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# The role of calcium in cell shrinkage and intracellular alkalinization by bradykinin in Ha-ras oncogene expressing cells

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In ras oncogene expressing cells, bradykinin leads to intracellular alkalinization by activation of the $Na^+/H^+$ exchanger. This effect is paralleled by oscillatory increase of intracellular calcium activity and cell shrinkage. Staurosporine (1 μmol/l) is not sufficient to prevent bradykinin induced intracellular alkalinization, thus pointing to a protein kinase C independent pathway for the activation of $Na^+/H^+$ exchange. The present study has been performed to elucidate, whether the increase of intracellular calcium contributes to cell shrinkage and activation of the $Na^+/H^+$ exchanger. To this end, the effects of the calcium ionophore ionomycin have been tested. Ionomycin leads to a dose dependent increase of intracellular calcium activity. At 100 nmol/l ionomycin intracellular calcium is increased from 114 ± 17 nmol/l to 342 ± 24 nmol/l ($n$ = 9), a value within the range of intracellular calcium concentrations following application of bradykinin. The calcium increase is paralleled by a decrease of cell volume by 12 ± 2% ($n$ = 5) and an increase of intracellular pH from 6.78 ± 0.02 to 6.90 ± 0.03 ($n$ = 11), values similar to those following application of bradykinin. The alkalinizing effect of ionomycin is completely abolished in the presence of the novel $Na^+/H^+$ exchange inhibitor HOE 694 (10 μmol/l), but is not inhibited by 1 μmol/l staurosporine. Inhibition of $K^+$ and $Cl^-$ channels by barium (5 mmol/l) and ochratoxin-A (5 μmol/l) prevents both ionomycin induced cell shrinkage and protein kinase C independent intracellular alkalinization. It is concluded that bradykinin leads to intracellular alkalinization mainly by increasing intracellular calcium concentration. Calcium triggers calcium sensitive $K^+$ channels, and presumably $Cl^-$ channels, the subsequent loss of cellular KCl leads to cell shrinkage which, in turn, activates $Na^+/H^+$ exchange.

Intracellular calcium; ras oncogene; Bradykinin; Cell volume; $Na^+/H^+$ exchanger

## 1. INTRODUCTION

In ras oncogene expressing NIH 3T3 fibroblasts bradykinin leads to sustained oscillations of intracellular calcium activity [1,2], cell shrinkage and intracellular alkalinization [3]. If bradykinin induced cell shrinkage is prevented by a reduction of extracellular osmolarity, the alkalinizing effect of bradykinin is blunted [3]. Since the $Na^+/H^+$ exchanger has been found to serve cell volume regulation in ras oncogene expressing NIH 3T3 fibroblasts and to be activated by cell shrinkage [4], it was concluded that bradykinin mediated cell shrinkage participated in the activation of the $Na^+/H^+$ exchanger [3]. The mechanism underlying cell shrinkage remained, however, elusive. Circumstantial evidence pointed to the involvement of intracellular calcium: previous studies have identified calcium sensitive $K^+$ channels in these cells [1] and the bradykinin induced oscillations of intracellular calcium activity are paralleled by oscillations of cell membrane potential [2,5,6]. Activation of $K^+$ channels is an element of cell volume regulation and has been shown to shrink cells in a variety of tissues (for review see [7–9]). Thus, it appeared conceivable that the bradykinin induced increase of intracellular calcium participates in the stimulation of the $Na^+/H^+$ exchanger. The present study has been performed to test this hypothesis. To this end, the effects of the calcium ionophore ionomycin on cell volume and intracellular pH have been investigated. As a result, ionomycin indeed shrinks the cells and leads to intracellular alkalinization. The alkalinization is abolished by HOE 694, a novel inhibitor of $Na^+/H^+$ exchange [10,11]. Both, ionomycin induced cell shrinkage and protein kinase C independent intracellular alkalinization are abolished in the presence of $K^+$ channel blocker barium and the putative $Cl^-$ channel blocker ochratoxin-A.

Thus, the bradykinin induced rise in intracellular calcium serves to activate $Na^+/H^+$ exchange via activation of ion channels, loss of cellular KCl and subsequent cell shrinkage.

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## 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 pointmutated at position 12, upon a 24 h treatment with 1 μmol/l dexamethasone [12]. As controls served transfected cells not treated with dexamethasone. The increase of the expressed protein is routinely controlled by Western blot analysis [4].

The cells were grown on glass coverslips in Dulbecco's modified Eagle's medium (DMEM) at 37°C, 5% $CO_2$/95% air supplemented

with 100 g/l fetal calf serum (FCS) [12]. Prior to the experiments the cells were exposed to low serum medium (5 g/l FCS) for 24 h.

