Regulation of intracellular pH by cell–cell adhesive interactions

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Abstract **As was shown in our previous work, the intracellular pH (pH_i) of cultured human fibroblasts depends on cell density. The pH_i is low in single cells, higher in cells, forming small groups and maximal in a sparse monolayer. On the other hand, the pH_i is low in areas of confluent monolayers. In the present work, we show that the effects of inhibitors of various pH-controlling mechanisms as well as inhibitors of key enzymes in signal transduction pathways depend on the local cell density. We have found that *N*-ethylmaleimide and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole, known as inhibitors of V-type H^+ ATPase, inhibit the elevation of pH_i induced by cell–cell contact interactions; meanwhile Cd^{2+} ions, which inhibit H^+ conductive pathway, cause an increase of pH_i in a confluent monolayer. Our data revealed also that the Na^+/H^+ antiporter does not play an essential role in the pH_i regulation by intercellular contacts.**

Inhibitors of phospholipase A_2 (4-bromophenacyl-bromide), phospholipase C (neomycin) and protein kinase C (H-7) dramatically change the way the pH_i is modulated by local cell density. It is suggested that cell–cell interactions regulate cell activities via modulation of pH_i, which is under positive control from phospholipase A2 and under negative control from protein kinase C.

Key words: Intracellular pH; Cell–cell interaction; Phospholipase A2; Protein kinase C; H^+-ATPase; Na^+/H^+ antiporter; H^+-conductance

1. Introduction

Many cell functions are regulated by cell–cell contact interactions. The mechanism by which information about the number of cells in the local environment is integrated in the cell and affects cell behavior remains to be elucidated. We have shown previously that the pH_i in cultured fibroblasts and attached neutrophils depends on adhesive interactions of cells with neighboring cells [1–4] and suppose that cell–cell contact interactions could affect various biochemical reactions in the cytoplasm by modulation of the pH_i. Changing of intracellular pH is a result of the signal transduction pathway from cell surface adhesive receptors to the cell interior. In the recent work we checked the involvement of key enzymes of signal transduction, such as phospholipase C, phospholiase A_2 and protein kinase C in pH_i regulation by cell–cell interactions. The aim of this work was also to clarify which of the known mechanisms of pH_i regulation contribute to the pH_i modulation by cellular contacts.

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Abbreviations: PLA2, phospholipase A2; PLC, phospholipase C; PKC, protein kinase C; Cl-NBD, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; NEM, *N*-ethylmaleimide.

2. Materials and methods

2.1. Materials

Bovine calf serum, lactalbumin hydrolysate and Eagle's medium were obtained from the Institute of Poliomyelitis (Moscow). Bicarbonate-free Hanks' solution, *N*-ethylmaleimide, amiloride, phorbol-12-myristate-13-acetate (PMA), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), bafilomycin, Cl-NBD, 4-bromophenacyl bromide, neomycin, nigericin were purchased from Sigma. Acetomethyl ether of 2′,7′-bis(2carboxyethyl)-5,(6)-carboxyfluorescein (BCECF) for measurement of the pH_i was obtained from Molecular Probes.

2.2. Cell culture

Human fibroblasts were grown in medium consisting of 45% Eagle's medium, 45% lactalbumin hydrolysate and 10% bovine calf serum. To obtain a confluent monolayer, cells were plated at high density (10^5 cells/ml) and grown for 4–5 days till confluency. Then cultures were 'wounded': part of the monolayer was mechanically removed and the cultures were incubated another 2–3 days. During this time cells from the edge of the monolayer have migrated in the free area of the substratum and areas of various cell densities were formed: (i) single cells non contacting each other; (ii) cells, contacting several neighboring cells; (iii) cells forming a loose monolayer at the edge of the wound; (iv) cells in a confluent monolayer. One hour before the pH_i measurement the medium was changed to bicarbonate-free Hank's solution buffered by 10 mM HEPES (pH 7.30) and the pH was measured. Various drugs were added in the concentration reported to be optimal for their specific effects. Amiloride (10 μM) was added to the cultures for 10 min or 1 h in DMSO, control cells were incubated in Hank's solution with the addition of the respective amount DMSO. To affect H^+ ATPase cells were incubated for 20 min in Hank's solution in the presence of either 100 μM of Cl-NBD, 100 μM of NEM or 1 μM bafilomycin. To affect PLA2, cells were incubated for 20 min in Hank's solution in the presence of 20 μM of 4-bromophenacyl bromide. To modulate the activity of PKC cells were incubated for 20 min in Hank's solution in the presence of 100 μM H-7 or 100 nM PMA. To affect PLC, cells were incubated for 20 min or 1 h in Hank's solution in the presence of 100 μM neomycin. To affect the ionic status of the cells, cultures were incubated for 30 min in Na-free medium, consisting of 10 mM HEPES, 108 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.09% glucose, or for 20 or 40 min in Hank's solution in the presence of 100 μM Cd^{2+} ions or for 10 min in Ca^{2+}-free Dulbecco medium.

