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Effects of bradykinin on cell volume and intracellular pH in NIH 3T3 fibroblasts expressing the ras oncogene

M. Ritter[a], E. Wöll[a], D. Häussinger[b] and F. Lang[c]

[a]*Institut für Physiologie der Universität Innsbruck, Fritz-Pregl-Str. 3, A-6010 Innsbruck, Austria,* [b]*Medizinische Klinik der Universität Freiburg, Hugstetterstr. 55, D-7800 Freiburg, Germany* and [c]*Physiologisches Institut der Universität Tübingen, Gmelinstr. 5, D-7400 Tübingen, Germany*

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BCECF fluorescence has been applied to determine intracellular pH (pH_i) in NIH 3T3 fibroblasts expressing the Ha-ras oncogene (+ras) and otherwise identical cells not expressing the oncogene (−ras). In +ras cells, pH_i is significantly more alkaline (6.79 ± 0.03 n = 12) than in −ras cells (6.64 ± 0.02, n = 8). Bradykinin (100 nmol/l) leads to intracellular alkalinization in both +ras (to 6.96 ± 0.04, n = 12) and −ras cells (to 6.85 ± 0.02, n = 8). The effect of bradykinin is completely abolished in the presence of dimethylamiloride (100 μmol/l), which does not modify pH_i in the absence of bradykinin. Similar to bradykinin, cell shrinkage by addition of 15 mmol/l NaCl to the extracellular fluid leads to intracellular alkalinization (by 0.08 ± 0.01, n = 15). Cell volume is significantly greater in +ras cells (2.7 ± 0.4 pl, n = 15) than in −ras cells (2.2 ± 0.4 pl, n = 15). Bradykinin leads to cell shrinkage in both +ras cells (by 7 ± 1%, n = 17) and −ras cells (by 5 ± 1%, n = 15). The effect of bradykinin on cell volume can be reversed by the reduction of extracellular NaCl concentration by 15 mmol/l NaCl in +ras cells and by 7 mmol/l NaCl in −ras cells. This maneuver completely abolishes (in −ras cells) or blunts (in +ras cells) the alkalinizing effect of bradykinin. In conclusion, +ras cells are more alkaline than −ras cells. Bradykinin leads to further intracellular alkalinization by activation of the Na^+/H^+-exchanger, at least in part secondary to hormone-induced cell shrinkage.

Intracellular pH; Na^+/H^+-exchanger; Cell volume; Ras oncogene; Bradykinin

1. INTRODUCTION

The expression of the Ha-ras oncogene has been reported to result in growth factor independent activation of the Na^+/H^+-exchanger followed by intracellular alkalinization [1–4]. The intracellular alkalinization has in turn been invoked to play a crucial role in the regulation of cell proliferation (for review see [5–8]).

Besides its involvement in the regulation of intracellular pH the Na^+/H^+-exchanger serves to regulate cell volume: it is turned on upon cell shrinkage and leads to intracellular accumulation of Na^+ thus leading to regulatory cell volume increase (for review see [9–11]). The expression of the Ha-ras oncogene has been shown to shift the cell volume regulatory set point of the Na^+/H^+-exchanger towards larger cell volumes [12].

The present study has been performed to further elucidate the interaction of cell volume and intracellular pH regulation in Ha-ras oncogene expressing cells. To this end cell volume and intracellular pH have been determined in cells expressing (+ras) and not expressing (−ras) the Ha-ras oncogene. Furthermore, the effect of bradykinin on cell volume and intracellular pH has been analyzed. The expression of the Ha-ras oncogene leads to elevation of bradykinin receptor density [13] as well as to increased bradykinin sensitivity of cellular inositoltrisphosphate formation [13–15]. Furthermore, bradykinin leads to sustained oscillations of K^+ channel activity secondary to intracellular calcium oscillations in +ras cells, whereas it leads to only a single transient activation of K^+ channels in −ras cells [16,17].

Correspondence address: M. Ritter, Institut für Physiologie der Universität Innsbruck, Fritz-Pregl-Str. 3, A-6010 Innsbruck, Austria. Fax: (43) (512) 58 1740.

2. MATERIALS AND METHODS

Experiments were performed on NIH 3T3 fibroblasts transfected with a transforming Ha-ras MMTV-LTR construct expressing the oncogene, which is point-mutated at position 12, upon a 24 h treatment with 1 μmol/l dexamethasone (+ras) [18]. As controls served transfected cells not treated with dexamethasone (−ras). The increase of the expressed protein is routinely controlled by Western blot analysis [12].

