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Interference of Ha-*ras* with inositol trisphosphate-mediated Ca^{2+}-release

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Expression of a transforming Ha-*ras* by dexamethasone in NIH3T3 cells transfected with a glucocorticoid-inducible Ha-*ras* construct results in a rapid desensitization of the intracellular Ca^{2+}-mobilizing system to bombesin. This effect precedes the down-modulation of inositol trisphosphate (IP_3) formation by several hours and is, therefore, not explained by an uncoupling of phosphoinositidase C. It is demonstrated that expression of Ha-*ras* attenuates the Ca^{2+}-release by IP_3 in permeabilized cells. The IP_3 concentration required for half-maximal Ca^{2+}-release is doubled in Ha-*ras* expressing cells. Maximal Ca^{2+}-release which is obtained with 2 μM IP_3 in control cells requires 10 μM IP_3 in cells expressing Ha-*ras*. The desensitization of the IP_3 receptors coincides with the desensitization of the Ca^{2+}-mobilizing system to bombesin. The results indicate that the Ha-*ras* mediated desensitization of the Ca^{2+}-releasing system to bombesin is – at least in part – caused by a decrease in the affinity of the IP_3 receptor to inositol trisphosphate.

NIH3T3 fibroblast; Harvey-*ras*; Calcium; Inositolphosphate; Bombesin

1. INTRODUCTION

Expression of a transforming Ha-*ras* has been shown to desensitize the intracellular Ca^{2+}-mobilizing system to serum growth factors or bombesin [1,2]. This phenomenon proved to be specific for transforming *ras* and was not observed in cells overexpressing the Ha-*ras* proto-oncogene [1]. Transforming *ras* genes have been shown to reduce the inositol phosphate generation in response to platelet-derived growth factor (PDGF) or prostaglandin $F_{2\alpha}$ and this effect has been attributed to a protein kinase C-mediated uncoupling of phosphoinositide hydrolysis by Ha-*ras* [3].

We have recently demonstrated, however, that the depression by Ha-*ras* of the bombesin-induced IP_3 response is not correlated to the down-modulation of the Ca^{2+} signal [4]. This was based on the finding that after expression of Ha-*ras* in a Ha-*ras* inducible system, the Ca^{2+}-mobilization is already maximally suppressed when the IP_3-response is still unchanged. Down-modulation of the IP_3 response was found to be a relatively late phenomenon occurring 3–4 h later than the depression of the Ca^{2+} signal. In view of these data it was postulated that Ha-*ras* interferes with IP_3-mediated Ca^{2+}-release at the level of the IP_3 receptor. This assumption was supported by data demonstrating that IP_3-mediated Ca^{2+}-release in permeabilized cells is indeed attenuated in cells expressing Ha-*ras* [4]. These findings, however, are in contrast to observations reported by Olinger et al. [5] who did not observe an inhibition by EJ-*ras* of the IP_3-mediated Ca^{2+}-release after microinjection of IP_3. Although the system employed by Olinger et al. differs in various aspects from the system employed by us, we considered this discrepancy unsatisfying and decided to study the IP_3-mediated Ca^{2+}-release as a function of the IP_3 concentration in order to get some information whether Ha-*ras* affects the affinity of the receptor to IP_3. Such a mechanism would explain the differences if Olinger et al. employed saturating IP_3 concentrations whereas non-saturating IP_3 concentrations might have been used in our studies.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; Dex, dexamethasone; EGTA, [ethylene-bis-(oxyethylene-nitrilo)]tetraacetic acid; FCS, fetal calf serum; PBS, Phosphate buffered saline; HBS, HEPES-buffered saline, HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; IP_3, inositol-1,4,5-trisphosphate; MMTV-LTR, mouse mammary tumor virus long terminal repeat.

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2. MATERIALS AND METHODS

2.1. *Materials*

1,4,5-Inositoltrisphosphate, dexamethasone, Na_3VO_4, saponin and bombesin were purchased from Sigma Chemicals, Munich, Germany. Myo-[2-^{3}H]inositol (12.8 Ci/mmol) was from New England Nuclear, Dreieich, Germany. $^{45}Ca^{2+}$ (10–40 mCi/mg) from Amersham, UK. Fura-2 was purchased from Molecular Probes, Eugene, OR. Inositol-free DMEM was from Amimed, Basel, Switzerland.

2.2. *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 MMTV-LTR as described [6]. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS in the presence of 5% CO_2. One day after plating, cells were made quiescent by incubation in inositol-free DMEM containing 0.5% FCS (in the case of

inositolphosphate measurement, see below) or DMEM plus 0.5% FCS (for Ca^{2+} determinations) for 48 h.

2.3. *Determination of cytosolic Ca^{2+}-concentrations*

$[Ca^{2+}]_i$ was determined by fluorescence spectrophotometry employing fura-2 as described [1,9].