2.3. pH measurement

Cells were incubated for 30 min in 5 μM of BCECF and the emission at 520 nm was measured with a microfluorimeter-equipped Zeiss microscope at two excitation wave-lengths (430 and 490 nm) as described in [5]. Calibration was performed as described in [6]. All the data shown are the mean values of pH_i–S.E. obtained from the data of measuring pH_i in 15–30 cells for each area.

3. Results and discussion

We have found previously and confirmed in the present work that the pH_i in cultured human fibroblasts depends on the local cell density [1–4]. By using the technique of a 'wounded' fibroblast monolayer we obtained areas of four different local cell density in one culture as described in section 2. By comparing the pH_i in these areas of the same preparation we avoided the possible influence of various diffusible factors released by cells

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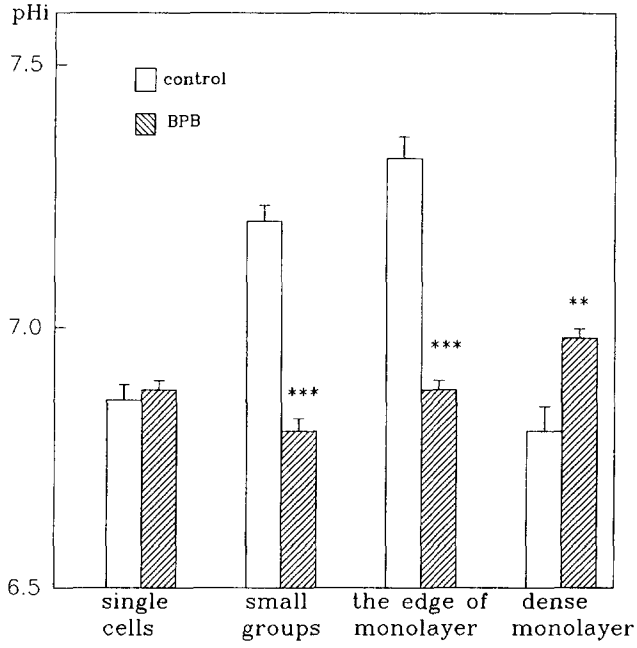

Fig. 1. The effect of 4-bromophenacyl bromide on the pH_i of fibroblasts in areas of various cell density. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, when compared to control values in the same area.

