 µl) and electroporated (280 V, 975 µF) in the presence of 2 µg of plasmid pGFP-N1 (Clontech, Palo Alto, CA, USA) and 18 µg of either pMT2-HA-V38R-Ras or pMT2-HA-N43R-Ras (24). Subsequently, cells were seeded on a glass cover slip (15,000 cells/30 µl) and allowed to attach for 30 min. Culture medium was added and the cells were grown for 48 h.

Detection of R-Ras mutants in CHO cells

GFP-positive and GFP-negative cells were separated by means of fluorescence activated cell sorting at 24 h after electroporation. The cells were cultured for another 24 h, homogenized, and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred overnight to polyvinylidene difluoride membranes (Immobilon P, Millipore, Bedford, MA, USA). For detection of HA-V38R-Ras and HA-N43R-Ras, blots were incubated overnight with the anti-HA monoclonal antibody 12ca5. Immunoreactive bands were detected with alkaline phosphatase-conjugated rabbit anti-mouse IgG.

Single cell Ca\(^{2+}\) imaging

Cells were loaded with indo-1 for 30 min at 37°C in DMEM containing 10% FCS, 3 µM indo-1/AM and 0.025% (w/v) pluronic F-127. Excess indo-1 was removed by washing twice with calcium-free HEPES/Tris medium containing 133 mM NaCl, 4.2 mM KCl, 1.0 mM
MgCl₂, 5.8 mM glucose, an amino acid mixture according to Eagle, 0.1% (w/v) bovine serum albumin and 10 mM HEPES, adjusted with Tris to pH 7.4. Cover slips (22 mm) were subsequently mounted in a Leiden chamber (25) and placed on the stage of an inverted microscope (Nikon, Diaphot), attached to a videorate confocal microscope (Noran instruments, Middleton, WI, USA). A water immersion objective (x40, NA 1.2) was used, allowing a field of view of about 15 cells. Within each field (165 x 155µm), cells of comparable size were selected. GFP-positive cells were identified by recording their green emission at 525 ± 25 nm after 488 nm excitation delivered by an Argon-ion laser (Omnichrome Inc., Chino, CA, USA). Specific excitation of indo-1 (351 nm) was provided by a high power argon ion laser (Coherent Enterprise, Santa Clara, CA, USA). Indo-1 fluorescence emission was monitored at 405 ± 45 nm and 485 ± 45 nm at 30 Hz by using a 455 nm DCLP dichroic mirror. The OZ hardware set-up and acquisition was controlled with Intervision software (Version 1.6, Noran instruments) running under IRIX 6.2 on an Indy workstation (Silicon Graphics Inc., Mountain View, CA, USA) equipped with 128 Mb of RAM. Fluorescence signals were collected in real-time (30 Hz, 5-10 min total recording time) from eight rectangular regions of interest (including a cell-free area for background correction) drawn on full-frame images (512 x 480 pixels). The zoom factor was 0.6 and the pixelsize was 0.323 µm as calibrated with a graticule (26). To reduce noise and to ensure that each cell was fully within the confocal volume no slit was applied (optical sections thickness of 10.53 µm). Between recordings, hardware settings (i.e. brightness, contrast and laser power) were kept constant. The laser power used (28 µW at the back of the objective lens) was minimal to prevent cytotoxic and/or heating artifacts. The cells were incubated for 2 minutes in calcium free HEPES/Tris medium containing 0.5 mM EGTA prior to the start of the recording. Subsequently, the cells were stimulated with either CCK₈ (0.1 nM or 100 nM)
or thapsigargin (1 µM). The fluorescence emission ratio at 405 and 485 nm was monitored as a measure of \([\text{Ca}^{2+}]_i\) after excitation at 351 nm.

For long-term recordings, cells were loaded with fura-2 in the presence of 3 µM fura-2/AM and 0.025% (w/v) pluronic F-127 as described above. Cover slips were mounted in a thermostatic (37°C) perfusion chamber placed on the stage of an inverted microscope (Nikon, Diaphot). Dynamic video imaging was carried out as described previously (27) using the MagiCal hardware and TARDIS software provided by Joyce Loebl (Dukesway, Team Valley, Gateshead, UK). By using an epifluorescent x40 oil immersion objective we were able to simultaneously monitor the cytosolic Ca\(^{2+}\) concentration in close to 50 individual cells. GFP-positive cells were identified by their green emission (525 ± 20 nm) at an excitation wavelength of 490 nm. Fura-2 emission was monitored at 492 nm during alternating excitation at 340 and 380 nm. The fluorescence emission ratio at 492 nm was monitored as a measure of \([\text{Ca}^{2+}]_i\) after excitation at 340 and 380 nm. CCK\(_8\) (0.1 nM) was added by means of a custom-made superfusion system.

**Inositol 1,4,5-trisphosphate measurements**

At 24 h after transfection, GFP-negative and positive cells were separated by means of fluorescence activated cell sorting and plated out in 12-well plates (100,000 cells/well). After another 24 hours of culturing, cells were washed in HEPES/Tris medium containing 1% (w/v) bovine serum albumin and stimulated by the addition of 125 µl HEPES/Tris medium containing the indicated concentration of CCK\(_8\). After 20 sec, trichloroacetic acid (31 µl, 50%) was added to stop the reaction. The cells were scraped off and transferred to an Eppendorf test tube. The samples were centrifuged for 4 min at 10,000 g and a 120 µl aliquot of the supernatant was removed. This aliquot was extracted three times with 2 ml of water-saturated diethyl ether. Subsequently, 75 µl was taken to which 2 µl KHCO\(_3\) (50%) was
added to increase the pH above 7.5. The inositol 1,4,5-trisphosphate content of the extract was determined by isotope dilution assay as previously described (28).