2.4. *Measurements of $^{45}Ca^{2+}$-uptake and IP_3-induced Ca^{2+}-release in permeabilized cells*

NIH3T3 cells, transfected with the transforming Ha-*ras* were grown in 35-mm culture dishes (6-well plates) at a density of $1.4–1.8\times10^5$ cells per dish. Where indicated, Ha-*ras* expression was induced by 1 μM dexamethasone for 2 or 24 h. $^{45}Ca^{2+}$-uptake and IP_3-induced Ca^{2+}-release experiments were performed as described [4]. At first the medium was removed and the cells were washed with 1 ml buffer A (buffer A: 20 mM NaCl, 100 mM KCl, 5 mM $MgSO_4$, 1 mM Na_2HPO_4, 25 mM HEPES, 1% BSA, pH 7.2). After a preincubation period of 10 min in buffer A in the presence of 1 mM EGTA, the experiment was started by incubation of the cells in 1 ml buffer B (buffer B: buffer A supplemented with 0.02% saponin, 3 mM ATP, 1 μCi $^{45}Ca^{2+}$/ml). Where indicated IP_3 and 0.1 mM vanadate were added. The experiment was stopped by removing the buffer B and washing 3 × with buffer A (−BSA). Then the cells were collected and one fraction was added to 9 ml of scintillation fluid and counted for $^{45}Ca^{2+}$-radioactivity (Beckman LS3801). The second fraction was used for determination of protein content as described [10].

2.5. *Isolation of inositolphosphates*

Inositolphosphates were analyzed by HPLC and quantified by mass measurement as described previously [4,11].

3. RESULTS AND DISCUSSION

NIH 3T3 were transfected with a transforming Ha-*ras* recombined in vitro with a MMTV-LTR sequence [6,7]. Administration of dexamethasone leads to an accumulation of p21*ras* within 1–3 h following addition of the hormone as previously described [4]. Figure 1 demonstrates that 3 h after expression of Ha-*ras* a significant depression of the bombesin-induced mobilization of Ca^{2+} from intracellular stores can be observed. The depression of the Ca^{2+} signal is not explained by a reduction of IP_3 formation. As shown in Fig. 1 the bombesin-induced increase in IP_3 generation is unaffected by Ha-*ras* 3 h following expression of the oncogene. In accordance with previous results from this laboratory [4] it has to be concluded that the rapid

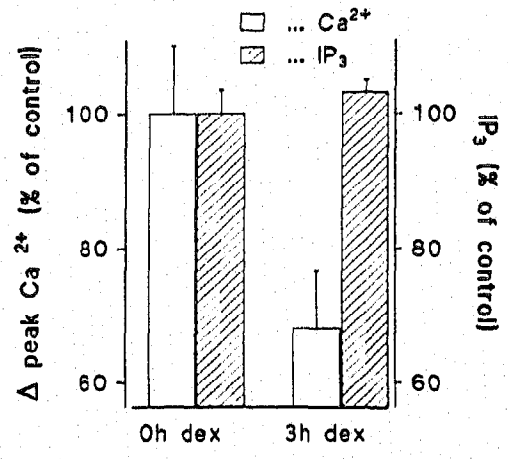

Fig. 1. Ca^{2+}-release and IP_3-formation by bombesin after the induction of the Ha-*ras* oncogene. NIH 3T3 cells were grown and prepared as described in section 2. The expression of the Ha-*ras* oncogene was induced by the addition of 1 μM dexamethasone. IP_3 was determined 10 s after stimulation of the cells by bombesin (basal value was 255±34 dpm/10^5 cells). Values of Δpeak Ca^{2+} were calculated as the difference between the maximum Ca^{2+} level after bombesin stimulation and the resting Ca^{2+} level in the absence of extracellular Ca^{2+} (HBS with 10 mM EGTA). The absolute value of Δpeak Ca^{2+} at time 0 was 380±25 nM. Bars indicate means ± standard error ($n \geq 5$).

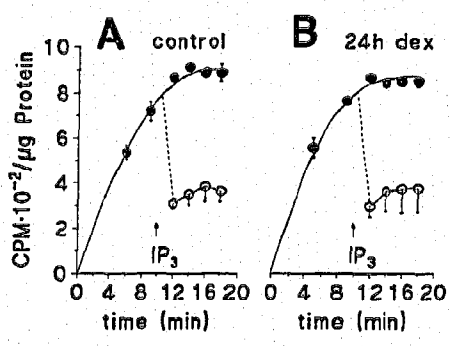

Fig. 2. $^{45}Ca^{2+}$-uptake and IP_3-induced $^{45}Ca^{2+}$-release of permeabilized NIH3T3 cells. NIH3T3 cells, transfected with the transforming Ha-*ras* were prepared for the experiment as described in section 2. Steady state distributions of ATP-dependent $^{45}Ca^{2+}$-uptake (●) are attained within approximately 12 min at 25°C. Addition of IP_3 (○; 10 μM, final concentration) in the presence of 0.1 mM vanadate immediately induces $^{45}Ca^{2+}$-release from intracellular, IP_3-sensitive stores. Data represent the mean ±SE ($n \geq 3$).

