Volume 243, number 1, 17–20 FEB 06680 January 1989

Growth stimulation by bombesin does not involve activation of Na^+/H^+ exchange in chick embryo otic vesicles in vitro

J.I. Gillespie, F. Giraldez* and J.R. Greenwell

Department of Physiological Sciences, The Medical School, Framlington Place, The University, Newcastle upon Tyne NE2 4HH, England and **Departamento de Bioquimica y Biologica Molecular y Fisiologia, Universidad de Valladolid, 47005-Valladolid, Spain*

Received 17 October 1988

The effect of the mitogen bombesin on pH_i in cells of the chick otic vesicle was studied using dual wavelength micro-spectrofluorimetric techniques. The results show that the bombesin did not produce an immediate alkalinization in quiescent otic vesicle cells nor a long-term change in pH_i in vesicles reactivated to grow in culture. The recovery of pH_i from an acid load, caused by an NH_4 pulse, involved both Na^+/H^+ exchange and $Na^+:HCO_3$ influx mechanisms, neither of which was influenced by bombesin. These observations do not fit with a general model whereby pH_i is a universal signal for DNA replication and cell proliferation.

pH, intracellular; Cell proliferation; Development; Na^+/H^+ antiport; $Na^+:HCO_3$ symport

1. INTRODUCTION

Current models to describe the mechanisms which activate cell proliferation involve a stimulation of Na^+/H^+ exchange and cytoplasmic alkalinisation [1–6]. In the sea urchin embryo an increase in intracellular pH (pH_i) has been shown to be an important step leading to replicative DNA synthesis and cell division [7]. However, in quiescent cultured cells the evidence for a direct link between the observed stimulation of Na^+/H^+, the resulting increase in pH_i and cell division is less clear [6].

Cell division, in the epithelium forming the developing inner ear of the 2-day-old avian embryo, can be arrested in vitro by incubation in serum free media and reactivated by serum, growth factors or mitogens [8]. This process requires protein kinase C activation and can be blocked by amiloride [9], suggesting a role for the Na^+/H^+ exchanger in cell activation of these cells. The present experiments use the potent mitogen bombesin to examine directly the involvement of pH_i and the Na^+/H^+ antiport in the stimulation of cell proliferation in cells of the isolated otic vesicle.

No difference in resting pH_i could be detected nor any stimulation of Na^+/H^+ exchange. These results question the universal nature of pH_i as a signal for stimulating DNA replication and cell division.

Correspondence address: J.I. Gillespie, Department of Physiological Sciences, The Medical School, Framlington Place, The University, Newcastle upon Tyne NE2 4HH, England

2. MATERIALS AND METHODS

Intact otic vesicles, free of surrounding mesenchyme, were isolated from chick embryos at stage 18 [10] as described [11]. Vesicles were used immediately on dissection, after culture in serum free, antibiotic free medium (M-199 medium with Hank's salts; Flow Laboratories) or after culture in media containing 200 nM bombesin + 10 U insulin [8]. Tissues were maintained at 37°C in an atmosphere with 5% CO_2.

Experiments were done on small epithelial segments (100 μm^2) micro-dissected from the otic vesicle epithelium and placed in a perfusion chamber (200 μl) on the stage of a dual emission micro-spectrofluorimeter [12]. Cells were loaded with the fluorescent pH sensitive dual emission dye dicyanohydroquinone (DCH) [13] by incubation with the esterified form diacetoxythalonitrile (ADB: Molecular Probes,

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

Oregon, USA, or Paesel, FRG) for 5 min. The experiments were done in Balanced Salt Solutions containing 140 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes buffer at pH 7.4. Na^+ free solutions were made using potassium or *N*-methylglutamine as substitute. HCO_3 was added where needed. Nominally HCO_3 free solutions were measured to contain 1 mM and nominally 5 mM HCO_3 contained 6 mM as measured using a blood gas analyser (ABL3: Radiometer, Copenhagen, Denmark). Vesicular growth was assessed by inspection at ×40 magnification. In some experiments images were recorded on video. Results are expressed as means ± 1 SE and the difference between means was determined using Student's *t*-test with a level of significance set at $P < 0.05$. Experiments were done at 30–35°C.