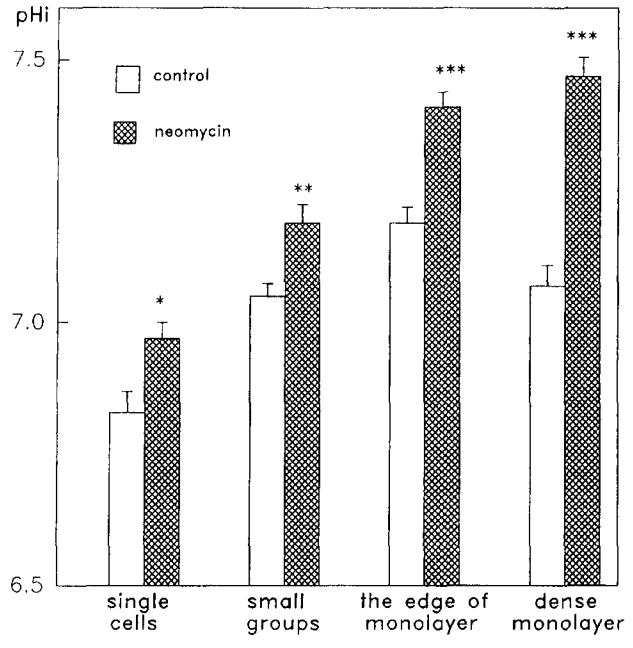

Fig. 2. The effect of neomycin on the pH_i of fibroblasts in areas of various cell density. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, when compared to control values in the same area.

on pH_i (unless they diffuse in the range of a few cell diameters). We have found that the establishing of intercellular contacts results in an increase of pH_i. pH_i is low in single cells, higher in cells forming small groups, and maximal in sparse monolayer. pH_i is low again in areas of a confluent monolayer. What are the mechanisms of intracellular pH regulation by cell density?

To evaluate the relative contribution of various pH_i-controlling systems as a function of local cell density we have measured the intracellular pH in these areas in control and drug-treated cultures using inhibitors of various pH_i-controlling pathways. Activation of the Na^+/H^+ antiporter was shown to be the main mechanism increasing intracellular pH upon stimulation of cells by soluble growth factors and mitogens ([7], for review). We measured the pH_i in various zones of cell density in the presence of an inhibitor of the Na^+/H^+ antiporter, amiloride [7]. After 10 min incubation of cell cultures with 10 μM of amiloride the pH_i was increased in the areas of a cell monolayer, at it's edge and to a lesser degree in small groups of cells (Table 1). The effect of amiloride was reversed and the pH_i dropped to the control values after 1 h of incubation although the drug remained in the media.

Substitution of Na^+ ions in the extracellular madium by K^+ ions did affect the pH_i in neither zones of various cell density

Table 1
The effect of inhibitors of various pH-regulating mechanisms on the pH_i in fibroblasts at different cell densities

Inhibitors	Single cells	Small groups	The edge of monolayer	Confluent monolayer
Inhibitors of Na/H antiporter:				
None	6.75 ± 0.03	6.87 ± 0.03	7.10 ± 0.02	6.98 ± 0.03
Na-free medium	6.83 ± 0.03	6.94 ± 0.03	7.10 ± 0.02	7.14 ± 0.02***
Amiloride, 10 min.	6.64 ± 0.03	6.98 ± 0.03*	7.36 ± 0.03***	7.36 ± 0.04***
Amiloride, 20 min.	6.59 ± 0.01***	6.77 ± 0.03	7.05 ± 0.03	7.05 ± 0.03
Inhibitors of V-type H-ATPase:				
None	6.97 ± 0.03	7.07 ± 0.03	7.25 ± 0.04	6.90 ± 0.03
Bafilomycin	7.06 ± 0.03	7.11 ± 0.04	7.31 ± 0.04	
NEM, 20 min.	6.79 ± 0.03***	6.90 ± 0.02***	6.98 ± 0.02***	6.97 ± 0.01
Cl-NBD, 20 min.	6.80 ± 0.01***	6.83 ± 0.01***	6.85 ± 0.01***	6.79 ± 0.01**
Inhibitor of H-conductance:				
None	6.87 ± 0.03	7.04 ± 0.03	7.19 ± 0.03	6.91 ± 0.03
Cd ions, 20 min.	6.87 ± 0.02	6.94 ± 0.03	7.26 ± 0.03	7.17 ± 0.03***
Cd ions, 40 min.	6.84 ± 0.02	7.04 ± 0.03	7.37 ± 0.04**	7.60 ± 0.09***
Ca-free medium	7.05 ± 0.03	7.12 ± 0.03	7.24 ± 0.02	7.29 ± 0.04

$^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, when compared to control values in the same area.