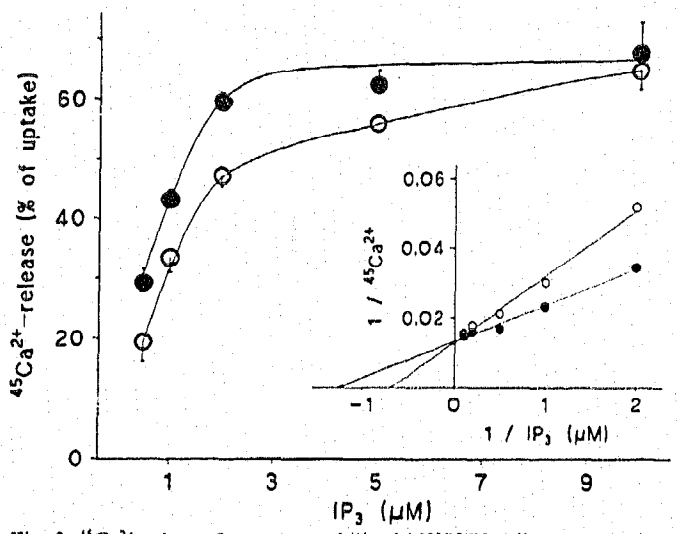

Fig. 3. $^{45}Ca^{2+}$-release from permeabilized NIH3T3 cells as a function of IP_3 concentration. NIH3T3 cells, transfected with the transforming Ha-*ras* were prepared for this experiment as described in the legend to Fig. 2. After attainment of steady state distributions of ATP-dependent $^{45}Ca^{2+}$-uptake, $^{45}Ca^{2+}$-release was stimulated by addition of 1,4,5-IP_3. $^{45}Ca^{2+}$-release was calculatred as the difference between the $^{45}Ca^{2+}$-uptake and the residual $^{45}Ca^{2+}$ after IP_3 stimulation (2 min incubation) at concentrations as indicated. Data (●, control; ○, 24 h after induction of the transforming Ha-*ras* by dexamethasone) represent the mean of at least 5 independent experiments ±SE. The reciprocal presentation of the data is depicted in the inset.

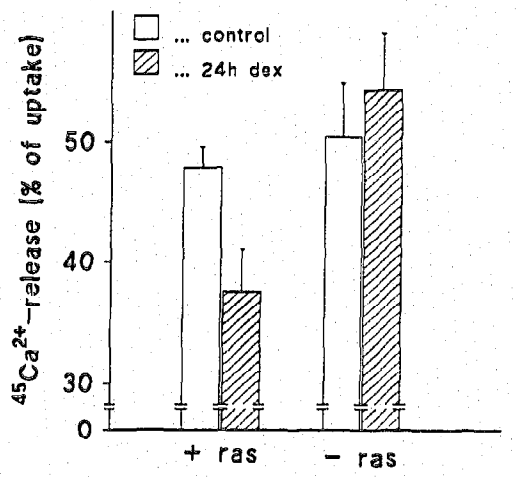

Fig. 4. $^{45}Ca^{2+}$-release by IP_3 from permeabilized NIH3T3 control cells and Ha-*ras* transfected NIH3T3 cells. NIH3T3 cells, either not transfected (−*ras*) or transfected with the transforming Ha-*ras* (+*ras*) were prepared for $^{45}Ca^{2+}$-release experiments as described in the legend to Fig. 2. Where indicated, the cells were treated with 1 μM dexamethasone for 24 h causing expression of the Ha-*ras* in the transfected cell line (+*ras*). $^{45}Ca^{2+}$-release was stimulated by addition of 1 μM IP_3. Data represent the mean ±SE ($n \geq 8$).

desensitization of the Ca^{2+}-mobilizing system to bombesin by Ha-*ras* is not the result of an uncoupling or an inhibition of phosphoinositidase C by Ha-*ras*.

