Ha-ras Activates the Na⁺/H⁺ Antiporter by a Protein Kinase C-independent Mechanism*

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In quiescent Ha-ras-transfected NIH 3T3 cells, addition of serum growth factors, bombesin or 12-O-tetradecanoylphorbol-13-acetate (TPA) leads to a dimethylamiloride-sensitive intracellular alkalization which can be inhibited by staurosporine, a potent inhibitor of protein kinase C. Expression of the transforming Ha-ras gene causes a growth factor-independent increase in cytoplasmic pH. This Ha-ras-induced alkalization is sensitive to dimethylamiloride but is not affected by staurosporine concentrations which prevent the pH response after addition of growth factors or TPA. Protein kinase C depletion by long term exposure to TPA eliminates the pH response to bombesin and phorbol ester but does not affect the Ha-ras-induced intracellular alkalization. It is concluded that expression of Ha-ras causes an activation of the Na⁺/H⁺ antiporter by an as yet unknown protein kinase C-independent mechanism.

In previous publications it has been demonstrated that expression of a transforming Ha-ras oncogene, not however, of the corresponding protooncogene, leads to a growth factor-independent activation of the Na⁺/H⁺ antiporter in NIH 3T3 fibroblasts (1, 2). Similar effects have been observed by others following microinjection of p21⁰ (3). Stimulation of the Na⁺/H⁺ antiporter and the resulting cytosolic alkalization can be observed under the influence of many growth factors and mitogenic agents and is considered as either essential or at least permissive for the proliferative response (for reviews, see Refs. 4 and 5). In a variety of systems, the stimulation of the Na⁺/H⁺ antiporter is mediated via protein kinase C (4, 5). This seems to be the case for growth factors which act through a stimulation of a phospholipase C, catalyzing the hydrolysis of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphate and diacylglycerol. The primary effect of diacylglycerol is considered to be the activation of protein kinase C (for a review, see Ref. 6). In the system described here, expression of the transforming Ha-ras leads to an increased formation of inositol phosphates (2, 7) and phospatic acid (8) suggesting an elevated generation of diacylglycerol which in turn should stimulate protein kinase C. Direct evidence for an activation of protein kinase C in Ha-ras-transformed cells has been described (9, 10). Thus, available evidence is consistent with the supposition that the stimulation of the Na⁺/H⁺ antiporter by the transforming Ha-ras is mediated through protein kinase C. However, the data presented here demonstrate that the Ha-ras-induced activation of the Na⁺/H⁺ antiporter is insensitive to staurosporine, a potent inhibitor of protein kinase C (11) and occurs in protein kinase C-depleted cells with the same efficiency as in undepleted controls.

**EXPERIMENTAL PROCEDURES**

**Materials—**2',7'-Bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)⁰ is a product from Molecular Probes (Eugene, OR); 5-N',N-Dimethylamiloride was a gift from Merck, Sharp & Dohme (Vienna, Austria). Nigericin, TPA, and bombesin were purchased from Sigma Chemicals, Munich, Germany. Staurosporine was donated by Boehringer-Mannheim, Germany. [γ-³²P]ATP was from Amersham Corp. (Vienna, Austria).

**Cell Culture—**NIH 3T3 fibroblasts were transfected with the transforming human Ha-ras oncogene subjected to transcriptional regulation by glucocorticoids by in vitro recombination with the MMTV-LTR as described by Jaggi et al. (12). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) in the presence of 5% CO₂. One day after plating, cells were made quiescent by incubation in DMEM containing 0.5% FCS for 24-48 h.

**Determination of the Cytosolic pH—**NIH 3T3 cells were grown on coverslips (9 × 18 mm). One day after plating, cells were growth arrested by incubating in low serum (0.5%) medium for at least 48 h. At this time point average cell count per coverslip was 0.5-1 × 10⁶ cells. pH (intracellular pH) was determined by fluorescence spectrophotometry employing BCECF. Fibroblasts attached to rectangular coverslips were washed in a HCO₃⁻-free HEPES-buffered saline (HBS) (140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 5.5 mM glucose, 20 mM HEPES/NaOH, pH 7.4). The cells were loaded with BCECF by incubation in HBS containing 2.5 µM BCECF acetoxymethylster for 10 min at 37 °C and subsequent washing (two times) with HBS. For fluorescence measurements one coverslip supporting the cells was placed into a 1 × 1 × 3-cm quartz cuvette containing 2 µl of HBS at 37 °C. The coverslip in the cuvette was oriented at a 90° angle relative to the excitation beam, while 0.6 cm² of the coverslip was illuminated. Fluorescence (excitation, 502 or 440 nm; emission, 530 nm) was detected with a SPECT (Edison, NJ) Fluorolog 2 spectrofluorometer (CM-1) equipped with two excited monochrometers and a chopper system. The cytoplasmic pH, values were calculated from the ratio of the fluorescence intensities I₅₀₃/₅₃₀ nm. At the end of each experiment the ratio of the fluorescence intensities I₅₃₀/₅₀₃ nm was calibrated to pH by using a nigericin calibration procedure as described (28), employing a buffer consisting of

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¹The abbreviations used are: BCECF, 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; MMTV-LTR, mouse mammary tumor virus long terminal repeat; TPA, 12-O-tetradecanoylphorbol-13-acetate; EGTA, (ethylene)bis(oxyethyl)tetraacetic acid; HBS, HEPES-buffered saline.
140 mM KCl, 10 mM NaCl, 20 mM Hepes/KOH, 10 μg/ml nigericin.

**Determination of Protein Kinase C Activity**—Protein kinase C was partially purified from NIH 3T3 cells by employing DEAE-cellulose chromatography according to a modified method described by Walton et al. (29). Protein was eluted with a gradient from 0.0 to 0.4 M NaCl in elution buffer (20 mM Tris/HCl, 20 mM mercaptoethanol, 0.1 mM EGTA, 0.1 mM EDTA, 2% glycerol, pH 7.4).