BCECF fluorescence has been used for determination of intracellular pH ($pH_i$), fura-2 fluorescence for determination of intracellular calcium ($Ca_i$). To this end, glass coverslips with incompletely confluent cell layers were incubated for 15 min with 3 μmol/l BCECF-acetoxymethylester (Molecular Probes, Eugene, OR, USA) or 2.5 μmol/l fura- 2-AM (Molecular Probes, Junction City, OR, USA and Calbiochem, Geneva, Switzerland). Measurements were made under an inverted phase-contrast microscope (IM-35; Zeiss, Oberkochen, Germany) equipped for epifluorescence and photometry (Hamamatsu, Herrsching, Germany) [13]. Light from a xenon arc lamp (XBO75; Osram, Berlin, Germany) was directed through a grey-filter (nominal transmission 3.16%, Oriel, Darmstadt, Germany), and alternatingly through interference filters (halfwidth 10 nm, Oriel, Darmstadt, Germany) of 488 nm and 440 nm (for $pH_i$) or 340 nm and 380 nm (for $Ca_i$), respectively, and a diaphragm and deflected by a dichroic mirror (FT510 ($pH_i$) or FT420 ($Ca_i$), Zeiss, Oberkochen, Germany) into the objective (Plan-Neofluar 63 × oil immersion; Zeiss, Oberkochen, Germany). The emitted fluorescence was directed through a 530 nm ($pH_i$) or 420 nm ($Ca_i$) 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 phototube (limitation to a circular area of 60 μm diameter). All fluorescence values were corrected for cellular autofluorescence. $pH_i$ was calibrated with the high potassium/nigericin technique [14]. $Ca_i$ was calculated from the ratio ($R$) of the fluorescence intensities at the two different excitation wave lengths (340 nm/380 nm) [1,15]:

$$Ca_i = K_d \cdot S_{f_2} (R - R_{min})/S_{b_2} (R_{max} - R)$$

where $R_{min}$ and $R_{max}$ are the fluorescence ratios corrected for autofluorescence at experimental conditions, at minimum and maximum calcium binding; $K_d$ is the dissociation constant for fura-2 (= 225); $S_{f_2}$ and $S_{b_2}$ are the proportionality coefficients for the fluorescence at 380 nm excitation of free and calcium bound dye, respectively. $R_{min}$ and $S_{f_2}$ were determined by exposure of the cells to 2 mmol/l $Mn^{2+}$ + 20 μmol/l A23187, $R_{max}$ and $S_{b_2}$ after exposure of the cells to either 20 μmol/l digitonin (Sigma, Munich, Germany) or 20 μmol/l A23187.

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 [4]. During measurement 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 extracellular perfusate was composed of (all numbers in mmol/l): NaCl 114, KCl 5.4, $MgCl_2$ 0.8, $CaCl_2$ 1.2, $Na_2HPO_4$ 0.8, $NaH_2PO_4$ 0.2, $NaHCO_3$ 20 and glucose 5.5. The solution was equilibrated with 5% $CO_2$/95% air (pH 7.4). All chemicals were obtained from Sigma, Munich, Germany, unless specified otherwise.

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 reported previously [3,4] expression of the Ha-ras oncogene leads to an increase in cell volume from 2.0 ± 0.05 pl ($n$ = 10) to 2.6 ± 0.04 pl ($n$ = 17) and to an increase in intracellular pH ($pH_i$) from 6.62 ± 0.05 ($n$ = 16) to 6.81 ± 0.02 ($n$ = 51). Cells expressing the ras oncogene respond to bradykinin with a further increase in $pH_i$ by 0.16 ± 0.05 pH units ($n$ = 6) [3]. After one hour of preincubation and the continuous presence of staurosporine (1 μmol/l) which has been shown to inhibit PKC activity in these cells [16] bradykinin still leads to intracellular alkalinization (by 0.15 ± 0.03 pH units, $n$ = 11; Fig. 1). Under control conditions the intracellular calcium concentration ($Ca_i$) in these cells is 114 ± 17 nmol/l ($n$ = 9). As shown in Fig. 2, ionomycin (1, 10, 100 and 1,000 nmol/l) leads to a dose dependent increase in both, $Ca_i$ (by 5 ± 12, 87 ± 28, 224 ± 33 and 936 ± 164 nmol/l, resp., $n$ = 8–9) and $pH_i$ (by 0.03 ± 0.02, 0.04 ± 0.01, 0.11 ± 0.02 and 0.20 ± 0.02 pH units, resp., $n$ = 6–12). The ionomycin (100 nmol/l) induced increase in $pH_i$ is completely abolished in the presence of 10 μmol/l HOE 694 ($\Delta pH_i$ = 0.01 ± 0.01, $n$ = 8; Fig. 3), a novel potent inhibitor of the $Na^+/H^+$ exchanger [10], but is not significantly modified after one hour of preincubation and the continuous presence of 1 μmol/l staurosporine ($\Delta pH_i$ = 0.11 ± 0.03, $n$ = 10; Fig. 5). Fig. 4 shows that the ionomycin induced increase in $pH_i$ is paralleled by a decrease in cell volume by 12 ± 2% ($n$ = 5). This decrease in cell volume ($\Delta$CV) is blunted in the presence of the $K^+$ channel blocker barium (10 mmol/l; $\Delta$CV = 7 ± 1%, $n$ = 6). In the presence of both, barium (5 mmol/l) and the putative $Cl^-$ channel blocker ochratoxin-A (5 μmol/l) [17] the ionomycin induced decrease in cell volume ($\Delta$CV = 4 ± 1%, $n$ = 6) is not significantly different from the volume of