Data analysis

Data was analyzed using Origin Pro 6.1 (Microcal, Northampton, MA, USA) and Image Pro Plus 4.1 image analysis software (Media Cybernetics, Silver Spring, MD, USA). The results presented are the mean ± S.E.M. Overall statistical significance was determined by analysis of variance (ANOVA). In case of significance, individual groups were compared according to Fischer and p-values < 0.05 were considered significant. For linear fits the least-squares algorithm was applied using both Pearson’s R and p-values as a measure for the quality of the fit. In all graphs, indo-1 ratio signals were normalized to the basal (prestimulatory) level.

Materials

CCK8 was obtained from Sigma (St. Louis, MO, USA), thapsigargin from LC services (Woburn, MA, USA) and tissue culture medium with additives from Gibco (Paisley, Scotland). Indo-1/acetoxyethyl ester, fura-2/acetoxyethyl ester and pluronic F-127 were purchased from Molecular Probes Inc. (Leiden, The Netherlands). D-myo \[^{3}H\] inositol 1,4,5-trisphosphate (51.4 Ci/mmol) was obtained from Amersham (Buckinghamshire, UK). All other chemicals were of reagent grade.
RESULTS

Co-expression of R-Ras and GFP in CHO cells

CHO cells expressing the rat CCK\textsubscript{A} receptor (CHO-CCK\textsubscript{A} cells) were co-transfected with GFP and either constitutively active HA-V38R-Ras or dominant negative HA-N43R-Ras. At 48 hrs post-transfection, cells were loaded with the fluorescent Ca\textsuperscript{2+} indicator indo-1 and visualized by confocal microscopy. Figure 1A shows a representative example of a cluster of GFP-positive (GFP\textsuperscript{+}) cells co-transfected with V38R-Ras. When identical hardware settings were used during acquisition, no significant differences in GFP intensity between V38R-Ras- and N43R-Ras-transfected cells were observed (Table 1). The corresponding indo-1 image (Fig. 1B) shows a second cluster of GFP-negative (GFP\textsuperscript{−}) cells. Importantly, these GFP\textsuperscript{−} cells, present on the same cover slip as the GFP\textsuperscript{+} cells, were used as a control for the effect of R-Ras expression on cellular Ca\textsuperscript{2+} handling. Under the experimental conditions used, no cross-talk between GFP and indo-1 fluorescence signals was observed.

To demonstrate that GFP expression reports expression of R-Ras, GFP\textsuperscript{+} and GFP\textsuperscript{−} cells were separated by fluorescence-activated cell sorting (FACS) at 24 h post-transfection and cultured for another 24 h. After this second culturing period, total cell lysates were prepared and subjected to Western blot analysis using the monoclonal anti-HA antibody 12ca5. Figure 1C shows that both R-Ras mutants were highly expressed in the GFP\textsuperscript{+} cells.

Morphology of R-Ras-expressing cells

To demonstrate alterations in cellular morphology induced by R-Ras, we compared both the cross-sectional area and morphology between GFP\textsuperscript{−} and GFP\textsuperscript{+} cells. Table 1 shows that the cross-sectional area was significantly increased in N43R-Ras-expressing cells (p<0.01). To detect more subtle morphological alterations we calculated the ‘formfactor’ $F$...
(perimeter²/4·π·area). For a round cell, the numerical value of $F$ is one. The data presented show that $F$ was significantly decreased in the R-Ras-expressing cells (Table 1, $p<0.01$). Importantly, however, $F$ was significantly smaller in N43R-Ras-expressing cells as compared to V38R-Ras-expressing cells ($p<0.05$). Taken together, these findings demonstrate that CHO cells which express N43R-Ras and, to a lesser extent, V38R-Ras are larger and rounder than GFP⁻ cells.

*V38R-Ras alters the kinetics of the thapsigargin-induced Ca²⁺ rise*

To assess possible effects of R-Ras on cellular Ca²⁺ handling, CHO-CCK₄ cells transiently expressing either N43R-Ras or V38R-Ras were treated with thapsigargin (Tg), a specific inhibitor of the sarco- and endoplasmic reticulum Ca²⁺-ATPase (SERCA). The cells were loaded with indo-1 and the Tg-induced changes in cytosolic Ca²⁺ concentration were monitored by means of high-speed confocal microscopy. Tg increases the cytosolic Ca²⁺ concentration by preventing the active re-uptake of the Ca²⁺ ions that continuously leak out of the endoplasmic reticulum (ER) Ca²⁺ store. In the absence of extracellular Ca²⁺, these Ca²⁺ ions are removed from the cytosol by the action of the plasma membrane Ca²⁺-ATPase (PMCA).

Figure 2A shows the effect of Tg (1 µM) on the average cytosolic Ca²⁺ concentration of 4 V38R-Ras expressing cells (filled circles) and 4 GFP⁻ cells (open circles) present on the same cover slip. The experiment was performed in the absence of extracellular Ca²⁺. Under these conditions, Tg transiently increased the cytosolic Ca²⁺ concentration in both V38R-Ras-expressing cells and the GFP⁻ cells. This observation shows that the rate of Ca²⁺ leak from the ER is considerably faster than the rate of cytosolic Ca²⁺ removal via the PMCA.

