Research article

Modulation of intracellular calcium and proliferative activity of invertebrate and vertebrate cells by ethylene

Sanja Perovic[1], Jürgen Seack[1], Vera Gamulin[2], Werner EG Müller*[1] and Heinz C Schröder[1]

Address: [1]Institut für Physiologische Chemie, Abteilung Angewandte Molekularbiologie, Universität, Duesbergweg 6, D-55099 Mainz, Germany and [2] Institut Rudjer Boskovic, Department of Molecular Genetics, Bijenicka c. 54, HR-10000 Zagreb, Croatia

E-mail: Sanja Perovic - perovic@mail.uni-mainz.de; Jürgen Seack - seacj000@web.de; Vera Gamulin - gamulin@rudjer.irb.hr; Werner EG Müller* - wmueller@mail.uni-mainz.de; Heinz C Schröder - hschroed@mail.uni-mainz.de

*Corresponding author

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Abstract

Background: Ethylene is a widely distributed alkene product which is formed enzymatically (e.g., in plants) or by photochemical reactions (e.g., in the upper oceanic layers from dissolved organic carbon). This gaseous compound was recently found to induce in cells from the marine sponge *Suberites domuncula*, an increase in intracellular Ca^{2+} level ($[Ca^{2+}]_i$) and an upregulation of the expression of two genes, the potential ethylene-responsive gene, *SDERR*, and a Ca^{2+}/calmodulin-dependent protein kinase.

Results: Here we describe for the first time, that besides sponge cells, mammalian cell lines (mouse NIH-3T3 and human HeLa and SaOS-2 cells) respond to ethylene, generated by ethephon, with an immediate and strong, transient increase in $[Ca^{2+}]_i$ level, as demonstrated using Fura-2 imaging method. A rise of $[Ca^{2+}]_i$ level was also found following exposure to ethylene gas of cells kept under pressure (SaOS-2 cells). The upregulation of $[Ca^{2+}]_i$ was associated with an increase in the level of the cell cycle-associated Ki-67 antigen. In addition, we show that the effect of ethephon addition to *S. domuncula* cells depends on the presence of calcium in the extracellular milieu.

Conclusion: The results presented in this paper indicate that ethylene, previously known to act as a mediator (hormone) in plants only, deserves also attention as a potential signaling molecule in higher vertebrates. Further studies are necessary to clarify the specificity and physiological significance of the effects induced by ethylene in mammalian cells.

Background

Ethylene is the chemically simplest plant hormone. This compound plays an important regulatory role in plant growth, development, and senescence; it is involved in a variety of stress responses in plants (for a review, see [1]). In the last years, much progress has been made in the isolation and characterisation of the genes and proteins participating in the ethylene signal transduction pathway in plants (for a review, see [2]). Calcium and protein phosphorylation/dephosphorylation processes may be involved in the transduction of the ethylene signal [3]. Ethylene is also among the mediators of programmed cell death in plants [4].

Recently we demonstrated for the first time that besides plants, certain animal cells, namely cells from a marine

sponge (*Suberites domuncula*), sensitively react to ethylene [5]. This gas is present, at a concentration of up to 100 pM, in seawater [6], where it can be produced from dissolved organic carbon by photochemical (especially ultraviolet light-induced) reactions [7,8,9]. We showed that primmorphs of *S. domuncula*, consisting of aggregates of dissociated sponge cells that are able to proliferate [10], respond to ethylene with an increase in $[Ca^{2+}]_i$ and a reduction of apoptosis induced by starvation [5]. In addition, in *S. domuncula* primmorphs an upregulation of the expression of two genes occurs following ethylene exposure, one of these genes, termed *SDERR* [5], is related to the ethylene-responsive plant gene *HEVER* [11]. The other gene encodes the Ca^{2+}/calmodulin-dependent protein kinase II [5]. The *SDERR* cDNA has been isolated and characterized [5].

The sponges (Porifera) are considered to form the first or one of the first metazoan phyla that diverged from the common ancestor of all Metazoa, the Urmetazoa [12]. They are provided with the same protein constituents known from higher animals, including molecules involved in cell recognition and signal transduction pathways (for a review, see [13]). Therefore, we asked if besides sponges, cells from higher vertebrates respond to ethylene. Here we demonstrate that various mammalian cell lines react to ethylene, generated by ethephon (or ethylene gas), with an upregulation of $[Ca^{2+}]_i$ level and an increased expression of the cell cycle-associated antigen Ki-67, used as a marker of cell proliferation.

