Stimulation of K⁺ Transport Systems by Ha-ras*

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The expression of Ha-ras in quiescent NIH3T3 cells carrying a glucocorticoid-inducible human Ha-ras gene (Val-Gly mutation at codon 12) stimulates total ⁸⁶Rb⁺ influx. This effect is predominantly due to an elevated ⁸⁶Rb⁺ uptake through an ouabain-resistant, furosemide-sensitive system. The ouabain-sensitive Na⁺/K⁺-ATPase is less affected. The transport which is resistant to both inhibitors is not altered by Ha-ras. Overexpression of the Ha-ras proto-oncogene causes only a marginal increase in total ⁸⁶Rb⁺ uptake. The stimulation of the furosemide-sensitive influx by Ha-ras is paralleled by an increase in mean cell volume which can be inhibited by furosemide. A rapid stimulation of the furosemide-sensitive Rb⁺ influx is also observed after addition of bombesin to growth-arrested cells. Furosemide inhibits the mitogenic response after expression of Ha-ras or addition of bombesin. Both the Ha-ras and the bombesin-induced stimulation of the furosemide-sensitive Rb⁺ transport can be blocked by protein kinase C depletion or the protein kinase C inhibitor staurosporine. In contrast to bombesin-induced phosphorylilinositol-4,5-bisphosphate hydrolysis which is down-modulated by Ha-ras, the stimulation of the furosemide-sensitive Rb⁺ influx by bombesin is elevated in Ha-ras-expressing cells. This is in accordance with the increased mitogenic activity of bombesin in Ha-ras-expressing cells.

An increased flux of K⁺ ions through the plasma membrane is one of the earliest events after stimulation of quiescent cells by most mitogens (1–5). The elevated K⁺ influx has been attributed to an activation of the Na⁺/K⁺-ATPase (3, 6). However, K⁺ may be taken up by a variety of mechanisms. Besides the ouabain-sensitive Na⁺/K⁺-ATPase, many cell types employ an additional ouabain-resistant, furosemide-sensitive K⁺ transport system (4, 7–12). The K⁺ influx by the furosemide-sensitive system was shown to be coupled to influxes of Na⁺ and Cl⁻ with a Na⁺:K⁺:Cl⁻ ratio of 1:1:2 (4, 10–13). This Na⁺/K⁺/2Cl⁻ cotransporter also seems to be involved in mitogenic signal transduction. This supposition is based on observations indicating a dramatic stimulation of the furosemide-sensitive K⁺ influx after addition of serum growth factors to growth-arrested NIH3T3 fibroblasts (5, 14, 15). Furthermore, a modulation of the cotransporter by TPA¹ has been reported as a requirement for the growth-promoting action of phorbol esters (16, 17).

Expression of transforming Ha-ras in growth-arrested fibroblasts has been shown to result in growth factor-independent cellular replication (18–20), an elevation of inositol phosphate formation (21–24), an activation of protein kinase C (25, 26), and a stimulation of Na⁺/H⁺ antiporter (20, 27, 28). Although none of these effects is ras-specific, they are consistent with a model in which Ha-ras, by a still unknown mechanism, causes a constitutive activation of a mitogenic signaling system. In view of the putative role of K⁺ transport systems in growth factor signal transduction, it seemed interesting to investigate whether any of them is involved in the mitogenic signaling initiated by Ha-ras. Results of these studies may contribute to a more detailed identification of the mitogenic pathway employing p21ras.

This paper demonstrates that expression of a transforming Ha-ras causes an increase in the influx of ⁸⁶Rb⁺ which is in part caused by stimulation of the Na⁺/K⁺-ATPase but is predominantly due to an activation of the furosemide-sensitive cotransporter. The activation of the cotransporter is paralleled by an increase in cell volume.

EXPERIMENTAL PROCEDURES

Materials—TPA, leupeptin, pepstatin, dexamethasone, furosemide, ouabain, and bombesin were purchased from Sigma, and staurosporine was from Boehringer Mannheim. ⁸⁶RbCl (1 μCi/ml) was from Du Pont-New England Nuclear. [⁶⁻¹⁴C]Thymidine (25 Ci/mmol), [³²P]labeled protein A, and autoradiography films (Hyperfilm-MP) were from Amersham Corp.