The duration of the Tg-induced Ca²⁺ transient was significantly shortened from 190 ± 12 s ($n = 38$ cells) in GFP⁻ cells to 121 ± 13 s ($n = 24$ cells; $p<0.001$) in V38R-Ras-expressing cells.
The effect was specific for V38R-Ras because the duration of the Tg-induced transient was not significantly altered in N43R-Ras-expressing cells (173 ± 6 s; n = 10). The amplitude of the transient (Fig. 2A) seemed to be decreased in V38R-Ras-expressing cells (2.63 ± 0.12; n = 24 cells) as compared to GFP cells (2.86 ± 0.10; n = 38 cells) and N43R-Ras expressing cells (3.10 ± 0.19; n = 10 cells) but this effect was not statistically significant.

The rising phase of the Tg-induced Ca$^{2+}$ transient continues as long as the rate of Ca$^{2+}$ leak from the ER exceeds that of active cytosolic Ca$^{2+}$ removal by the PMCA. The kinetics of this phase was adequately described by a sigmoid (boltzmann) equation ($y = A_2+(A_1-A_2)/(1+\exp((x-x_0)/\tau))$) (Fig. 2B). The time constant $\tau$, which is inversely proportional to the rate of Ca$^{2+}$ rise, was smaller in V38R-Ras-expressing cells (4.42 ± 0.09 s; $R^2 = 0.99$; n = 4) as compared to the corresponding GFP cells (7.54 ± 0.012 s; $R^2 = 0.99$; n = 4) present on the same cover slip. This shows that the rate of Ca$^{2+}$ rise is 1.7-fold increased in V38R-Ras-expressing cells.

The decay phase of the Tg-induced Ca$^{2+}$ transient starts when the rate of Ca$^{2+}$ leak from the ER becomes smaller than the rate of PMCA-mediated Ca$^{2+}$ removal from the cytosol. The kinetics of this phase was adequately described by a monoexponential equation ($y = y_0+A.e^{-t/\mu}$) (Fig. 2C). The time constant $\mu$, which is inversely proportional to the rate of Ca$^{2+}$ decay, was markedly smaller in V38R-Ras-expressing cells (49.7 ± 0.4 s; $R^2 = 0.93$; n = 4) as compared to GFP cells (200.3 ± 15.0 s; $R^2 = 0.98$; n = 4). This shows that the rate of Ca$^{2+}$ decay is 4-fold increased in V38R-Ras-expressing cells.

Calculation of the integrated area underneath the Ca$^{2+}$ transient, as a measure of the amount of Ca$^{2+}$ released into the cytosol, revealed a significant decrease from 162 ± 10 AU.s (n = 38) and 169 ± 15 AU.s (n = 10) in GFP cells and N43R-ras-expressing cells, respectively, to 93 ± 8 AU.s (n = 24) in V38R-Ras-expressing cells (Table 1).
**V38R-Ras alters the kinetics of the CCK\textsubscript{8} (100 nM)-induced single Ca\textsuperscript{2+} rise**

In CHO-CCK\textsubscript{A} cells, CCK-induced cytosolic Ca\textsuperscript{2+} signals arise from Ins(1,4,5)P\textsubscript{3}-mediated Ca\textsuperscript{2+} release from the endoplasmic reticulum (23), paralleled by capacitative Ca\textsuperscript{2+} entry across the plasma membrane (29). To investigate whether R-Ras affects stimulus-induced Ca\textsuperscript{2+} release from the endoplasmic reticulum, cells were stimulated with 100 nM CCK\textsubscript{8} in the absence of extracellular Ca\textsuperscript{2+}. At this concentration, CCK\textsubscript{8} evokes a single Ca\textsuperscript{2+} transient that is not followed by repetitive Ca\textsuperscript{2+} transients (Ca\textsuperscript{2+} oscillations). Figure 2 (D and F) shows that both GFP\textsuperscript{+} and GFP\textsuperscript{-} cells displayed a single Ca\textsuperscript{2+} transient that consisted of a rapid increase followed by a first phase of slow decay and a second phase of fast decay to basal levels. V38R-Ras-expressing cells displayed a Ca\textsuperscript{2+} transient that was less wide (Fig. 2D) and rose more slowly (Fig. 2E) than that in the corresponding GFP\textsuperscript{-} cells. The amplitude was only slightly decreased from 3.02 ± 0.10 (n = 58) in GFP\textsuperscript{-} cells to 2.80 ± 0.11 (n = 44) in V38R-Ras-expressing cells or 3.08 ± 0.15 (n = 19) in N43R-Ras-expressing cells (Table 1). However, this decrease was not statistically significant. Detailed analysis of the rate of Ca\textsuperscript{2+} rise revealed a \(\tau\) value that was increased for V38R-Ras-expressing cells (\(\tau = 0.12 ± 0.01\) s; \(R^2 = 0.99; n = 4\)) as compared to the corresponding GFP\textsuperscript{-} cells (\(\tau = 0.07 ± 0.01\) s; \(R^2 = 0.99; n = 4\)) present on the same cover slip (Fig. 2E). This demonstrates that the rate of Ca\textsuperscript{2+} rise is reduced in V38R-Ras-expressing cells. In N43R-Ras-transfected cells, the kinetics of the decline (Fig. 2F) and rising phase (Fig. 2G) of the Ca\textsuperscript{2+} transient were identical between the GFP\textsuperscript{+} and GFP\textsuperscript{-} cells. This shows that GFP expression in itself had no effect on the shape of the CCK\textsubscript{8}-induced Ca\textsuperscript{2+} transient. Importantly, 100 nM CCK\textsubscript{8} completely released the thapsigargin-sensitive intracellular Ca\textsuperscript{2+} store (Fig. 2H).