Results

Effect of ethylene on $[Ca^{2+}]_i$ level in mammalian cells

As shown in Figure 1A, addition of 1 mM of the ethylene-releasing agent, ethephon, to mouse NIH-3T3 fibroblasts resulted in a marked increase in intracellular calcium level ($[Ca^{2+}]_i$), as documented by the consecutive record of the fluorescence images obtained in experiments using the Ca^{2+} indicator dye Fura-2. A rapid and strong shift from dark blue to light-blue, green, orange and red was seen in all cells immediately (0.5 min) after ethylene treatment (Figure 1A,b2) which started 5 min after the beginning of the experiment. Subsequently the $[Ca^{2+}]_i$ levels dropped slowly, yielding light-blue (Figure 1A,d2-b3) and finally dark-blue cell images (Figure 1A,c3-c4). In Figure 1A,d4, a photo of the cells taken at the end of the experiment by Nomarski phase contrast interference optics is shown. Increased $[Ca^{2+}]_i$ levels were also found in human cell lines (HeLa and SaOS-2 cells) following ethylene exposure. However, in the case of human osteosarcoma SaOS cells the increase in $[Ca^{2+}]_i$ level was not observed in all cells examined; some cells showed a drastic rise in $[Ca^{2+}]_i$ level, while other cells seemed to be unaffected by the ethylene treatment (Figure 1B).

A quantitative analysis of the changes following addition of ethephon to NIH-3T3 cells revealed that, at 1 mM ethephon, the fluorescence ratio 340/380 nm increased from 1.00 ± 0.01 to 1.67 ± 0.09; this corresponds to 239 nM of $[Ca^{2+}]_i$ (Figure 2A). The increase in the 340/380 nm ratio was weaker (from 1.09 ± 0.02 to 1.31 ± 0.05, corresponding to 187 nM of $[Ca^{2+}]_i$) when 0.3 mM ethephon was added to the cells. Lower concentrations of ethylene (0.1 mM ethephon; Figure 2A) had no effect on $[Ca^{2+}]_i$ level. The rapid rise of $[Ca^{2+}]_i$ level occurring after addition of 0.3 or 1 mM ethephon was followed by a slow decrease (tailing) of the fluorescence ratio (Figure 2A).

Decomposition of ethephon in solution at increasing pH results in the production of H_3PO_4. Therefore, in control experiments, 0.5 mM of H_3PO_4 was added instead of ethephon (Figure 2A). The results revealed that H_3PO_4 did not cause any change of $[Ca^{2+}]_i$ level. Similarly no change in $[Ca^{2+}]_i$ level was found in assays without ethephon or any other compounds (not shown).

The extent of upregulation of $[Ca^{2+}]_i$ level following ethylene exposure considerably varied between the cell lines studied. Only a relatively small but significant ($p < 0.001$) increase in $[Ca^{2+}]_i$ level (increase in 340/380 nm ratio from 0.66 ± 0.01 to 0.74 ± 0.01) was observed in HeLa cells treated with a high concentration of 2 mM ethephon (Figure 2B), while osteosarcoma SaOS-2 cells responded to ethylene exposure (increase in 340/380 nm ratio from 0.90 ± 0.01 to 1.03 ± 0.05) already at a low concentration of 0.3 mM ethephon (Figure 2C). Exposure of cells (SaOS-2 cells) to ethylene (generated by ethephon addition) in Locke's solution depleted of $CaCl_2$ resulted in only a small but not significant effect on $[Ca^{2+}]_i$ level, indicating that the response of mammalian cells requires the presence of extracellular calcium (results not shown).

To ensure that the response observed after ethephon addition is specific to ethylene and not to ethephon itself, we examined the effect of ethylene gas on $[Ca^{2+}]_i$ level in SaOS-2 cells. For these experiments cells kept under pressure of 1 physical atmosphere (atm) were used as previously described for sponge primmorphs [12]. As shown in Figure 3B and 3C, exposure of the cells to a pressure of 1 atm for 10 min caused a slight increase in $[Ca^{2+}]_i$ level. Injection of ethylene gas into the pressure chamber (kept under pressure of 1 atm) resulted in an additional, strong increase in $[Ca^{2+}]_i$ level compared to control (incubation period, 10 min; Figure 3C and 3D). Determination by gas chromatography revealed that under the conditions used the amount of dissolved ethylene gas was 7.9 µM. Interestingly, in contrast to ethephon addition (Figure 1B), the rise of $[Ca^{2+}]_i$ level induced by