Cell Culture—NIH3T3 fibroblasts were transfected with the transforming human Ha-ras oncogene or the Ha-ras proto-oncogene subjected to the transcriptional regulation by glucocorticoids by in vitro recombination with the mouse mammary tumor virus long terminal repeat as described previously (30). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum in the presence of 5% CO₂.

¹⁸⁶Rb⁺ Uptake Measurement: Basic Procedure—Fibroblasts were seeded onto 35-mm culture dishes (6-well plates, Falcon) at a density of 0.8–1.5 X 10⁴ cells/dish. One day after plating, the medium was replaced by Dulbecco’s modified Eagle’s medium containing 0.5% fetal calf serum. Quiescent subconfluent cultures were obtained under these conditions after 48 h. The expression of the Ha-ras oncogene or the Ha-ras proto-oncogene was induced by addition of 1 μM dexamethasone (final concentration). When indicated, cells were preincubated for 2 min with ouabain (2 mM) and/or furosemide (1 mM). Then the medium was removed, and the ⁸⁶Rb⁺ uptake experiments were started by adding 1 ml of prewarmed (37 ℃) assay buffer A (buffer A; 10 mM HEPES, 140 mM NaCl, 5.0 mM KCl, 1.0 mM CaCl₂, 0.5 mM MgCl₂, pH 7.4, 0.8–2.0 μCi or ⁸⁶Rb⁺/ml, supplemented with 0.3 mM furosemide and/or 2 mM ouabain if indicated). After an incubation period of 2, 4, or 6 min, the ⁸⁶Rb⁺ uptake was terminated by sucking off the assay buffer A and rinsing

The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 4-morpholinopropanesulfonic acid.

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the monolayer two times with ice-cold buffer B (buffer B: 100 mM MgCl₂, 5 mM MOPS, pH 7.4) to remove the extracellular isotope, followed by a final wash procedure with phosphate-buffered saline (PBS: 140 mM NaCl, 2.7 mM KCl, 4.6 mM Na₂HPO₄, 1.7 mM Na-H₂PO₄, pH 7.4). After the last wash solution was removed, the cell layer was rinsed with 1 ml of ice-cold buffer B (buffer B: 100 mM Tris/HCl, 5 mM EDTA, 10 mM MgCl₂, 5 mM MOPS, pH 7.4) to remove the suspension. 0.2 ml of this suspension was used to determine the cell number using a Coulter counter. The residual part was added to 10 ml of scintillation liquid and counted for "Rb" radioactivity using a liquid scintillation counter (Beckman LS 3801). "Rb" uptake rates are expressed as dpm/min/10⁶ cells, calculated from the regression line for the 30-s measured data points corresponding to 2-, 4-, and 6-min incubation times. "Rb" uptake measurements were limited to early, linear ranges (≤5 min; the linear range was up to 10 min (data not shown)). Identical results for "Rb" uptake were obtained when the experiments were performed in culture medium instead of buffer A (data not shown), which makes it reliable to have physiological conditions in the uptake assay.

"Rb" Uptake after Growth Factor Stimulation by Bombesin—Cells were prepared for "Rb" uptake as described above. Bombesin (10 μM, final concentration) was added to buffer A for the times indicated, and "Rb" was present during the last 2 min of the assay. "Rb" uptake rates were expressed as dpm/min/10⁶ cells, calculated from the 2-min "Rb" uptake period.