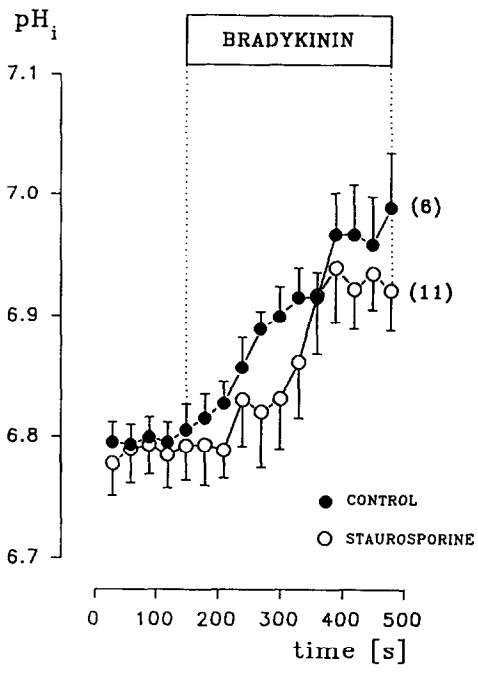


Fig. 1. Effect of 100 nmol/l bradykinin on intracellular pH ($pH_i$) of ras oncogene expressing NIH 3T3 fibroblasts. Closed symbols show the time course of alkalinization under control conditions. Open symbols show the time course after one hour of preincubation and in continuous presence of 1 μmol/l staurosporine. Arithmetic means ± S.E.M., numbers in parenthesis indicate numbers of independent experiments.