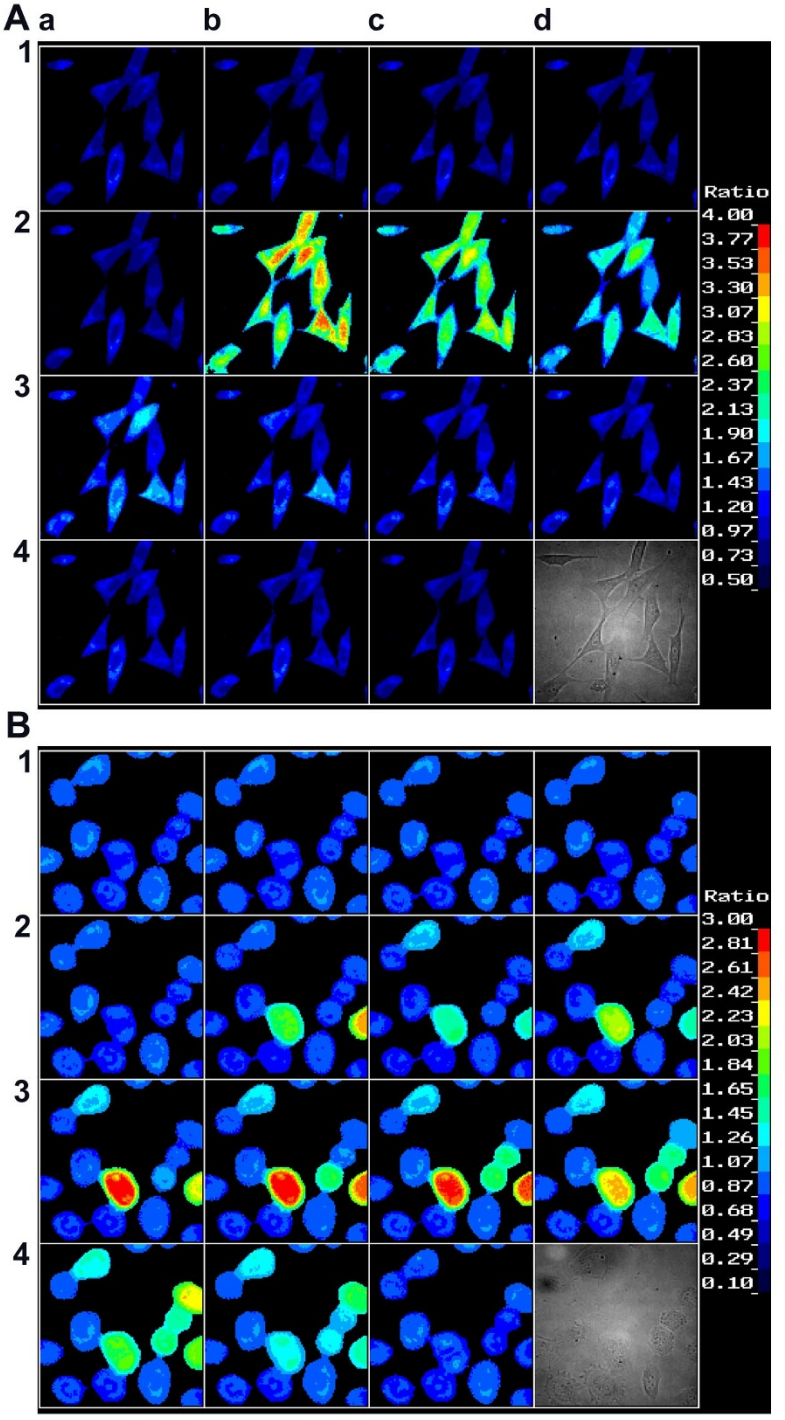

Figure 1

Effect of ethylene on $[Ca^{2+}]_i$ level in NIH-3T3 cells (A) and SaOS-2 cells (B). Cells were loaded with Fura-2 and analyzed by the fluorescence ratio-imaging system as described in Materials and Methods. Ethylene was generated by addition of 1 mM ethephon (time: 5.0 min). Fluorescence images were recorded at time zero (a1) and after 1.5 (b1), 3 (c1), 4.5 (d1), 5 (a2), 5.5 (b2), 6 (c2), 6.5 (d2), 7 (a3), 7.5 (b3), 8 (c3), 8.5 (d3), 10 (a4), 11.5 (b4), and 13.5 min (c4). In (d4) the cells inspected are shown by Nomarski phase contrast interference optics. The spectrum color scale ranges from blue (low $[Ca^{2+}]_i$) to red (high $[Ca^{2+}]_i$). Magnification, 400-fold.