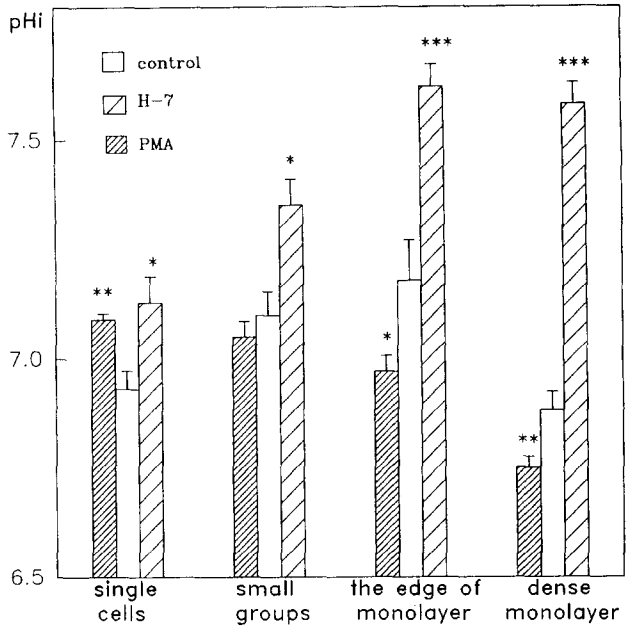

Fig. 3. The effect of H-7 and PMA on the pH_i of fibroblasts in areas of various cell density. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, when compared to control values in the same area.

(Table 1), but increased it in confluent monolayer. These results indicated that the Na^+/H^+ antiporter is not responsible for the pH_i dependence on cell density, although it's activity may contribute to the decrease in pH_i upon establishing many cell–cell contacts in a confluent monolayer.

We have checked whether the inhibition of H^+-ATPase affects cell density-dependent modulation of the pH_i. Plasma membrane-localized H^+-ATPase was found to be operative at physiological pH_i levels and to contribute to the maintenance of a steady-state pH_i in adherent resident macrophages [8,9] and osteoclasts [10]. We have treated cell cultures with the H^+-ATPase inhibitors [11] Cl-NBD, NEM and bafilomycin (the latter reported to be a specific inhibitor of vacuolar type H^+-ATPase). We have found that both NEM and Cl-NBD (100 μM, 10 min) significantly decreased the pH_i (Table 1), so that the pH_i became almost the same in cells in all zones. Bafilomycin (1 μM, 10 min), demonstrated to inhibit cytoplasm alkalinization after artificial acidification [12,13], was ineffective in our experiments (Table 1). Earlier it was reported to be of no effect on the pH_i in adherent macrophages [9]. Whether H^+-ATPase in these cells was resistant to bafilomycin, or NEM and Cl-NBD have some other mechanism of action remains to be studied.

To estimate the role of the H^+ conductive pathway ([14], for review) in cell contact regulation of the intracellular pH, we studied the effect of Cd^{2+} ions on the pH_i in areas of various cell density. Incubation of cell cultures with 100 μM of Cd^{2+} for 20 min increased the pH_i in confluent monolayers (Table 1). Another 20 min of incubation made this effect even more pronounced (Table 1). Thus H^+ conductance does not play a role in maintaining the elevated level of pH_i in zones of high local cell density, but can contribute to the decrease of pH_i in confluent monolayers, acting for example as a proton influx mechanism. Although the opening of H^+ channels may result in both inward and outward currents [14,15], only outward H^+ currents were detected over a wide range of conditions preventing excessive acidification of the cytoplasm [14–19].

Ca^{2+} plays an important role in regulating the pH_i: Ca^{2+} entry was reported to 'mimic the effect of proton influx' in snail neurons [15,20]. In our experiments Ca^{2+}-free media did not affect the pH_i in any cell density zones, but in the dense monolayer, where the pH_i was increased relative to the control (Table 1). The effect of Ca^{2+}-free media on the pH_i was similar to the effect of Cd^{2+} and may be caused by the same mechanism since Cd^{2+} was reported to block Ca^{2+} channels [21] and thus interfere with Ca^{2+} entry.