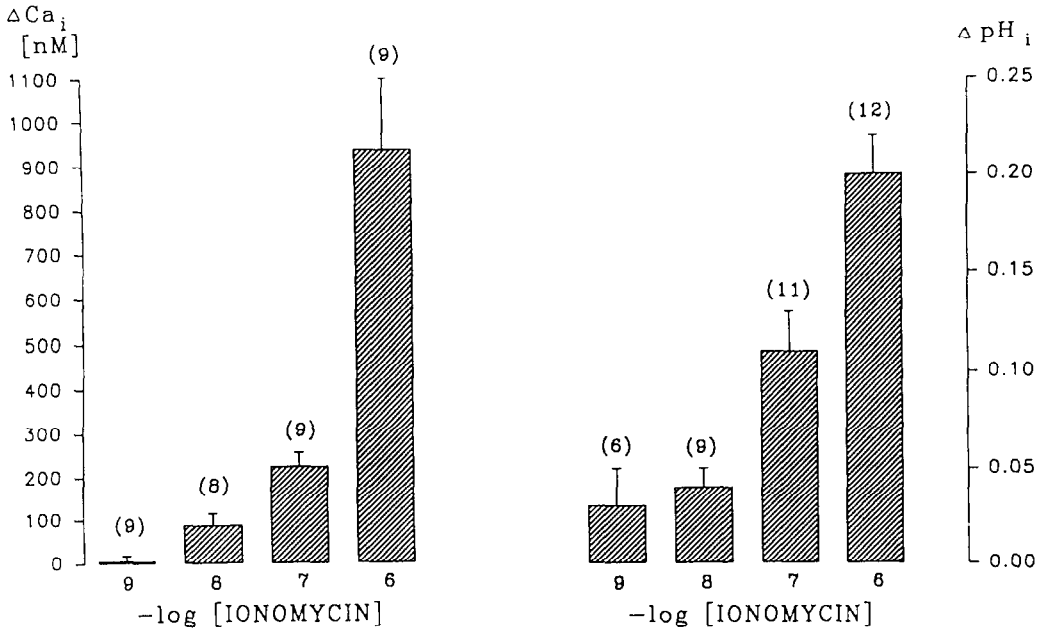


Fig. 2. Dose–response relationship for ionomycin. (Left panel) Increase in intracellular calcium concentration ($\Delta Ca_i$) in NIH 3T3 fibroblasts expressing the ras oncogene by 1, 10, 100 and 1,000 nmol/l ionomycin. (Right panel) Increase in intracellular pH ($\Delta pH_i$) in NIH 3T3 fibroblasts expressing the ras oncogene by 1, 10, 100 and 1,000 nmol/l ionomycin. Arithmetic means ± S.E.M., numbers in parentheses indicate numbers of independent experiments.

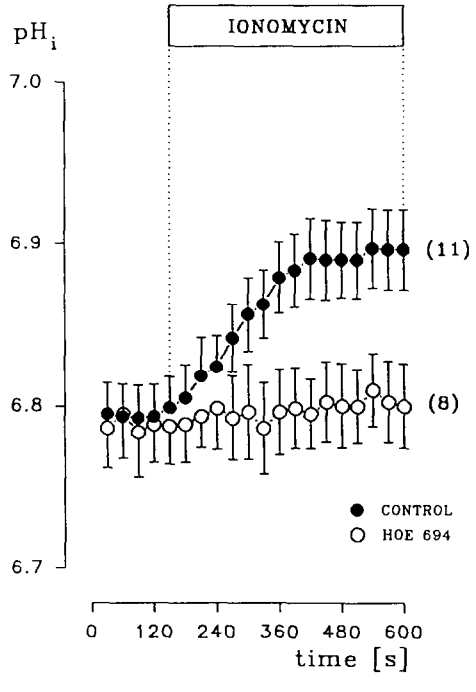


Fig. 3. Effect of 100 nmol/l ionomycin on intracellular pH ($pH_i$) in NIH 3T3 fibroblasts expressing the ras oncogene. Experiments were performed in both, the absence (closed symbols) and continuous presence of $Na^+/H^+$ exchange inhibitor HOE 694 (10 μmol/l). Arithmetic means ± S.E.M., numbers in parentheses indicate numbers of independent experiments.

untreated cells ($\Delta CV = 3 \pm 1\%$, $n = 5$). Similarly the intracellular alkalinization observed in the presence of staurosporine is completely abolished by barium (5 mmol/l) and ochratoxin-A (5 μmol/l; $\Delta pH_i = -0.01 \pm 0.02$, $n = 8$; Fig. 5).

## 4. DISCUSSION

A variety of mitogenic signals has been shown to activate the $Na^+/H^+$ exchanger, leading – under appropriate conditions – to intracellular alkalinization, one of the putative prerequisites for the initiation of cell proliferation (for review see [18–21]). Similarly, the expression of the Ha-ras oncogene leads to growth factor independent activation of the $Na^+/H^+$ exchanger followed by intracellular alkalinization [16,22–24]. The activation of the $Na^+/H^+$ exchanger by mitogenic signals is thought to be secondary to protein phosphorylation by PKC [18,25]. However, in some reports the alkalinization induced by mitogenic signals [26–30], expression of Ha-ras oncogene [16] or cell shrinkage [31,32] has been shown to be independent of PKC activation. As shown in this study bradykinin induced alkalinization in Ha-ras oncogene expressing NIH 3T3 fibroblasts is similarly insensitive to treatment of the cells with staurosporine, pointing to an alternative, PKC independent pathway of $Na^+/H^+$ exchanger activation. As reported previously the alkalinization is paralleled by cell shrinkage [3] and oscillations of $Ca_i$ [2]. The hormone induced cell shrinkage has been shown to participate in the acti-