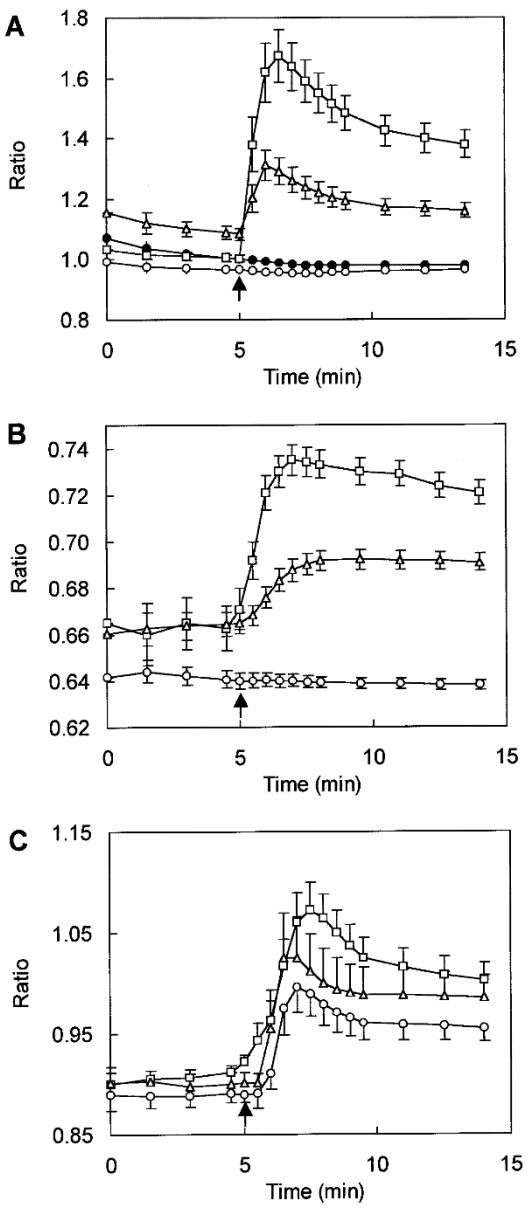

Figure 2

Changes of $[Ca^{2+}]_i$ level in NIH-3T3, HeLa and SaOS-2 cells following ethylene treatment. A. NIH-3T3 cells were treated with 0.1 mM (○), 0.3 mM (△) and 1 mM ethephon (□) 5 min after starting the experiment. In one control experiment, 0.5 mM of H_3PO_4 was added (●). **B.** HeLa cells were treated with 0.3 mM (○), 1 mM (△) and 2 mM ethephon (□) 5 min after starting the experiment. **C.** SaOS-2 cells were treated with 0.3 mM (○), 1 mM (△) and 2 mM ethephon (□) 5 min after starting the experiment. The values for the ratios of the 340/380 nm images are shown. The arrows mark the time at which ethephon was added to the cells. The results are expressed as mean values ± S.E.; n = 40.

ethylene gas was observed in nearly all cells examined. The time kinetics of change of $[Ca^{2+}]_i$ following exposure to ethylene gas revealed a steady increase in $[Ca^{2+}]_i$ level starting 1 min after ethylene injection for up to 20 min (increase in 340/380 nm ratio from 1.10 ± 0.01 to 1.40 ± 0.01; the latter value corresponds to 201 nM of $[Ca^{2+}]_i$; $p < 0.001$; Figure 4). The minimum concentration of dissolved ethylene resulting in a change of $[Ca^{2+}]_i$ level was at 3.2 µM. In the presence of 0.5 µM and 1.4 µM of dissolved ethylene gas, no effect was observed. The initial decrease in $[Ca^{2+}]_i$ level after ethylene injection (Figure 4) is most likely caused by the drop in pressure after opening the switch on the tube connecting the incubation chamber with the syringe filled with ethylene gas.

Effect of ethylene on mammalian cell proliferation

To determine the effect of ethylene on the proliferative activity of the cell lines studied, the level of the cell cycle-associated nuclear antigen Ki-67 was determined 10 h after treatment with ethylene (addition of 1 mM ethephon) or after incubation of the cells for the same time period without ethylene treatment. This antigen which is expressed only in proliferating cells was detected using a mouse monoclonal antibody (clone Ki-S5). As summarized in Table 1, the proliferative activity of NIH-3T3, HeLa and SaOS-2 cells expressed as Ki-67 labeling index (percentage of Ki-67 positive cells over the total number of cells) was significantly higher after ethylene exposure compared to untreated cells.

Effect of ethylene on $[Ca^{2+}]_i$ level in sponge cells

In parallel to mammalian cells, the effect of ethylene on the $[Ca^{2+}]_i$ level in primmorphs (aggregates of dissociated cells) from S. domuncula was determined. As shown in Figure 5, incubation of S. domuncula cells with 1 mM ethephon in Ca^{2+}- and Mg^{2+}-free artificial seawater (CMFSW) did not result in a significant change in $[Ca^{2+}]_i$ level (340/380 nm ratio). However, if the experiment was performed with cells kept in Ca^{2+}- and Mg^{2+}-containing seawater (SW) a significant ($p < 0.001$) change of $[Ca^{2+}]_i$ level in response to ethylene was observed (increase in 340/380 nm ratio from 1.64 ± 0.02 to 1.70 ± 0.01; the latter value corresponds to 243 nM of $[Ca^{2+}]_i$; Figure 5). No change of the $[Ca^{2+}]_i$ level was shown by cells without ethylene treatment (not shown). These results indicate that the presence of Ca^{2+} in the surrounding medium is essential to obtain a response of sponge cells after ethylene exposure.

Effect of ethephon on cell viability

In the concentration range used ethephon displayed no effect on cell viability as determined in L5178y mouse lymphoma cells and HeLa cells. The cells were grown in the presence of 0.001 to 1000 µM ethephon. The ED_{50} was >1 mM.