Analysis of the second (fast) phase of Ca\textsuperscript{2+} decay revealed a \(\mu\) value that was of the same order of magnitude for N43R-Ras-expressing cells (27.8 ± 0.2 s; \(R^2 = 0.79; n = 4\)) and the
corresponding GFP- cells (63.3 ± 0.8 s; R² = 0.96; n = 4) and for V38R-Ras-expressing cells (25.2 ± 0.4 s; R² = 0.98; n = 4) and the corresponding GFP- cells (31.2 ± 1.2; R² = 0.98; n = 4). Of note, these μ values were similar to that obtained with V38R-Ras-expressing cells following Tg treatment (49.7 ± 0.4 s; R² = 0.93; n = 4) but markedly lower than those obtained with GFP- cells following Tg treatment (200.3 ± 15.0 s; R² = 0.98; n = 4).

Finally, calculation of the integrated area underneath the Ca²⁺ transient revealed a significant decrease from 187 ± 17 AU.s (n = 58) and 177 ± 13 AU.s (n = 19) in GFP- cells and N43R-ras-expressing cells, respectively, to 133 ± 7 AU.s (n = 44) in V38R-Ras-expressing cells (Table 1).

CCK₈-induced Ins(1,4,5)P₃ formation is not altered in R-Ras-expressing cells

CCK₈ acts through Ins(1,4,5)P₃ to increase the cytosolic free Ca²⁺ concentration in CHO-CCKₐ cells. Figure 3 shows the dose-response curve for the effect of CCK₈ on the cellular Ins(1,4,5)P₃ content, measured at 20 s following the onset of stimulation. Neither V38R-Ras (A) nor N43R-Ras (B) interfered with the CCK₈-induced production of Ins(1,4,5)P₃.

V38R-Ras does not alter the kinetics of the CCK₈ (0.1 nM)-induced oscillatory Ca²⁺ rises

To assess possible effects of V38R-Ras expression on physiologically relevant Ca²⁺ signals, we studied the kinetics of the CCK₈-induced repetitive Ca²⁺ rises (Ca²⁺ oscillations) in CHO-CCKₐ cells. When added at a 1000-fold lower concentration (0.1 nM), CCK₈ readily induced oscillatory Ca²⁺ rises (Fig. 4A; (30)). Of note, this measurement was performed in the absence of extracellular Ca²⁺. Under this condition, the amplitude of the Ca²⁺ oscillations gradually decreased as a function of time. The inset of the figure shows that the rate of Ca²⁺ release (dotted line) was much slower for the last (b) than for the first (a) Ca²⁺ oscillation.
These findings are indicative for a gradual decrease of the Ca\textsuperscript{2+} content of the ER due to the action of the PMCA removing part of the released Ca\textsuperscript{2+} out of the cell during each oscillation. Analysis of the first oscillatory Ca\textsuperscript{2+} rise revealed no significant differences in amplitude and width between V38R-Ras-expressing cells and GFP\textsuperscript{−} cells (Table 1). As far as the width is concerned, this is in sharp contrast with the findings for the single CCK\textsubscript{8} (100 nM)-induced Ca\textsuperscript{2+} transient (see above). However, the kinetics of the first CCK\textsubscript{8} (0.1 nM)-induced oscillatory Ca\textsuperscript{2+} rise appeared to be completely different from that of the CCK\textsubscript{8} (100 nM)-induced single transient. Thus, whereas the amplitude of the first oscillation was only 0.8-fold lower, its width was 5.8-fold smaller (Table 1).

Analysis of the rate of Ca\textsuperscript{2+} rise revealed τ values 0.44 ± 0.09 s (R\textsuperscript{2} = 0.99; n = 4) and 0.39 ± 0.05 s (R\textsuperscript{2} = 0.96; n = 3) for V38R-Ras-expressing cells and corresponding GFP\textsuperscript{−} cells, respectively. These values were markedly higher than those obtained for the CCK\textsubscript{8} (100 nM)-induced single transient (0.12 s and 0.07 s for V38R-Ras-expressing cells and corresponding GFP\textsuperscript{−} cells, respectively).

Moreover, analysis of the decay phase revealed µ values of 5.6 ± 0.8 s (R\textsuperscript{2} = 0.96; n = 3) and 5.3 ± 0.6 s (R\textsuperscript{2} = 0.96; n = 4) for V38R-Ras-expressing cells and corresponding GFP\textsuperscript{−} cells that were considerably smaller than those obtained for the decay phase of the CCK\textsubscript{8} (100 nM)-induced single transient (25.2 s and 31.2 s for V38R-Ras-expressing cells and corresponding GFP\textsuperscript{−} cells, respectively). These differences are compatible with the idea that the \textit{Ins}(1,4,5)P\textsubscript{3}-operated Ca\textsuperscript{2+} release channels remain open at 100 nM CCK\textsubscript{8}, leading to the removal of all releasable Ca\textsuperscript{2+} by the action of the (slower) PMCA, whereas these channels rapidly close at 0.1 nM CCK\textsubscript{8}, allowing the (faster) SERCA pump to re-sequester the larger part of the released Ca\textsuperscript{2+} in the ER during each oscillation.
Reduced frequency of stimulus-induced repetitive Ca\(^{2+}\) rises in V38R-Ras-expressing cells