The cells were grown on coverglasses in Dulbecco's modified Eagle's medium (DMEM) at 37°C, 5% CO_2 and 95% air supplemented with 100 g/l fetal calf serum (FCS) [18]. Prior to the experiments the cells were exposed to low serum medium (5 g/l FCS) for 48 h.

For the determination of intracellular pH, coverglasses with incompletely confluent cell layers were incubated for 15 min with 3 μmol/l BCECF-acetoxymethylester (Molecular Probes, Eugene, OR, USA). Measurements were made under an inverted phase-contrast microscope (IM-35, Zeiss, Germany) equipped for epifluorescence and photometry (Hamamatsu, Herrsching, Germany) [19]. Light from a xenon arc lamp (XBO75, Osram, Berlin, Germany) was directed through a grey-filter (nominal transmission 3.16%, Oriel, Darmstadt, Germany), a 488 nm or 440 nm, respectively, interference filter (halfwidth 10 nm; Oriel, Darmstadt, Germany) and a diaphragm and was deflected by a dichroic mirror (FT425, Zeiss, Germany) into the objective (Plan-

Neofluar 63× oil immersion, Zeiss, Germany). The emitted fluorescence was directed through a 530 nm cutoff filter to a photomultiplier tube (R4829, Hamamatsu, Herrsching, Germany). To reduce the region from which fluorescence was collected a pinhole was placed in the image plane of the phototubus (limitation to a circular area of 60 μm diameter). Fluorescence values were corrected for cellular autofluorescence, intracellular pH was calibrated with the high potassium/nigericin technique [20].

Cell volume was measured by cell sizing using a Coulter counter (model ZM, Coulter Electronics, UK) adapted with a Coulter channelyzer (model Coulter S-plus). The cell volume was calculated from the medians of volume distribution curves [12]. During the measurements cells were kept at 37°C. Absolute cell volumes were obtained using latex beads (13.7 μm diameter, Coulter Electronics, UK) as standards. Shortly before the cell volume measurements, the cells were dispersed by gentle treatment with calcium and magnesium free, trypsin/EDTA containing balanced salt solution and resuspended in isotonic electrolyte solution. The isotonic solution was composed of (all numbers mmol/l): 114 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 1.2 $CaCl_2$, 0.8 Na_2HPO_4, 0.2 NaH_2PO_4, 20 $NaHCO_3$ and 5.5 glucose. The solution was equilibrated with 5% carbon dioxide and 95% air (pH 7.4). Osmolarity was increased by increasing the extracellular NaCl concentration by 7 or 15 mmol/l, respectively, and decreased by reducing the extracellular NaCl concentration by 7 or 15 mmol/l, respectively.

Where indicated, bradykinin (100 nmol/l), dimethylamiloride (100 μmol/l) or ethylisopropylamiloride (10 μmol/l) have been added.

Applicable data are expressed as arithmetic means ± standard error of the mean (S.E.M.). Statistical analysis was made by paired or unpaired *t*-test, where applicable. Statistically significant differences were assumed at $P < 0.05$.

3. RESULTS

As illustrated in Fig. 1, the intracellular pH is significantly more alkaline in NIH 3T3 fibroblasts expressing the ras oncogene (+ras), than in otherwise identical cells not expressing the oncogene (−ras). Bradykinin (100 nmol/l) leads to a similar intracellular alkalinization in +ras and −ras cells, i.e. from 6.79 ± 0.03 to 6.96 ± 0.04 by 0.17 ± 0.02 in +ras cells (n = 12), and from 6.64 ± 0.02 to 6.85 ± 0.02 by 0.21 ± 0.02 in −ras cells (n = 8). The effect of bradykinin is completely abolished in

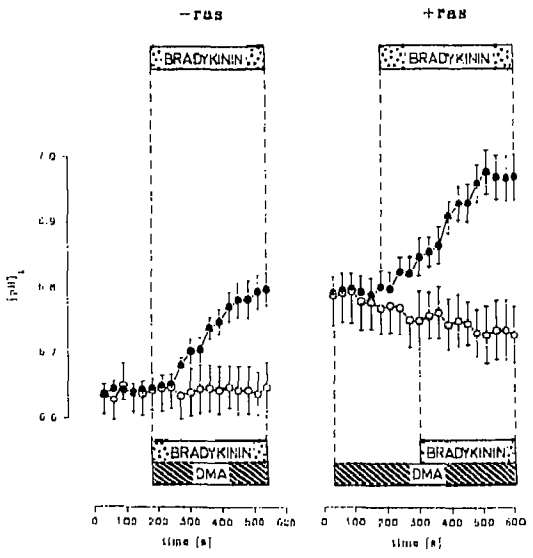

Fig. 1. Effect of 100 nmol/l bradykinin on intracellular pH of NIH 3T3 fibroblasts in the presence (open symbols) and absence (closed symbols) of 100 μmol/l dimethylamiloride. (Left panel) Effect in cells not expressing the ras oncogene (−ras). (Right panel) Effect in cells expressing the ras oncogene (+ras). Arithmetic means ± S.E.M., numbers in parenthesis indicate numbers of independent experiments.