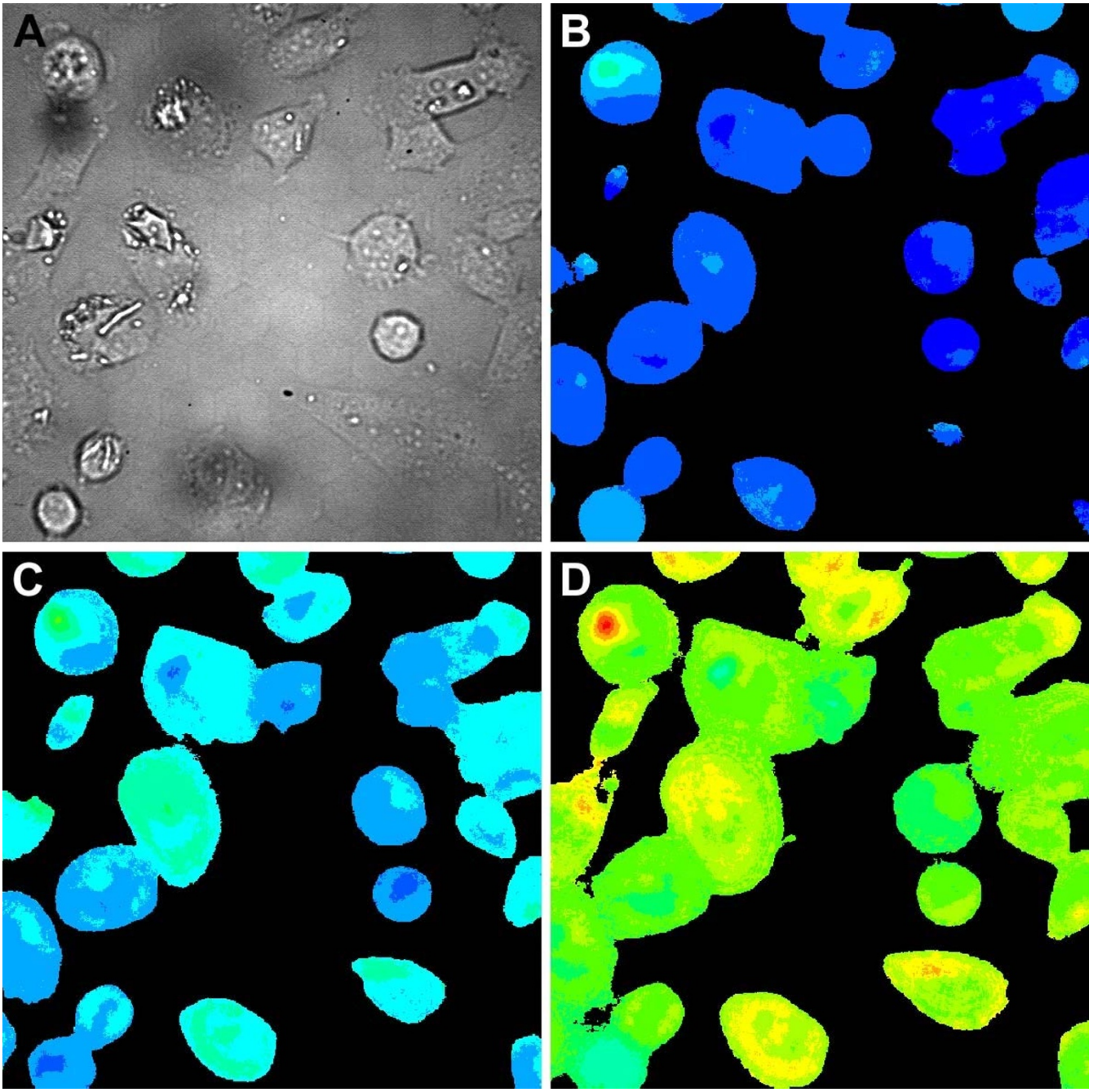

Figure 3

Effect of ethylene gas on $[Ca^{2+}]_i$ level in SaOS-2 cells kept under pressure. Cells grown on coverslip were loaded with Fura-2 and transferred into a pressure chamber as described in Materials and Methods. **A.** Cells inspected by Nomarski phase contrast interference optics. **B.** Untreated cells. **C.** Cells kept under pressure (1 atm) for 10 min. **D.** Cells kept under pressure (1 atm) 10 min after ethylene gas injection into the pressure chamber. Magnification, 200-fold. For further details, see Figure 1.