To demonstrate a possible effect of V38R-Ras-expression on the temporal characteristics of the cytosolic Ca\(^{2+}\) oscillations, we stimulated the cells with 0.1 nM CCK\(_8\) in the presence of 1 mM extracellular Ca\(^{2+}\). The latter prevented depletion of the endoplasmic reticulum Ca\(^{2+}\) store and allowed recording of Ca\(^{2+}\) oscillations during prolonged periods of time. Cells were loaded with fura-2 and video-imaging microscopy was used to monitor the CCK\(_8\)-induced Ca\(^{2+}\) changes. Figure 5 shows that the oscillation frequency was significantly reduced in V38R-Ras-expressing cells but not in N43R-Ras-expressing cells (p<0.01; n = 307, 31 and 157 cells for GFP, V38R-Ras and N43R-Ras, respectively).
DISCUSSION

Evidence in the literature has implicated both R-Ras and intracellular Ca\textsuperscript{2+} in programmed cell death (1,12,13,16,17,21,31) and integrin-mediated cell adhesion (7,8,19,20). This prompted us to investigate the possibility that R-Ras might exert its effects by altering the activities of proteins and/or organelles involved in cellular Ca\textsuperscript{2+} handling. To study the effects of R-Ras, CHO cells stably expressing the cholecystokinin (CCK)-A receptor were co-transfected with GFP and either constitutively active V38R-Ras or dominant negative N43R-Ras. Separation of GFP\textsuperscript{+} and GFP\textsuperscript{-} cells by fluorescence-activated cell sorting followed by Western blot analysis revealed that GFP\textsuperscript{+} cells indeed expressed the HA-tagged R-Ras protein.

V38R-Ras causes cell rounding and enlargement

For fluorescence measurements, cells were seeded on a glass cover slip immediately after transfection and grown for 48 h. This procedure provided us with the unique opportunity to simultaneously monitor the cytosolic Ca\textsuperscript{2+} changes in R-Ras-expressing (GFP\textsuperscript{+}) cells and the corresponding sham-transfected (GFP\textsuperscript{-}) cells present on the same cover slip. Detailed analysis of the size and morphology of the R-Ras-expressing cells revealed that N43R-Ras and, to a lesser extent, V38R-Ras caused cell enlargement and rounding. This is in agreement with the observation that inactivation of R-Ras by clostridial cytotoxins caused cell rounding and detachment (32).

V38R-Ras expression decreases both the ER Ca\textsuperscript{2+} content and the frequency of the CCK\textsubscript{8}-induced cytosolic Ca\textsuperscript{2+} rises

Cells transiently expressing either V38R-Ras or N43R-Ras were loaded with indo-1 or fura-2 and the changes in cytosolic free Ca\textsuperscript{2+} concentration were monitored by means of high-speed confocal or conventional video-imaging microscopy, respectively. To start with, the cells
were treated with Tg, a potent and selective inhibitor of the Ca\textsuperscript{2+} pump of the ER Ca\textsuperscript{2+} store (SERCA). Inhibition of this pump prevents re-uptake of Ca\textsuperscript{2+} ions that continuously leak out of the ER into the cytosol. We have previously shown that in the absence of active Ca\textsuperscript{2+} pumping this Ca\textsuperscript{2+} leak process is adequately described by a monoexponential equation (33,34). The present study shows that Tg evoked a rapid increase in cytosolic Ca\textsuperscript{2+} when added in the absence of extracellular Ca\textsuperscript{2+} to prevent capacitative Ca\textsuperscript{2+} uptake. This indicates that initially the rate of passive Ca\textsuperscript{2+} leak exceeds that of active cytosolic Ca\textsuperscript{2+} removal via the PMCA. After having reached its maximum, cytosolic Ca\textsuperscript{2+} slowly decreases to prestimulatory values. This suggests that during the entire down-stroke of the Tg-induced Ca\textsuperscript{2+} transient, Ca\textsuperscript{2+} is released from the ER thus slowing down the rate of Ca\textsuperscript{2+} decay.

Expression of V38R-Ras markedly decreased the duration of the Tg-induced Ca\textsuperscript{2+} transient. The integrated area underneath the cytosolic Ca\textsuperscript{2+} peak, which reflects the amount of Ca\textsuperscript{2+} released into the cytosol, was markedly (40 %) decreased in V38R-Ras-expressing cells as compared to N43R-Ras-expressing cells and GFP cells. This demonstrates that V38R-Ras expression causes a marked reduction of the ER Ca\textsuperscript{2+} content. Moreover, V38R-Ras caused a significant increase in the rate of Ca\textsuperscript{2+} rise, suggesting an increased Ca\textsuperscript{2+} leak across the ER membrane. Finally, V38R-Ras expression increased the rate of Ca\textsuperscript{2+} decay. This latter effect is compatible with an accelerated ER Ca\textsuperscript{2+} release during the rising phase and consequently reduced Ca\textsuperscript{2+} release during the decay phase resulting in a reduced slowing down of the rate of Ca\textsuperscript{2+} decay. Based on the data obtained with Tg we postulate that expression of V38R-Ras increases the ER Ca\textsuperscript{2+} leak, thereby decreasing the steady-state ER Ca\textsuperscript{2+} content.