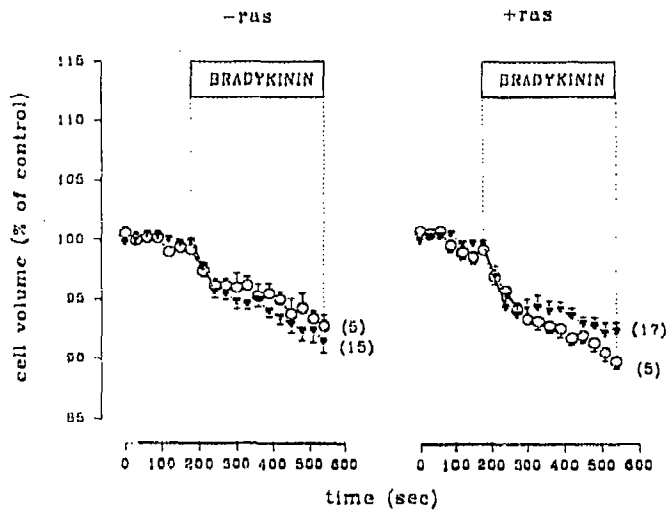

Fig. 2. Effect of 100 nmol/l bradykinin on cell volume of NIH 3T3 fibroblasts in the presence (open circles) and absence (closed triangles) of 10 μmol/l ethylisopropylamiloride. (Left panel) Effect in cells not expressing the ras oncogene (−ras). (Right panel) Effect in cells expressing the ras oncogene (+ras). Arithmetic means ± S.E.M., numbers in parenthesis indicate numbers of independent experiments.

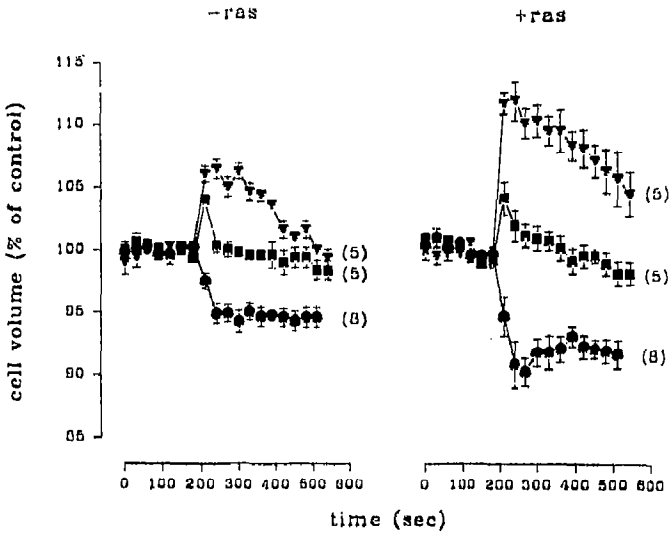

Fig. 3. Effects of 100 nmol/l bradykinin and of reduced extracellular NaCl concentration on cell volume of NIH 3T3 fibroblasts. Effect of bradykinin alone (closed circles), of reduced extracellular NaCl concentration alone (closed triangles) and of both, bradykinin and reduced extracellular NaCl concentration combined (closed squares). (Left panel) Effects in cells not expressing the ras oncogene (−ras). In these cells NaCl has been reduced by 7 mmol/l. (Right panel) Effects in cells expressing the ras oncogene (+ras). In these cells NaCl has been reduced by 15 mmol/l. Arithmetic means ± S.E.M., numbers in parenthesis indicate numbers of independent experiments.

the presence of dimethylamiloride (100 μmol/l), which does not significantly modify pH_i in the absence of bradykinin. Cell volume is significantly greater in +ras cells (2.7 ± 0.5 pl, n = 17) than in −ras cells (2.2 ± 0.4 pl, n = 15). As illustrated in Fig. 2, bradykinin leads to cell shrinkage in both, +ras cells (by 7 ± 1%, n = 17) and −ras cells (by 5 ± 1%, n = 15). Ethylisopropylamiloride (10 μmol/l) does not significantly modify cell volume in the presence (Fig. 2) or absence of bradykinin (0 ± 2%, n = 4).