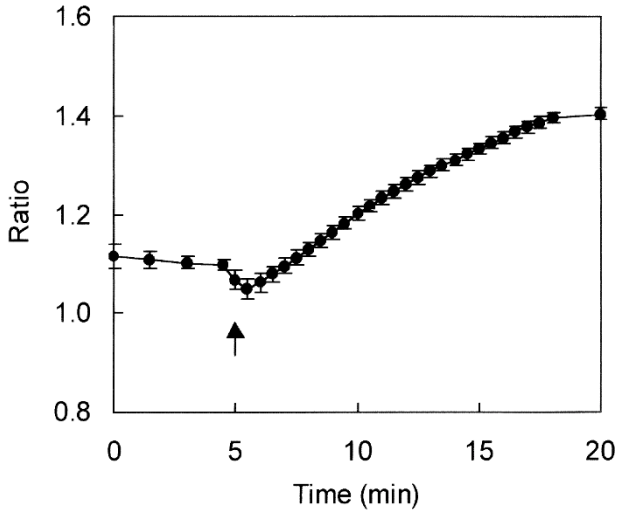

Figure 4

Changes of $[Ca^{2+}]_i$ level in SaOS-2 cells kept under pressure (1 atm) following ethylene gas exposure. Ethylene gas was injected into the pressure chamber 5 min after starting fluorescence measurements (arrow). The values for the ratios of the 340/380 nm images are shown. The results are expressed as mean values ± S.E.; n = 20.

Table 1: Effect of ethylene on proliferative activity of mammalian cells. The proliferative activity of the cells is expressed as Ki-67 labeling index. Results are means ± S.D. of 8 independent experiments.

Cell line	Ki-67 labeling index (%)	
	Without ethephon	Addition of ethephon (1 mM)
NIH-3T3	42 ± 14	68 ± 11**
HeLa	38 ± 6	47 ± 6*
SaOS-2	37 ± 7	49 ± 10*

Level of significance: * $p \leq 0.01$, ** $p \leq 0.001$.

Discussion

Previously we found that low concentrations of ethylene present in seawater [6] significantly reduce the extent of apoptosis caused by starvation in primmorphs of the marine sponge *S. domuncula* [5]. Primmorphs, which contain proliferating cells, are formed by aggregation of dissociated sponge cells [10]. In invertebrate (sponge) cells, the mode of action of ethylene, a well-known growth hormone in plants (for a review, see [14]), is still uncertain but seems to be associated with calcium metabolism [5].

In plants, ethylene has important regulatory functions; its production can be elicited by various stress factors (for a review, see [14]), including stress by oxygen radicals [15, 16]. The molecular basis of the ethylene signal transducing system has been studied mainly in *Arabidopsis* (for reviews, see [17,18,19]). A specific receptor to which ethylene binds has been identified [20].

In view of our finding that ethylene responsive pathways exist in sponges, the phylogenetic oldest metazoan phylum, we examined if cells derived from higher Metazoa (human and animal cell lines) respond to ethylene too. This alkene was produced in the culture fluid by addition of ethephon [21], which hydrolyzes in aqueous solution at pH >3.5 under formation of ethylene [22].

Using the Fura-2 imaging method, we could demonstrate that all mammalian cell lines studied reacted to ethylene, generated from ethephon, by an increase in $[Ca^{2+}]_i$ level. The rise of $[Ca^{2+}]_i$ level occurred immediately after addition of ethephon to the medium, indicating that ethylene causes a fast effect on cell metabolism. However, the threshold value and the extent of the ethylene-induced effect on $[Ca^{2+}]_i$ level strongly differed among the cells examined. NIH-3T3 fibroblasts and osteosarcoma SaOS-2 cells sensitively responded to lower concentrations of ethylene produced by 0.3 mM ethephon, while the effect on HeLa cells was rather weak but significant (at 1 and 2 mM ethephon). In addition the amount of cells responding to ethylene within the total cell population varied among the cell lines examined.

Exposure of mammalian cells kept under pressure (SaOS-2 cells) to ethylene gas, instead of ethylene generated by ethephon, caused also a strong but more protracted increase in $[Ca^{2+}]_i$ level compared to ethephon addition, most likely due to the different kinetics of changes of concentration of dissolved ethylene. This result and the finding that H_3PO_4 generated during ethephon hydrolysis does not change $[Ca^{2+}]_i$ level demonstrate that the effects observed are specifically induced by ethylene. In addition, ethephon displayed no effect on cell viability.

The increase in $[Ca^{2+}]_i$ level induced by ethylene exposure may be associated with changes in cell proliferation. Therefore, as a measure of the proliferative activity of the cells, the Ki-67 labeling index was determined. The Ki-67 antigen, a dimeric, non-histone protein with a molecular weight of 345-395 kDa is specifically expressed by proliferating cells; it is absent in resting cells [23]. Using the Ki-S5 antibody, we determined that the expression of Ki-67 was significanly increased following ethylene exposure, indicating that ethylene may activate cell proliferation. At this time point (10 h after treatment with