Cell Number and Cell Volume Measurements—Cell numbers and cell volumes were measured by cell sizing using a Coulter counter (model 2M, Coulter Electronics, Luton, United Kingdom) adapted with a Coulter Channelyzer (Coulter model 5-plus calibration unit). Fibroblasts were seeded onto 35-mm dishes and dishes were cultured on tissue culture plastic until 70% confluent (Falcon) at a density of 0.8-1.5×10⁵ cells/dish. One day after plating, the medium was replaced with Dulbecco's modified Eagle's medium containing 0.5% fetal calf serum. Quiescent subconfluent cultures were obtained after 48 h of starvation. After removing the medium, the cells were collected by a 10-min incubation in PBS supplemented with 0.1% trypsin/EDTA. Subsequently, the cell suspension was homogenized, and the cells were kept in PBS (25°C) for 10 min before starting the volume analysis. The mean cell volume was calculated from the cell volume distribution curves. During the measurements, cells were kept in PBS at 25°C. Absolute cell volumes were obtained using latex beads (8.7 μm diameter, Coulter Electronics) for calibration.

[3H]Thymidine Incorporation Experiments—NIH3T3 fibroblasts transfected with the transforming Ha-ras oncogene were plated as described above in 8.5-cm dishes (Falcon) at a density of approximately 3×10⁵ cells/dish. One day after plating, cells were growth-arrested by incubation in low-serum (0.5%) medium for at least 48 h. According to the times as indicated, 5 μM bombesin (final concentration), 1 μM dexamethasone (final concentration), or a combination of dexamethasone and bombesin was added. DNA synthesis was measured by adding [3H]thymidine (10 μCi/ml) to the medium for the last 3 h of the incubation period. At the end of the labeling period, the medium was removed and the cells were rinsed twice with 1 ml of ice-cold PBS and harvested by treatment with trypsin. Aliquots of 300 μl of homogenates were transferred to 96-well filter plates (multiscreen HA, Millipore) and collected under a vacuum. The cells were precipitated by addition of 250 μl of 5% trichloroacetic acid. The filter discs were punched out using a filter punch apparatus (Millipore), transferred into scintillation vials containing 4 ml of scintillation liquid, and counted for H-radioactivity in a liquid scintillation counter (Beckman LS 3801). The cell numbers were determined with an electronic counter (Coulter Electronics).

Western Blot Analysis—Fibroblasts were grown as described above in 8.5-cm dishes (Falcon) to a density of approximately 1.5-2×10⁶ cells/dish. Oncogene expression was induced by 1 μM dexamethasone 1, 3, or 5 h before harvest. At each time indicated, cells from 10 dishes were collected for the preparation of membrane extracts. The cells were swollen for 10 min in a lysis buffer containing 10 mM Tris/HCl, 5 mM EDTA, 10 μM leupeptin, and 10 mM pepstatin before homogenisation with 15 strokes in a tight-fitting glass Potter. Nuclei were pelleted at 3000 × g, and the crude membrane preparation was finished by centrifugation of the resulting supernatant for 1 h at 100,000 × g. Homogenization of the membrane pellets in lysis buffer was done by sonification for 10 s with a Branson Sonifier at 25 watts. Protein content of the samples was determined as described previously. Equal amounts of protein were loaded onto 12% SDS/PAGE (5%) gels and electrophoretically separated on SDS-15% polyacrylamide gel 16-cm gels according to Laemmli (24). Subsequent electrophoretic transfer of proteins was done on Immobilon polyvinilidene difluoride transfer membranes (Millipore, Bedford, MA) according to Pefersen (45) in a Bio-Rad Trans-Blot system for 90 min at a constant current of 3 mA/cm² gel. After the transfer, p21 protein was immuno-detected by exposure to rabbit polyclonal p21-ras antibody with subsequent treatment with 125I-labeled protein A (Amersham Corp.) and autoradiography at −70°C for 24 h on Hyperfilm-MP (Amersham Corp.).