vation of the $Na^+/H^+$ exchanger and may thus represent the alternative pathway. The pulsatile increase in $Ca_i$ leads to the activation of calcium sensitive $K^+$ channels, oscillations of the cell membrane potential [1] and the subsequent loss of cellular ions and is expected to shrink the cells. As shown in this study an increase in $Ca_i$, induced by the calcium ionophore ionomycin, is indeed tightly coupled to an increase in $pH_i$ and to cell shrinkage. At a concentration of 100 nmol/l ionomycin increases $Ca_i$ and $pH_i$ and decreases cell volume to values similar to those observed with bradykinin [2,3]. The ionomycin induced alkalinization is inhibited by the novel blocker of the $Na^+/H^+$ exchanger, HOE 694, but remains unaffected by treatment of the cells with staurosporine and is thus not due to a calcium mediated activation of PKC [33]. Furthermore, ionomycin has been shown to lead to activation of $Ca^{2+}$ dependent $K^+$ channels resulting in hyperpolarization or, under certain conditions, even oscillations of the cell membrane potential [5,11]. Accordingly, inhibition of $K^+$ channels by barium blunts the ionomycin induced cell shrinkage. However, a complete inhibition of cell shrinkage requires the combined action of both, barium and ochratoxin-A, which has recently been shown to be a very potent $Cl^-$ channel blocker [17]. This manoeuvre is also sufficient to inhibit ionomycin induced intracellular alkalinization in staurosporine treated cells. Thus both $K^+$ and $Cl^-$ channels might be involved in calcium mediated cell shrinkage and activation of the $Na^+/H^+$ exchanger.

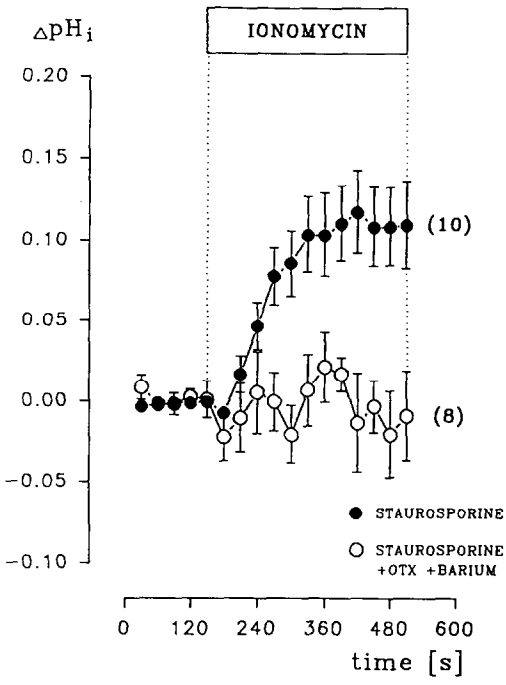


Fig. 5. Increase in intracellular pH ($\Delta pH_i$) by 100 nmol/l ionomycin in NIH 3T3 fibroblasts expressing the ras oncogene. Closed circles show experiments after one hour preincubation and continuous presence of 1 μmol/l staurosporine. Open circles show experiments after one hour of preincubation and continuous presence of 1 μmol/l staurosporine together with 5 mmol/l barium and 5 μmol/l ochratoxin-A. Arithmetic means ± S.E.M., numbers in parentheses indicate numbers of independent experiments.

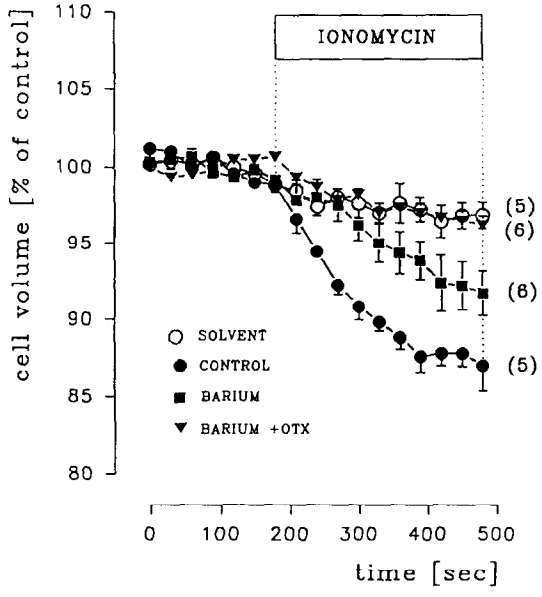


Fig. 4. Effect of 100 nmol/l ionomycin on cell volume of NIH 3T3 fibroblasts expressing the ras oncogene. Effect of ionomycin alone (closed circles), ionomycin in combination with 10 mmol/l barium (closed squares) and ionomycin together with 5 mmol/l barium plus 5 μmol/l ochratoxin-A (closed triangles). Open circles refer to cells treated with the solvent only. Arithmetic means ± S.E.M., numbers in parentheses indicate numbers of independent experiments.

In conclusion, in Ha-ras oncogene expressing NIH 3T3 fibroblasts bradykinin leads to intracellular alkalinization utilizing a PKC independent pathway by increasing intracellular calcium activity, activation of $K^+$ and $Cl^-$ channels, cell shrinkage and subsequent volume regulatory activation of the $Na^+/H^+$ exchanger. The calcium mediated cell shrinkage – most likely by activation of ion channels – may, therefore, represent an important step in the action of mitogenic signals.

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