Protein kinase C activity was determined by measuring 32P incorporation from [γ-32P]ATP into H1 histone according to Fabbro et al. (26). Briefly, the reaction mixture (125 μl) contained 0.133 μM [γ-32P]ATP (30 Ci/mmol), 40 mM Tris/HCl, pH 7.4, 1 mM CaCl2, 700 μM EGTA, 50 μg of histone, 8 μg/ml L-α-phosphoryl-L-serine, and 0.8 μg/ml 1,2-dioctanoyl-rac-glycerol (CR80). The reaction time was 15 min at 32°C. The reaction was stopped by addition of 1 ml of 20% trichloroacetic acid. Protein was precipitated on Whatman GF/F filters and counted in a liquid scintillation counter. Where indicated, protein kinase C was partially purified by employing DEAE-cellulose chromatography of cell extracts according to Kreutter et al. (27). DNA was partially purified and determined as described under “Experimental Procedures.”

**RESULTS**

Addition of dexamethasone to quiescent NIH 3T3 cells transfected with a MMTV-LTR Ha-ras construct leads to a rapid induction of LTR-ras-transcription. The accumulation of p21<sup>ras</sup> is detectable within 1 h after addition of the glucocorticoid and reaches a maximum after 24 h of ~21<sup>ras</sup> is detectable within 1 h after addition of the glucocorticoid and reaches a maximum after 24 h (12). As shown in Fig. 1, expression of Ha-ras causes a progressive cytosolic alkalinization which can be blocked by dimethylamiloride (DMA), an inhibitor of the Na<sup>+</sup>/H<sup>+</sup> antiporter. A DMA-sensitive cytosolic alkalinization is also seen after addition of serum growth factors, bombesin or TPA to quiescent 3T3 cells (Fig. 3). The data indicate that Ha-ras causes a growth factor-independent activation of the Na<sup>+</sup>/H<sup>+</sup> antiporter.

**Fig. 1.** Effect of Ha-ras expression on intracellular pH.

Cells were growth-arrested by 24-h incubation in DMEM containing 0.5% FCS. Oncogene expression was induced by addition of 1 μM dexamethasone at time zero. Where indicated, DMA was added to a final concentration of 50 μM. Bars indicate means ± S.E. (n ≥ 6).

**Fig. 2.** Inhibition of protein kinase C from Ha-ras-transfected NIH 3T3 cells by staurosporine. Protein kinase C (PKC) was partially purified and determined as described under “Experimental Procedures.”

**Fig. 3.** Effect of staurosporine on intracellular alkalinization by Ha-ras, fetal calf serum, bombesin, or TPA. Cells were growth arrested by incubation at 0.5% FCS as described under “Experimental Procedures.” Where indicated, FCS (5%), bombesin (5 μM), or TPA (0.5 μM) were added. Ha-ras was expressed by addition of 1 μM dexamethasone. In all cases pH<sub>i</sub> increase was recorded until a new steady state level was obtained, i.e. 0.1–5 min after serum, bombesin, or TPA stimulation. pH<sub>i</sub> response after Ha-ras expression was determined 26 h after addition of dexamethasone. Data represent means ± S.E. (n ≥ 10). Addition of 50 μM (final concentration) DMA completely blocks alkalinization in all cases. The values obtained for the combination FCS + DMA are shown. Identical results are obtained for combinations of bombesin, TPA, or dexamethasone with DMA (not shown). Where indicated, staurosporine was added at a final concentration of 2.5 nM 1 h before stimulation by serum, bombesin, TPA, or dexamethasone. Left to right: Control, FCS, addition of 5% FCS; Stauro. (1 h) + FCS, addition of 2.5 nM staurosporine + 5% FCS; DMA + FCS, addition of 5% FCS + 50 μM DMA; Bombesin, addition of 5 μM bombesin; Stauro. (1 h) + Bomb., addition of 2.5 nM staurosporine + 5 μM bombesin; TPA, addition of 0.5 μM TPA; Stauro. (1 h) + TPA, addition of 2.5 nM staurosporine + 0.5 μM TPA; Dexamethasone, Ha-ras expression by 1 μM dexamethasone; Stauro. + Dexamethasone, staurosporine + dexamethasone; Stauro. (24 h) + TPA, staurosporine at time zero, TPA after 24 h.

**Fig. 4.** Protein kinase C depletion by pretreatment with TPA. Cells were preincubated with 0.25 μM TPA for 24 h. Cell extracts were prepared and further fractionated by chromatography on DEAE-cellulose as described under “Experimental Procedures.” The fraction numbers 1–9 represent the NaCl concentrations of 0.04, 0.07, 0.09, 0.11, 0.12, 0.14, 0.17, 0.24, and 0.4 M. The figure shows the elution profile of protein kinase C activities from controls (C) or TPA-pretreated cells (B).
pHi was determined 20 h after administration of the hormone. Data recorded. Ha-ras was expressed by addition of 1 nM TPA, addition of 0.5 μM bombesin in protein kinase C-depleted cells; TPA, addition of 1 mM dexamethasone to reflect transformation-linked metabolic alterations which may be responsible for the activation of protein kinase C by Ha-ras are not ras-specific phenomena but seem to reflect transformation-linked metabolic alterations which are produced by other oncogenes like v-mos as well (7, 25). Obviously, Ha-ras activates an additional, still unknown mechanism to stimulate the Na+/H+ antiporter. The elucidation of this mechanism may reveal early ras-initiated signals which have not been considered so far and which may be relevant for the stimulation of cellular repulsion or transformation by this oncogene.

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