Reduction of extracellular NaCl concentration leads to cell swelling followed by regulatory cell volume decrease (Fig. 3). The effect of bradykinin on cell volume can be reversed by simultaneous reduction of extracellular NaCl concentration by 15 mmol/l NaCl in +ras cells and by 7 mmol/l NaCl in −ras cells (Fig. 3).

Mimicking bradykinin induced cell shrinkage by addition of 7 mmol/l NaCl (−ras cells) or 15 mmol/l NaCl (+ras cells), respectively, to the extracellular fluid results in intracellular alkalinization in both, −ras cells (by 0.05 ± 0.01, n = 8) and +ras cells (by 0.08 ± 0.01, n = 15). Cell swelling, on the other hand, following reduction of extracellular NaCl concentration by 15 mmol/l (+ras cells) or 7 mmol/l NaCl (−ras cells), respectively, does not significantly modify pH_i (Fig. 4). If, in parallel to the addition of bradykinin, extracellular NaCl is reduced by 15 mmol/l, alkalinization of +ras cells is significantly blunted (Fig. 4). Thus, prevention of cell shrinkage blunts the alkalinizing effect of bradykinin. In −ras cells the bradykinin induced alkalinization is abolished, if bradykinin is added in parallel to a reduction of extracellular NaCl by 7 mmol/l (Fig. 4).

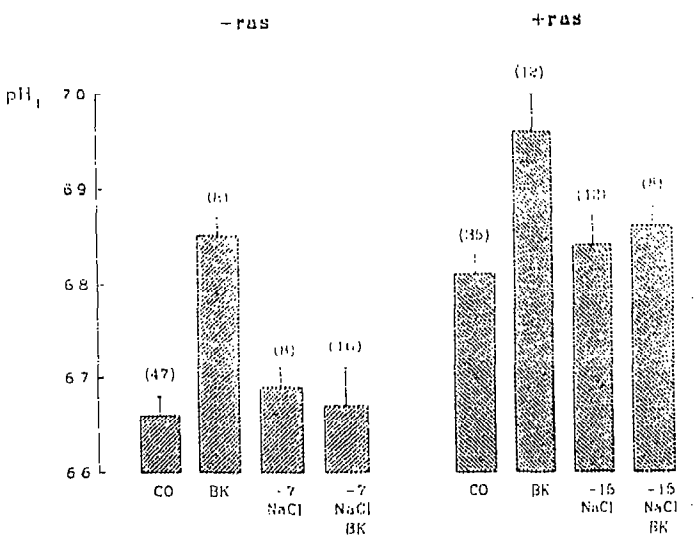

Fig. 4. Intracellular pH (pH_i) in the absence of bradykinin and in isotonic extracellular fluid (Co), following the addition of 100 nmol/l bradykinin (BK), following reduction of extracellular osmolarity (−NaCl) and following simultaneous reduction of extracellular osmolarity and application of 100 nmol/l bradykinin (−NaCl/BK). (Left panel) Effect in cells not expressing the ras oncogene (−ras). In these cells NaCl has been reduced by 7 mmol/l. (Right panel) Effect in cells expressing the ras oncogene (+ras). In these cells NaCl has been reduced by 15 mmol/l. Arithmetic means ± S.E.M., numbers in parenthesis indicate numbers of independent experiments.

4. DISCUSSION

The present observations confirm previous reports demonstrating that the expression of the Ha-ras oncogene leads to intracellular alkalinization [1–4] and increase of cell volume [12,21]. Inhibition of the Na^+/H^+-exchanger by dimethylamiloride or ethylisopropylamiloride did not lead to rapid acidification or decrease of cell volume, respectively. Obviously, 24 h after initiation of Ha-ras oncogene expression, the new set points for intracellular pH regulation and cell volume regulation have been approached and the carrier is thus turned off. Subsequent inhibition of the silent carrier does not modify intracellular pH. Bradykinin stimulates the Na^+/H^+-exchanger in both, ras oncogene expressing and non-expressing cells. The respective alkalinization can be inhibited by dimethylamiloride.