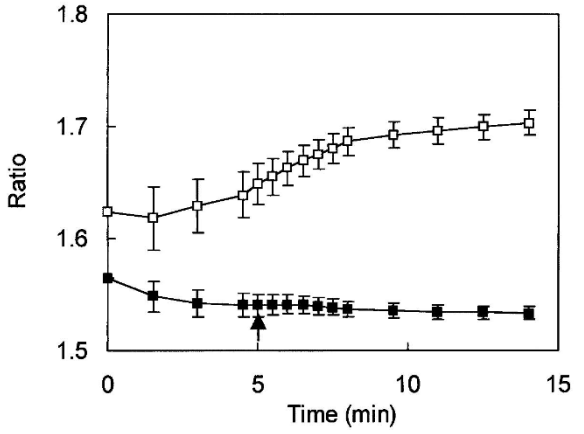

Figure 5

Changes of $[Ca^{2+}]_i$ level in primmorphs (aggregates of dissociated cells) from the sponge *Suberites domuncula* following ethylene treatment. *S. domuncula* cells were kept either in Ca^{2+}- and Mg^{2+}-free artificial seawater (CMFSW) (■) or in Ca^{2+}- and Mg^{2+}-containing seawater (SW) (□). The cells were treated with 1 mM ethephon 5 min after starting the experiment. The values for the ratios of the 340/380 nm images are shown. The arrow marks the time at which ethephon was added to the cells. The results are expressed as mean values ± S.E.; n = 30.

ethylene) $[Ca^{2+}]_i$ has recovered basal levels (not shown in Figure 2A,B,C).

The concentrations at which the ethylene-releasing agent ethephon induced a shift of $[Ca^{2+}]_i$ level in mammalian cells are in the same range or close to that at which sponge cells reacted (1 mM ethephon). However, the increase in $[Ca^{2+}]_i$ level induced by this compound in at least some mammalian cell lines was even stronger than in sponge cells. From these results we conclude that besides invertebrate (sponge) cells, mammalian cells are sensitive to ethylene. In addition, we could demonstrate that the response of sponge cells following ethylene exposure depends on the presence of Ca^{2+} in the surrounding medium; no effect was observed if this metal ion was absent in the external milieu.

At present it is unknown if ethylene binds to a membrane receptor in mammalian cells, and sponge cells too. Therefore, the mechanism of the effect of ethylene resulting in an increase in intracellular level of calcium, one important messenger in intracellular signal transduction pathways, is still unclear. In sponge cells a Ca^{2+}/calmodulin-dependent protein kinase II is up-regulated after ethylene exposure [5]. In mammalian cells, it is known that Ca^{2+} mediates the Ca^{2+}/calmodulin-dependent protein kinase II cascade [24], resulting in prevention of apoptosis [25]. The second gene which was found to be upregulated in sponges after ethylene exposure is the proposed ethylene-responsive gene, *SDERR*, which has been isolated from *S. domuncula* [5].

It should be noted that the minimal concentration of ethylene required to evoke the raise of $[Ca^{2+}]_i$ level in mammalian cells (3.2 μM) is rather high compared to the threshold concentration in plants, which may be as low as 0.1 μl/liter; this corresponds to an aqueous solution of 0.65 nM ethylene at 25°C [14]. Therefore it cannot be excluded that the ethylene-induced effects in mammalian cells are non-specific due to the anesthetic effect of the gas which is not mediated by a receptor. Future studies have to show whether animal cells possess a specific receptor for ethylene gas as found in plant cells [20]. These studies are necessary to demonstrate the physiological significance of the observed effects.

In summary, our results show that mammalian cells respond to ethylene with an increase in $[Ca^{2+}]_i$ and cell proliferation (increased expression of cell-cycle-associated Ki-67 protein). In sponges the effects of ethylene on cell physiology are associated with an upregulation of an ethylene-responsive gene, *SDERR* [5]. At present, it is unknown if similar proteins exist also in cells from higher animals and humans.

Conclusions

Measurements of intracellular calcium level ($[Ca^{2+}]_i$) in various mammalian cell lines (mouse NIH-3T3 and human HeLa and SaOS-2 cells) revealed that ethylene, produced by ethephon, caused a significant upregulation of $[Ca^{2+}]_i$ in these cells. A similar effect was found in cells kept under pressure after exposure to ethylene gas. These data support previous findings showing an upregulation of $[Ca^{2+}]_i$, as well as an increased expression of an ethylene-responsive gene, *SDERR*, in invertebrate cells (primmorphs of the marine sponge *S. domuncula*) [5]. These results indicate that ethylene is not only an important mediator of many biological processes in plants but may also have some modulatory effects on intracellular signaling pathways in animals.

Materials and Methods

Materials

Fura-2-acetoxymethyl ester (Fura-2-AM) was obtained from Molecular Probes (Leiden, The Netherlands); (2-chloroethyl)phosphonic acid (ethephon), (3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) (MTT) and natural Ca^{2+}- and Mg^{2+}-containing seawater (SW) were purchased from Sigma (Deisenhofen, Germany); and anti-Ki-67 (Ki-S5) mouse monoclonal antibody was from Roche Diagnostics (Man-

nheim, Germany). The sources of all other chemicals used were as described previously [26, 27]. The composition of Ca^{2+}- and Mg^{2+}-free artificial seawater (CMFSW) was given earlier [28].