In search for alternative mechanisms which could be responsible for the attenuation of the bombesin-induced Ca^{2+}-release by Ha-*ras*, the effect of the oncogene on IP_3-mediated Ca^{2+}-release was investigated. In Fig. 2 (panels A and B) the uptake of $^{45}Ca^{2+}$ into saponin-permeabilized cells is depicted. Under the conditions

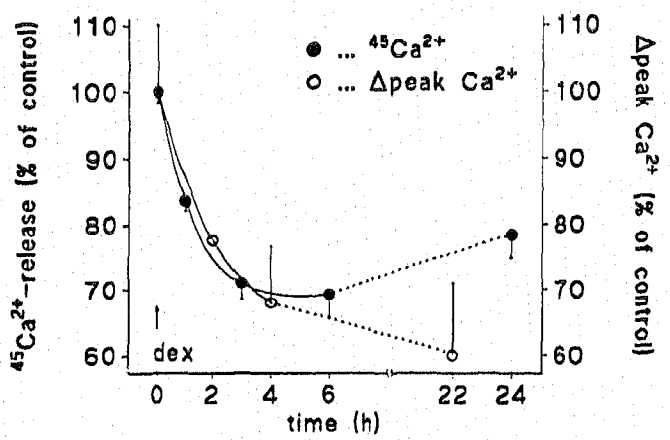

Fig. 5. Time course of $^{45}Ca^{2+}$-release by IP_3 and Ca^{2+}-mobilization by bombesin after induction of p21*ras*. NIH3T3 cells transfected with the transforming Ha-*ras* were grown and prepared for determination of the cytosolic Ca^{2+} concentration of the $^{45}Ca^{2+}$-release as described in the legends to Figs. 1 and 2. p21*ras* was induced by addition of 1 μM dexamethasone (dex) for times as indicated. Data, (●) $^{45}Ca^{2+}$-release by IP_3, (○) Ca^{2+}-mobilization by bombesin, represent the mean of at least 3 independent experiments ±SE.

used, $^{45}Ca^{2+}$-uptake is ATP-dependent and occurs predominantly (≥80%) into antimycin-resistant, non-mitrochondrial compartments (data not shown). As can be seen, cells overexpressing Ha-*ras* (panel B) accumulate Ca^{2+} to the same level as corresponding controls (panel A) and addition of 10 μM IP_3 releases approx. 60% of the total stored $^{45}Ca^{2+}$. Figure 3 shows the Ca^{2+}-release as a function of the IP_3 concentration. Expression of Ha-*ras* does not affect the total IP_3-sensitive Ca^{2+} pool. At saturating IP_3 concentrations, identical amounts of Ca^{2+} can be released in cells expressing p21*ras* and in non-induced controls. However, cells overexpressing Ha-*ras* require higher IP_3 concentrations for maximal Ca^{2+}-release than the corresponding controls. In control cells, 2 μM IP_3 is sufficient for maximal Ca^{2+}-release whereas 10 μM IP_3 is required to reach this level after expression of Ha-*ras*. The data from Fig. 3 suggest a decrease in the affinity to IP_3 in *ras*-expressing cells. Employing the double reciprocal plot shown in Fig. 3, the concentration for half-maximal Ca^{2+} release was determined as 0.68 μM IP_3 for the controls and 1.27 μM IP_3 for Ha-*ras*-expressing cells respectively. The decrease in IP_3 sensitivity is not a direct effect of dexamethasone. This is shown in Fig. 4 demonstrating that addition of dexamethasone to non-transfected cells (−*ras*) does not interfere with IP_3-mediated Ca^{2+}-release.

The Ha-*ras*-mediated desensitization of the Ca^{2+}-mobilizing system to bombesin is a relatively early phenomenon following induction of p21*ras* by dexamethasone. As shown in Fig. 5 the time course of the desensitization of the $^{45}Ca^{2+}$-release to 1 μM IP_3 closely follows the time course for the reduction of Ca^{2+} mobilization in response to bombesin in *ras*-expressing cells. This is in accordance with the kinetics of the appearance of p21*ras* itself [4]. The extent of the desensitization to IP_3 does not seem to account completely for the depression of the Ca^{2+} signal by Ha-*ras*. The data strongly suggest, however, that the observed Ha-*ras*-mediated decrease in IP_3 affinity contributes to the reduction of the Ca^{2+} signal in response to bombesin in Ha-*ras*-expressing cells. The permeabilized cells employed here may not totally reflect the biological situation in intact cells. Experiments with isolated ER-vesicles could yield clearer information on the actual IP_3 concentration at the corresponding receptors. Furthermore, information by which the mechanism of Ha-*ras* interferes with IP_3-mediated Ca^{2+}-release are necessary. It is still unclear whether the phenomenon described here is a direct effect of *ras* or whether it represents a phenomenon shared by many cells progrssing from G_0 into G_1 – perhaps associated with an activation of protein kinase C. It should be emphasized, however, that *ras*-like small G-proteins have been shown to be involved in intracellular Ca^{2+} transport [8]. It cannot be excluded, therefore, that *ras* is indeed biologically involved in the regulation of Ca^{2+}- signaling mechanisms.

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