CCK\textsubscript{8}, when added at a relatively high concentration of 100 nM, depleted the ER Ca\textsuperscript{2+} store. This indicates that at this concentration it causes the sustained opening of the inositol 1,4,5-trisphosphate-operated Ca\textsuperscript{2+} release channels. The rate of Ca\textsuperscript{2+} rise obtained with CCK\textsubscript{8} was considerably higher than that obtained with Tg (\(\tau\) values of 0.07 s and 7.5 s for CCK\textsubscript{8} and Tg,
respectively). This difference in rate of Ca\textsuperscript{2+} rise is in agreement with the idea that CCK\textsubscript{8} induces a significantly larger leak than Tg. The down-stroke of the CCK\textsubscript{8}-induced Ca\textsuperscript{2+} transient consisted of a first phase of slow decay and a second phase of fast decay to prestimulatory levels. The rate of Ca\textsuperscript{2+} decay during the second (fast) phase was markedly higher in CCK\textsubscript{8}-stimulated cells (\(\mu\) values of 30-60 s and 200 s for CCK\textsubscript{8} and Tg, respectively). This result is compatible with the idea that in these cells, due to a faster depletion of the ER Ca\textsuperscript{2+} store, no Ca\textsuperscript{2+} is released during the second (fast) phase of Ca\textsuperscript{2+} decay.

The integrated area underneath the cytosolic Ca\textsuperscript{2+} peak was significantly decreased in V38R-Ras-expressing cells as compared to N43R-Ras-expressing cells and GFP\textsuperscript{−} cells. This substantiates our conclusion that V38R-Ras causes a reduction of the ER Ca\textsuperscript{2+} content. Expression of V38R-Ras decreased rather than increased the rate of Ca\textsuperscript{2+} rise during the CCK\textsubscript{8} (100 nM)-induced single Ca\textsuperscript{2+} transient. This apparent paradox can be accounted for if it is assumed that CCK\textsubscript{8} induces a significantly larger leak than V38R-Ras. Because in V38R-Ras-expressing cells the ER Ca\textsuperscript{2+} content is decreased, less Ca\textsuperscript{2+} will flow through the CCK\textsubscript{8}-induced leak. Evidence that the CCK\textsubscript{8}-induced leak is independent of the expression of V38R-Ras is derived from the observation that the decay rate during the second (fast) phase is virtually the same for V38R-Ras-expressing cells, N43R-Ras-expressing cells and GFP\textsuperscript{−} cells.

When added at a 1000-fold lower concentration of 0.1 nM, CCK\textsubscript{8} induced oscillatory changes in Ca\textsuperscript{2+}. This indicates that at this CCK\textsubscript{8} concentration opening of the inositol 1,4,5-trisphosphate-operated Ca\textsuperscript{2+} channels is only transient. The rate of Ca\textsuperscript{2+} rise (\(\tau\) value of 0.4 s) was slower than that obtained with 100 nM CCK\textsubscript{8}. When the channels close, Ca\textsuperscript{2+} is rapidly removed by the concerted action of the SERCA and the PMCA. The Ca\textsuperscript{2+} removal rate (\(\mu\) value of 5.3 s) was markedly faster than that obtained with 100 nM CCK\textsubscript{8}, demonstrating that
under oscillatory conditions Ca$^{2+}$ is largely pumped back into the ER. V38R-Ras expression did not alter the kinetics of the CCK$_8$-induced Ca$^{2+}$ oscillations but significantly reduced their frequency. The lack of effect of V38R-Ras on the rate of Ca$^{2+}$ rise and amplitude of the oscillatory Ca$^{2+}$ rises is most likely explained by the cytosolic Ca$^{2+}$ dependence of the SERCA pumping Ca$^{2+}$ back into the ER at a rate depending on the ambient Ca$^{2+}$ concentration. But, whereas the ER Ca$^{2+}$ content has no effect on the kinetics of the oscillatory Ca$^{2+}$ rises it decreases their frequency (35,36).

Possible implications of the V38R-Ras-induced reduction in ER Ca$^{2+}$ content

The present study provides evidence that V38R-Ras expression reduces the ER Ca$^{2+}$ content by increasing the passive Ca$^{2+}$ leak across the ER membrane. A similar observation was reached following overexpression of the anti-apoptotic protein Bcl-2 in HeLa cells (22,37,38) and HEK-293 cells (21). It was concluded that Bcl-2 exerted its effect by increasing the Ca$^{2+}$ leak rather than decreasing the activity of the ER Ca$^{2+}$ pumps. The data presented in this study provide evidence for a similar mechanism of action of V38R-Ras. The finding that an increase in ER Ca$^{2+}$ content, realized by SERCA overexpression, increased spontaneous apoptosis (39), strengthens the idea that the anti-apoptotic action of Bcl-2 is mediated through its effect on the ER Ca$^{2+}$ content. In this context, the present finding that V38R-Ras decreases the ER Ca$^{2+}$ content provides a good explanation for the anti-apoptotic effect observed with activated mutants of R-Ras under certain experimental conditions (16,17). The latter study provided evidence for the involvement of the phosphatidylinositol 3-kinase pathway in the mechanism of action of R-Ras. Intriguingly, recent studies have implicated this pathway in agonist-induced upregulation of Bcl-2 (40,41) and the caspase inhibitor cIAP-2 (41). Based on these findings, it is tempting to speculate that activation of R-Ras promotes the phosphatidylinositol 3-kinase-mediated upregulation of Bcl-2, which, in turn, causes a decrease in ER Ca$^{2+}$ content by
increasing the ER Ca\(^{2+}\) leak via a hitherto unknown mechanism. However, it should be noted that other studies have shown that R-Ras stimulates the process of apoptosis under conditions where Ras is protective (1,12,13). The present study does not provide an explanation for this pro-apoptotic effect of R-Ras.

Lowering of the ER Ca\(^{2+}\) content has been demonstrated to trigger the process of capacitative Ca\(^{2+}\) entry across the plasma membrane (42). In case the reduced ER Ca\(^{2+}\) content is due to an increased ER Ca\(^{2+}\) leak, this would lead to an elevation of the cytoplasmic Ca\(^{2+}\) concentration. However, in the case of Bcl-2 overexpression it has been demonstrated that the capacitative Ca\(^{2+}\) entry was also down-regulated thus preventing a sustained increase of the resting cytosolic Ca\(^{2+}\) concentration (37).