Mammalian cells and sponge primmorphs

Mouse embryonic NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium containing streptomycin, penicillin and 10% fetal calf serum (FCS). HeLa cells were propagated in RPMI 1640 medium with 10% FCS. Human osteosarcoma SaOS-2 cells were cultivated in McCoy's 5A medium, supplemented with 1.5 mM L-glutamine, 0.1 g/l gentamicin and 15% FCS. All cell cultures were kept in a fully humidified atmosphere and 5% CO_2/air at 37°C.

Specimens of the marine sponge *Suberites domuncula* (Porifera, Demospongiae, Hadromerida) were collected in the Northern Adriatic near Rovinj (Croatia) and kept in aquaria in Mainz (Germany) at a temperature of 17°C. Sponge primmorphs were prepared from dissociated sponge cells as described [10].

Ethylene production

Ethephon was dissolved in 1x phosphate-buffered saline pH 2.5 yielding a stock solution of 69 mM and kept at 4°C. At this pH, an aqueous solution of ethephon is stable. At higher pH values (> pH 3.5), ethephon hydrolyses under formation of free ethylene and phosphoric acid (H_3PO_4) [22].

Calcium measurements

The concentration of intracellular calcium ($[Ca^{2+}]_i$) was determined using the Ca^{2+}-indicator dye Fura-2-AM; the fluorescence ratio at 340 and 380 nm was measured as described [29, 30]. "Chambered coverglass" incubation chambers (Lab-Tek, Nunc) were coated with poly-L-lysine. Mammalian cells were loaded in the dark with 6-8 µM Fura-2-AM in medium containing 1% FCS and 1% bovine serum albumin at 37°C for 1 h; sponge cells (primmorphs) were loaded with 10-12 µM Fura-2-AM in CMFSW containing 1% bovine serum albumin at 17°C for 2 h. Subsequently, the cells were washed twice with medium supplemented with 10% (HeLa and NIH-3T3 cells) or 15% FCS (SaOS-2 cells) and incubated further at 37°C for 1 h; sponge cells were washed with CMFSW and incubated further at 17°C for 1 h. These time periods were sufficient to load the cells with Fura-2-AM (inactive Fura-2) and for hydrolysis of the acetoxymethyl ester (active Fura-2). For the experiments mammalian cells were incubated with ethephon (0.1 to 2 mM) in Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose and 10 mM Hepes, pH 7.4); sponge cells were incubated in SW or CMFSW. Treatment of the cells with ethephon was performed 5 min after starting measurement of $[Ca^{2+}]_i$, which proceeded for 13.5 min. Control assays were performed by addition of 0.5 mM H_3PO_4 instead of ethephon. Fluorescence images were analysed using an inverted-stage Olympus IX70 microscope and a computerized imaging system as described before [30]. Calibration was performed with the "Fura-2 Calcium Imaging Calibration Kit" according to the instructions of the manufacturer (Molecular Probes). One unit of fluorescence ratio 340/380 nm equals ≈ 143 nM $[Ca^{2+}]_i$.

To determine the effect of ethylene gas on $[Ca^{2+}]_i$ of SaOS-2 cells, cells on coverglass (2×10^5 cells/cm^2 in 100 µl medium; after loading with Fura-2-AM and activation as above; diameter of the glass plates, 1 cm) were transferred into a pressure chamber as described previously [5]. The pressure in the chamber was generated by air (1 atm). Varying amounts of ethylene gas in air were injected using a syringe (10 ml; approximately a 5-fold excess compared to total gas space of the system) 5 min after starting measurement of $[Ca^{2+}]_i$, which was performed for 20 min.

The amount of ethylene dissolved in medium within the pressure chamber after injection of ethylene gas was determined in a Shimadzu GC9A apparatus using a FID detector. A column (size 3 × 600 mm) filled with aluminium oxide was used and the runs were performed at 70°C.

Immunostaining of Ki-67 antigen

Cells are fixed with 4% paraformaldehyde and incubated with anti-Ki-67 (Ki-S5) antibody for 30 min at room temperature. After washing, the cells were incubated with anti-mouse Ig-fluorescein for 30 min at room temperature. Analysis was performed by fluorescence microscopy. The proliferative activity of the cells is expressed as the percentage of Ki-67 positive cells over the total number of cells.

Cell viability

The viability of HeLa cells and L5178y mouse lymphoma cells [31] was determined by the MTT colorimetric assay [32]. Evaluation (at 595 nm) was performed using an ELISA plate reader (BioRad 3550) equipped with the program NCIMR IIIB. The effective dose, which inhibits cell proliferation by 50% (ED_{50}) was estimated by logit regression [33].

Statistics

The results were analyzed by Student's *t*-test [33].

Abbreviations

$[Ca^{2+}]_i$, intracellular concentration of Ca^{2+}; CMFSW, Ca^{2+} and Mg^{2+}-free artificial seawater; FCS, fetal calf se-

rum; MTT, 3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; SW, Ca^{2+}- and Mg^{2+}-containing seawater.

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