RESULTS

Addition of dexamethasone to NIH3T3 cells transfected with a mouse mammary tumor virus long terminal repeat Ha-ras construct leads to an induction of p21 expression as detected by Western blot analysis within 3 h after addition of the hormone (Fig. 1). Some biological consequences of p21 accumulation in this cell system have been described previously (20, 29). The effect of Ha-ras on K⁺ influx measured by the uptake of the K⁺ congener "Rb" is shown in Fig. 2, a-c. "Rb" influx in growth-arrested NIH3T3 cells occurs by two saturable major transport systems. Depending on the cell lines studied, 30-60% of the total Rb⁺ influx can be inhibited by ouabain and 45-60% by furosemide. Maximal inhibitory concentrations were found to be 2 mM for ouabain and 0.3 mM for furosemide (data not shown). In the presence of both inhibitors, a leak flux of approximately 9-12% can be registered. Expression of the transforming Ha-ras by 1 μM dexamethasone causes an approximately 2-fold stimulation of total Rb⁺ influx activity. This effect is predominantly due to a 3.7-fold increase in the activity of the ouabain-resistant transport system. As a combination of ouabain plus furosemide depresses the Rb⁺ influx by 90% to the level of the prestimulatory leak flux, it can be concluded that the ouabain-resistant fraction represents the furosemide-sensitive flux plus the leak flux. Since the leak flux is unchanged, the elevated level of the ouabain-resistant Rb⁺ influx is made up by the furosemide-sensitive uptake.

The expression of the Ha-ras proto-oncogene by dexamethasone leads to a similar but significantly smaller effect on Rb⁺ influx. Addition of the same dexamethasone concentration to growth-arrested nontransfected NIH3T3 cells results in a small reduction of the Rb⁺ influx. The data of Fig. 2, a-c demonstrate a Ha-ras-induced stimulation of K⁺ influx which is predominantly due to an activation of the furosemide-sensitive Na⁺/K⁺/2Cl⁻ cotransporter and significantly stronger under the influence of the transforming Ha-ras than under the influence of the proto-oncogene. The difference between cells expressing the transforming Ha-ras and those expressing the proto-oncogene cannot be explained by different levels of p21 accumulation. As can be seen in Fig. 1, the proto-oncogene product accumulates to higher levels than the transforming p21. This has been reported to be a result of a negative effect of the transforming but not the normal Ha-ras on the transcription of the Ha-ras constructs (30).

![Fig. 1. Accumulation of p21 in Ha-ras-transfected NIH3T3 cells.](image-url)
Fig. 2. $^{86}$Rb$^+$ uptake by growth-arrested NIH3T3 fibroblasts. A, NIH3T3 control cells; B, NIH3T3 cells transfected with the Ha-ras proto-oncogene; C, NIH3T3 cells transfected with the transforming Ha-ras oncogene. Cells were grown, prepared, and assayed for $^{86}$Rb$^+$ uptake experiments as described under "Experimental Procedures." Where indicated, the proto-oncogene or the transforming oncogene was induced by addition of 1 μM dexamethasone (dex). The $^{86}$Rb$^+$ influx in the absence (○) or the presence (●) of 1 mM furosemide is shown. Data represent the mean of at least five independent determinations or the mean ± S.E. (n = 5).

Fig. 3 shows the stimulation of the Rb$^+$ uptake as a function of time after addition of dexamethasone to cells containing the transforming Ha-ras. A significant activation is observed 3 h after administration of the hormone. This is in reasonable agreement with the appearance of immunologically detectable p21$^{ras}$ (Fig. 1).

The exact biological role of the furosemide-sensitive K$^+$ transport system is unknown. Evidence supporting a role of this system in cell volume regulation has been presented previously (10, 31–33). It was investigated, therefore, whether the ras-induced stimulation of cotransport activity is accompanied by cell volume alterations. Fig. 4 demonstrates that this is indeed the case. Mean cell volume of G0-arrested cells increases 3 h following induction of p21$^{ras}$ by dexamethasone. This volume change can be completely blocked by furosemide, thus indicating that the activation of the furosemide-sensitive system is responsible for the effect on cell volume.

A stimulation of the cotransporter has been reported to occur within 2 min after addition of serum growth factors to quiescent NIH3T3 cells (5). Fig. 5 exhibits similar kinetics following addition of bombesin to Ha-ras-transfected fibroblasts, suggesting an implication of this system in mitogenic signaling. In order to study the role of protein kinase C in the growth factor-mediated stimulation of the cotransporter, protein kinase C-depleted cells produced by a prolonged exposure to TPA were employed. Previous experiments had indicated that after a 6-h treatment with 150 nM TPA, protein kinase
Cotransporter activity between Ha-ras-expressing and non-expressing cells is completely eliminated (Fig. 5A). These data indicate that the activation of the cotransporter by bombesin, as well as by Ha-ras, is mediated by protein kinase C.