In a variety of tissues, the Na^+/H^+-exchanger is activated by cell shrinkage and its activation leads to cellular accumulation of Na^+ with subsequent increase of cell volume (for review see [9–11]). Thus, the activation of the Na^+/H^+-exchanger by bradykinin could be expected to swell the cells. The opposite is true: despite the activation of the Na^+/H^+-exchanger, bradykinin shrinks the cells. This shrinkage may be due to a cellular ion loss secondary to the activation of ion channels. In NIH 3T3 fibroblasts not expressing the ras oncogene bradykinin leads only to a transient activation of K^+ channels [16] hardly explaining the sustained decrease of cell volume. As is evident from experiments in MDCK cells [22], the transient activation of K^+ channels is paralleled by a more sustained activation of anion channels, which may indeed account for the sustained loss of cellular ions.

The bradykinin induced intracellular alkalinization can be inhibited by simultaneous reduction of extracellular osmolarity just sufficient to prevent cell shrinkage. Thus, it appears that the activation of the Na^+/H^+-exchanger by bradykinin is at least partially the result of cell shrinkage.

In conclusion, the expression of Ha-ras oncogene leads to both, intracellular alkalinization and increase of cell volume, pointing to a shift of the respective set points for the Na^+/H^+-exchanger. In both, ras oncogene expressing and non-expressing cells, bradykinin activates the Na^+/H^+-exchanger and leads to intracellular alkalinization at least in part by hormone induced cell shrinkage.

REFERENCES

[1] Doppler, W., Jaggi, R. and Groner, B. (1987) Gene 54, 147–153.
[2] Hagag, N., Lacal, J.C., Graber, M., Aaronson, S. and Viola, M.V. (1987) Mol. Cell. Biol. 7, 1984–1988.

[3] Maly, K., Überall, F., Loferer, H., Doppler, W., Oberhuber, H., Groner, B. and Grunicke, H. (1989) J. Biol. Chem. 264, 11839–11842.
[4] Maly, K., Oberhuber, H., Doppler, W., Hoflacher, J., Jaggi, R., Groner, B. and Grunicke, H. (1989) Adv. Enzyme Regul. 27, 121–131.
[5] Counillon, L. and Pouysségur, J. (1992) Cell. Physiol. Biochem. 2 (in press).
[6] Gillies, R.J., Martinez-Zaguilan, R., Peterson, E.P. and Perona, R. (1992) Cell. Physiol. Biochem. 2 (in press).
[7] Nelson, N., Beltrán, C., Supek, F. and Nelson, H. (1992) Cell. Physiol. Biochem. 2 (in press).
[8] Oberleithner, H. (1992) Cell. Physiol. Biochem. 2 (in press).
[9] Grinstein, S. and Dixon, J. (1989) Physiol. Rev. 69, 417–481.
[10] Hoffmann, E.K. and Simonsen, L.O. (1989) Physiol. Rev. 69, 315–382.
[11] Lang, F., Völkl, H. and Häussinger, D. (1990) Comp. Physiol. 4, 1–25.
[12] Lang, F., Ritter, M., Wöll, E., Weiss, H., Häussinger, D., Hoflacher, J., Maly, K. and Grunicke, H. (1992) Pflügers Arch. 420, 424–427.
[13] Downward, J., de Gunzburg, J., Riehl, R. and Weinberg, R.A. (1988) Proc. Natl. Acad. Sci. USA 85, 5774–5778.
[14] Downward, J. and Weinberg, R.A. (1988) Cold Spring Harbor Quant. Biol. 53, 883–892.
[15] Parries, G., Hoebel, R. and Racher, E. (1987) Acad. Sci. 84, 2648–2652.
[16] Lang, F., Friedrich, F., Kahn, E., Wöll, E., Hammerer, M., Waldegger, S., Maly, K. and Grunicke, H. (1991) J. Biol. Chem. 266, 4938–4942.
[17] Wöll, E., Waldegger, S., Lang, F., Maly, K. and Grunicke, H. (1992) Pflügers Arch. 420, 208–212.
[18] Jaggi, R., Salmons, B., Muellener, D. and Groner, B. (1986) EMBO J. 5, 2609–2612.
[19] Almers, W. and Neher, E. (1985) FEBS Lett. 192, 13–18.
[20] Ganz, M.B., Boyarsky, G., Sterzel, R.B. and Boron, W.F. (1989) Nature 337, 648–651.
[21] Meyer, M., Maly, K., Überall, F., Hoflacher, J. and Grunicke, H. (1991) J. Biol. Chem. 266, 8230–8235.
[22] Ritter, M. and Lang, F. (1990) J. Physiol. 443, 45–54.