To find out the role of the key elements of signal transduction cascade in pH_i modulation by cell–cell contacts we studied the effect of inhibitors of phospholipase C, phospholipase A2 and protein kinase C on the pH_i in areas of various cell density. The inhibitor of PLA2 4-bromophenacyl bromide (20 μM, 20 min) decreased pH_i in zones of all cell densities but in single cells (Fig. 1), thus making the pH_i become independent of cell density. An inhibitor of PLC, neomycin (100 μM, 20 min) increased the pH_i in all cell density zones and abolished the descent of pH_i in confluent monolayer (Fig. 2).

Incubation of cell cultures with the inhibitor of PKC H-7 (100 μM, 20 min) increased the pH_i in cells of all density zones. This effect was most pronounced in cells in zones of high local density, especially in confluent monolayer (Fig. 3). After treatment with H-7 the pH_i increased with the increase of cell density. Activation of PKC with PMA (20 min, 100 nM) had an opposite effect to the above described PKC inhibition: the pH_i was increased in single cells and decreased in monolayers. Thus the pH_i became to be inversely correlated with the local cell density (Fig. 3).

It seems that occupation of cell adhesive receptors by contacts with neighboring cells induces PLA2 activity, which results in the increasing of the pH_i. The intercellular contacts also activate PLC and PKC which execute the negative regulation of pH_i.

In conclusion, we have found that the effects of drugs which inhibit various cellular enzymes on pH_i depend on the local cell density. A complex interplay of enzymes involved in signal transduction seems to depend on the loca cell density which is translated in modulating the pH_i depending of the number of cell–cell contacts. The pH_i in its turn affects intercellular chemical reactions providing the way for cell neighbors to exercise control over cell functions.

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References

[1] Galkina, S.I., Sud'ina, G.F. and Margolis, L.B. (1992) Exp. Cell Res. 200, 211–214.
[2] Galkina, S.I., Sud'ina, G.F. and Margolis, L.B. (1992) Biol. Membr. 5, 925–935.
[3] Galkina, S.I., Sud'ina, G.F. and Margolis, L.B. (1994) Biol. Membr. 7, 17–26.
[4] Sud'ina, G.F., Galkina, S.I., Barski, O.A. and Margolis, L.B. (1994) FEBS Lett. 336, 201–204.
[5] Paradiso, A.M., Tsien, R.J. and Machen, T.E. (1984) Proc. Natl. Acad. Sci. USA 81, 7436–7440.

[6] Thomas, J.A., Buchsbaum, R.N., Zimniak, K.A. and Racker, E. (1979) Biochemistry 18, 2210–2218.
[7] Grinstein, S., Rotin, D. and Mason, M.J. (1989) Biochim. Biophys. Acta 988, 73–97.
[8] Tapper, H. and Sundler, R. (1992) Biochem. J. 281, 239–244.
[9] Tapper, H. and Sundler, R. (1992) Biochem. J. 281, 245–250.
[10] Baron, R., Neff, L., Louvard, D. and Courtoy, P.J. (1985) J. Cell Biol. 101, 2210–2222.
[11] Forgac, M. (1989) Physiol. Rev. 69, 765–796.
[12] Nanda, A., Gukovskaya, A., Tseng, J. and Grinstein, S. (1992) J. Biol. Chem. 267, 22740–22746.
[13] Swallow, S.J., Grinstein, S., Sudsbury, R.A. and Rotstein, O.D. (1993) 158, 453–460.
[14] DeCoursey, T.E. and Cherny, V.V. (1994) J. Membr. Biol. 141, 203–223.
[15] Thomas, R.S. (1989) Ann. NY Acad. Sci. 574, 287–293.
[16] Henderson, L.M., Chappel, J.B. and Jones, O.T.G. (1987) Biochem. J. 246, 325–329.
[17] Kapus, A., Romanec, R. and Grinstein, S. (1994) J. Biol. Chem. 269, 4736–4745.
[18] Nanda, A. and Grinstein, S. (1991) Proc. Natl. Acad. Sci. USA 88, 10816–10820.
[19] Kapus, A., Szaszi, K. and Ligeti, E. (1992) Biochem. J. 281, 697–701.
[20] Ahmed, Z. and Connor, J.A. (1980) J. Gen. Physiol. 75, 403–426.
[21] Th'evenod, F. and Jones, S.W. (1992) Biophys. J. 63, 162–168.