The data from Fig. 5 also demonstrate that expression of the transforming Ha-ras does not down-modulate the response of the cotransporter to bombesin. This is noteworthy as Ha-ras depresses the inositol phosphate and Ca²⁺ responses to bombesin in these cells, a phenomenon which has also been observed after stimulation by platelet-derived growth factor or serum growth factors (22, 34–36). The mitogenic response to bombesin, however, like that of the cotransporter, is still present in Ha-ras-expressing cells and adds to the mitogenic activity of Ha-ras (Fig. 6).

In order to investigate whether the activation of the furosemide-sensitive K⁺ transport is essential for the mitogenic response, the effect of furosemide on the stimulation of cell proliferation by bombesin or Ha-ras was determined. The studies (Fig. 7) revealed that concentrations of furosemide which block the activity of the cotransporter depress Ha-ras- or bombesin-induced cellular replication. These data demonstrate that the stimulation of the Na⁺/K⁺/2Cl⁻ cotransporter is essential for the mitogenic response to Ha-ras or bombesin. If this conclusion is correct, a removal of external K⁺ should eliminate the growth-promoting effects of Ha-ras or bombesin. This is indeed the case (data not shown). However, the cells die after 3–4 h in a K⁺-free isotonic medium, probably because several other vital K⁺-dependent functions including the Na⁺/K⁺-ATPase are impaired. Therefore, the furosemide sensitivity of the mitogenic response represents a more specific marker for the biological significance of the cotransporter than removal of extracellular K⁺.
Expression of the transforming Ha-ras has been shown to cause an activation of the Na$^+$/H$^+$ antiporter (20, 27, 28). Considering this effect, a secondary stimulation of the Na$^+$/K$^+$-ATPase as a result of the increased Na$^+$ influx might have been expected (1–3). The surprising result of the studies presented here is the predominant Rb$^+$ influx through the furosemide-sensitive cotransporter in response to Ha-ras expression. The activation of the furosemide-sensitive transport significantly exceeds the Rb$^+$ influx by the ouabain-sensitive Na$^+$/K$^+$ pump. This furosemide-sensitive K$^+$ transport system has been shown by others to represent a Na$^+$/K$^+$/2Cl$^-$ cotransporter (4, 10–13). The exact biological function of the cotransporter is not clear. Available evidence supports a role of this system in cell volume control (10, 31–33). In accordance with this assumption, the stimulation of the furosemide-sensitive Rb$^+$ influx by Ha-ras is paralleled by a furosemide-sensitive increase in mean cell volume. Whether the alteration in cell volume is merely a byproduct of the increase in cytosolic ion concentration or whether the volume change exerts signaling function by itself cannot be decided yet. Evidence for a stimulation of inositol phosphate formation in response to swelling has been presented previously (37). The data shown here indicate that the activation of the furosemide-sensitive K$^+$ transport by Ha-ras is a protein kinase C-mediated effect. Ha-ras has been shown to activate protein kinase C (25, 26, 28). This process seems to be essential for the stimulation of cellular replication by the oncogene (38–40) and probably utilizes diacylglycerol to activate protein kinase C which is not generated by phosphoinositide hydrolysis (41–42). Thus, the activation of the cotransporter seems to be implicated in an established mitogenic signaling system initiated by Ha-ras. This conclusion is in agreement with observations published by others (5, 14–17) which support the notion that the activation of the furosemide-sensitive cotransporter is involved in mitogenic signal transduction. This suppression is further supported by the observation that furosemide is capable of inhibiting the mitogenic response following expression of the transforming Ha-ras or addition of bombesin. The fact that the transforming p21ras is remarkably more effective in stimulating the cotransporter than overexpression of the ras protein is not generated by phosphoinositide C-independent mitogenic signaling system for bombesin which is sensitized by Ha-ras remains to be seen.

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