Possible implications of the V38R-Ras-induced decrease in frequency of stimulus-induced cytosolic Ca\(^{2+}\) oscillations

V38R-Ras expression did not significantly alter the amplitude and duration of the CCK\(_8\)-induced cytosolic Ca\(^{2+}\) oscillations. This means that R-Ras does not signal to its downstream effectors through modulation of the amplitude and/or duration of the cytosolic Ca\(^{2+}\) rises. However, the frequency of the cytosolic Ca\(^{2+}\) oscillations appeared to be reduced by 30% in V38R-Ras-expressing cells. This is in agreement with theoretical studies predicting a decrease in oscillation frequency when the ER Ca\(^{2+}\) content is reduced at a constant inositol 1,4,5-trisphosphate concentration (35,36). In accordance with this idea, measurement of the CCK\(_8\)-stimulated production of inositol 1,4,5-trisphosphate revealed no differences between V38R-Ras-expressing cells and GFP or N43R-Ras-expressing cells. Interference with frequency-encoded Ca\(^{2+}\) signals will lead to altered activation profiles of downstream effectors. Previous work has shown that constitutively active V38R-Ras keeps cellular integrins in an active state thus allowing attachment to surfaces coated with integrin ligands (7). Importantly, the cytoskeletal
reorganizations that occur during integrin-induced cell adhesion are controlled by cytosolic signals that cause periodic activation and inactivation of Rho GTPases. Recent evidence shows that the Ca$^{2+}$-dependent enzyme calpain cleaves RhoA and that cleaved RhoA inhibits integrin-induced stress fiber assembly and cell spreading (19). Because of the Ca$^{2+}$-dependence of calpain and previously reported effects of alterations in intracellular Ca$^{2+}$ concentration on integrin-mediated adhesion (20), it is tempting to speculate that the periodic activation and inactivation of RhoA is regulated by a frequency-encoded cytosolic Ca$^{2+}$ signal. A reduction in frequency of this signal by V38R-Ras might lead to reduced activation of calpain and, as a consequence, reduced cleavage of RhoA. Cell spreading will no longer be inhibited and cell detachment and migration will be inhibited.

In conclusion, the data presented show that activation of R-Ras increases the Ca$^{2+}$ leak across the endoplasmic reticulum membrane thus decreasing both the Ca$^{2+}$ content of this intracellular Ca$^{2+}$ store and, as a consequence, the frequency of the stimulus-induced oscillatory Ca$^{2+}$ rises. We propose that the reduction in endoplasmic reticulum Ca$^{2+}$ content may underly the anti-apoptotic effect of R-Ras described by Suzuki and co-workers (16,17). Furthermore, we propose that the decrease in frequency of the stimulus-induced cytosolic Ca$^{2+}$ rises may inhibit calpain activation, which, in turn, leads to inhibition of cell detachment and migration thus favouring integrin-mediated cell attachment and spreading.
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FIGURE LEGENDS

Figure 1: Co-expression of R-Ras and GFP in CHO cells.

CHO-CCKA cells, co-transfected with GFP and V38R-Ras, were loaded with the fluorescent ratiometric Ca\textsuperscript{2+} dye, indo-1, and imaged at 48 h post-transfection. (A) Fluorescence image recorded at 525 nm (excitation: 488 nm), showing the GFP-positive (GFP\textsuperscript{+}) cells. Dotted lines represent sham-transfected GFP negative (GFP\textsuperscript{-}) cells. (B) Fluorescence image of the same cells at 405 nm (excitation: 351 nm) depicting indo-1-loaded cells. (C) Western blot (representative of three independent experiments) of total cell lysates from GFP\textsuperscript{-} and GFP\textsuperscript{+} cells showing increased expression of R-Ras mutants in GFP\textsuperscript{+} cells. GFP\textsuperscript{-} and GFP\textsuperscript{+} cells were separated by means of fluorescence-activated cell sorting. The 'nt' lane represents total cell lysate from non-transfected cells.

Figure 2: Tg- and CCK\textsubscript{8}-induced single Ca\textsuperscript{2+} transients in V38R-Ras- and N43R-Ras-expressing cells.

CHO-CCKA cells, co-transfected with GFP and V38R-Ras, were loaded with the fluorescent ratiometric Ca\textsuperscript{2+} dye, indo-1, and monitored at 48 h post-transfection by means of high-speed confocal imaging microscopy. The recordings shown are the averages (± SEM) of four GFP\textsuperscript{-} cells and either four N43R-Ras-expressing cells or four V38R-Ras-expressing cells present on the same cover slip. The experiments were performed in the absence of extracellular Ca\textsuperscript{2+}. For clarity, only 120 evenly spaced data-points are displayed. For details, see text and in table 1. (A) Thapsigargin (Tg; 1 μM) evoked a single Ca\textsuperscript{2+} rise the duration of which was decreased in V38R-Ras-expressing cells as compared to the corresponding GFP\textsuperscript{-} cells. (B) V38R-Ras expression enhanced the rate of Ca\textsuperscript{2+} rise during the rising phase of the Ca\textsuperscript{2+} transient. (C) The rate of Ca\textsuperscript{2+} decay was decreased in V38R-Ras-expressing cells. (D) The duration of the CCK\textsubscript{8}-

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induced single Ca$^{2+}$ transient was decreased in V38R-Ras-expressing cells. (E) V38R-Ras expression decreased the rate of Ca$^{2+}$ rise. (F) CCK$_8$ (100 nM) evoked similar Ca$^{2+}$ transients in N43R-Ras-expressing cells and GFP$^-$ cells. (G) N43R-Ras expression did not alter the rate of Ca$^{2+}$ rise. (H) CCK$_8$ (100 nM) completely emptied the intracellular Ca$^{2+}$ store as indicated by the inability of Tg to increase Ca$^{2+}$ in CCK$_8$-stimulated cells.

**Figure 3: CCK$_8$-induced inositol 1,4,5-trisphosphate production in V38R-Ras- and N43R-Ras-expressing cells.**

CHO-CCK$_A$ cells, co-transfected with GFP and either N43R-Ras or V38R-Ras, were cultured for 24 h. GFP$^-$ and GFP$^+$ cells were separated by means of fluorescence-activated cell sorting and cultured for another 24 h. Cells were stimulated with the indicated concentration of CCK$_8$ for 20 s, after which the reaction was quenched by the addition of trichloroacetic acid. The inositol 1,4,5-trisphosphate content of the extract was determined by isotope dilution assay. (A) V38R-Ras expression and (B) N43R-Ras expression did not alter CCK$_8$-induced inositol 1,4,5-trisphosphate production. The data presented are the mean ± SEM of three independent measurements.

**Figure 4: CCK$_8$-induced cytosolic Ca$^{2+}$-oscillations in CHO-CCK$_A$ cells.**

CHO-CCK$_A$ cells, co-transfected with GFP and V38R-Ras, were loaded with the fluorescent ratiometric Ca$^{2+}$ dye, indo-1, and monitored at 48 h post-transfection by means of high-speed confocal imaging microscopy. (A) Superfusion with CCK$_8$ (0.1 nM) was started at the indicated time. The cell was stimulated in the absence of extracellular Ca$^{2+}$. Under this condition, the amplitude of the Ca$^{2+}$ oscillations gradually decreased as a result of store depletion. Eventually, the Ca$^{2+}$ oscillations stopped. (B) The rate of Ca$^{2+}$ increase during the rising phase of the Ca$^{2+}$ oscillation, indicated by the dotted line, was markedly decreased during...
the later peaks (b) as compared to the first peak (a). This decrease is compatible with a decreased Ca$^{2+}$ content of the store. Details on the kinetics of the first Ca$^{2+}$ oscillation are given in the text.

**Figure 5: Frequency of CCK$_8$-induced cytosolic Ca$^{2+}$-oscillations in V38R-Ras- and N43R-Ras-expressing cells.**

CHO-CCK$_A$ cells, co-transfected with GFP and V38R-Ras, were loaded with the fluorescent ratiometric Ca$^{2+}$ dye, fura-2, and monitored at 48 h post-transfection by means of digital imaging microscopy. The cells were incubated in the presence of extracellular Ca$^{2+}$ and stimulated with 0.1 nM CCK$_8$. The figure shows that the oscillation frequency was significantly decreased in V38R-Ras-expressing cells as compared to N43R-Ras-expressing cells and GFP$^-$ cells. No difference was observed between N43R-Ras-expressing cells and GFP$^-$ cells. The data presented are the mean ± SEM of 157 N43R-Ras-expressing cells, 318 V38R-Ras-expressing cells and 307 GFP$^-$ cells. * p< 0.01.
Table 1: Morphology and calcium dynamics in control and R-Ras expressing cells

| PARAMETER                  | CONTROL (GFP⁻) | V38R-Ras (GFP⁺) | N43R-Ras (GFP⁺) |
|----------------------------|----------------|-----------------|-----------------|
| **Cell morphology**        |                |                 |                 |
| GFP intensity (grey-level) | Not detectable | 130±11          | 157±10          |
| Area of cell (µm²)         | 331±13         | 429±46          | 475±25          |
| Formfactor $F$ (A.U.)      | 4.30±0.35      | 2.51±0.2**      | 1.83±0.06**     |
| **Calcium handling**       |                |                 |                 |
| Surface area of transient (A.U.·s) | n.d.          | 187±17          | 133±7**         |
| Width of transient (s)     | 19±2**³        | 110±6           | 75±4**²        |
| Amplitude of transient     | 2.48±0.08      | 3.02±0.10       | 2.8±0.11       |
| N (transients/oscillations) | 16³            | 58              | 44             |

Formfactor $F$ was defined as $\left(\frac{\text{perimeter}^2}{4 \cdot \pi \cdot \text{area}}\right)$ and has a minimal value of one for a perfect circular shape. The amplitude was defined as $\frac{\text{Rmax}}{\text{Ro}}$ with $\text{Ro}$ and $\text{Rmax}$ being the pre-stimulatory and maximal indo-1 ratio respectively. **Legend:** ³only first transient, ⁴compared to control, ⁵compared to V38-Ras cells, ⁶compared to N43-Ras cells, ⁷compared to control cells treated with 100 nM CCK₈, *: p<0.05, **: p<0.01, ***: p<0.001.
Koopman et al., Fig.1
A

V38

pmol IP₃/mg protein

log [CCK₈] (M)

GFP-positive

GFP-negative

B

N43

pmol IP₃/mg protein

log [CCK₈] (M)

Koopman et al., Fig.3
Figure 5: Comparison of oscillation frequency (1/min+SEM) among control, V38, and N43 conditions. The graph shows a significant difference between V38 and N43 conditions. 

Koopman et al., Fig.5
R-RAS alters CA2+ homeostasis by increasing the CA2+ leak across the endoplasmic reticular membrane

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