3. RESULTS AND DISCUSSION

The pH_i of freshly dissected cells was 7.28 ± 0.03 ($n = 9$). In restricted cells, cultured in serum free media for 24 h, pH_i was 7.36 ± 0.06 ($n = 19$), not significantly different from fresh tissue. In cells, restricted for 24 h and re-activated by culturing for a further 24 or 48 h in media containing bombesin, pH_i was 7.41 ± 0.06 ($n = 13$) again not significantly different from fresh or restricted tissue. These observations suggest that depression of cell proliferation and subsequent reactivation does not, with prolonged exposure, lead to alterations in pH_i.

The possibility that the action of bombesin may activate a transient change in pH_i was investigated by applying 100 nM bombesin + insulin to cells that were arrested by culturing in serum free medium for 24 h. Two experiments are shown in fig.1A on cells bathed in nominally HCO_3 free medium. In (a) there was small sustained acidification of approx. 0.2 pH units and in (b) there was a transient acidification of 0.1 pH units followed by a slow acidification. It is interesting to note that in these experiments (and 5 others) we never observed an alkalinisation, even if cells were acidified prior to bombesin by an exposure to low external pH.

Growth factors, although not affecting pH_i, may have effects on the kinetics of the pH_i regulating mechanisms [2]. Examination of the pH regulatory mechanism in the otic vesicle cells, by studying the recovery of pH_i after an acid loading, revealed two regulatory mechanisms. A detailed study of these will be the subject of a subsequent paper. Briefly, the recovery of pH_i, in the absence of HCO_3 was not complete, Na dependent and inhibited by amiloride suggesting the involvement of Na^+/H^+ exchange. Full recovery was HCO_3 dependent, required extracellular Na and was not affected by Cl removal or amiloride, suggesting the operation of an Na^+:HCO_3 influx mechanism of the type described by Boron and Boulpaep [14]. Recently, the latter mechanism was also found in chick embryonic somitic cells [15] and neural tube cells [16].

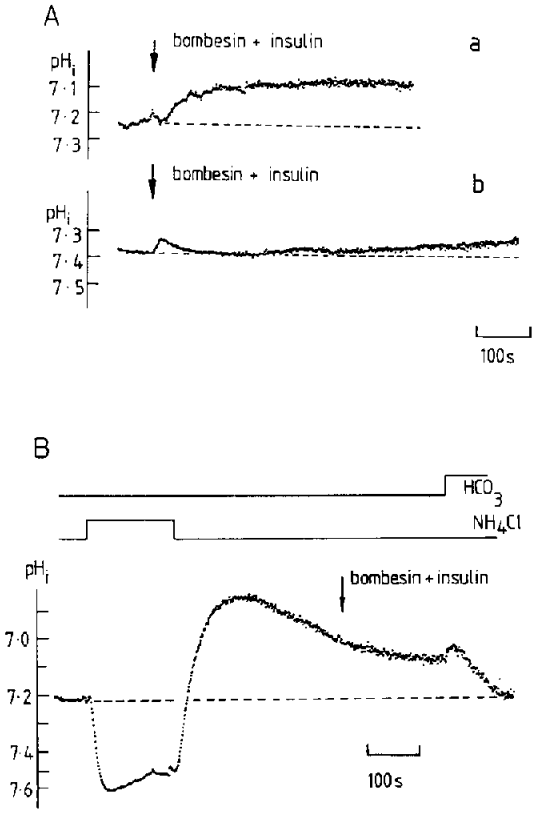

Fig.1. (A) Effect of bombesin on pH_i in cells from quiescent otic vesicles. The two traces correspond to different experiments. The arrows indicate the times at which the solutions were changed from the standard solution to one containing 200 nM bombesin + 10 IU insulin. Vesicles were made quiescent by incubation in serum free media for 24 h. (B) Recovery of pH_i after an acid load in cells from a quiescent otic vesicle. The cells were superfused with the standard solution and acidified with a pulse of 30 mM NH_4Cl for the time indicated. Where shown bombesin (200 nM) + insulin (10 IU) and HCO_3 were added to the superfusion solutions.

The effect of bombesin on the Na^+/H^+ antiport was then examined by measuring the rate of extrusion of protons after an acid load in HCO_3 free

solutions. An experiment of this kind, where the cells were acidified using an NH_4Cl prepulse, is shown in fig.1B. It can be seen that bombesin did not accelerate the rate of recovery from the acid load in the absence of HCO_3 where the Na^+/H^+ exchange is most likely to be operating. The failure of pH_i to recover to the resting pH, in the absence of HCO_3, suggests that the set point for the Na^+/H^+ exchange, after a short-term exposure to bombesin, remains more acid than the resting pH.

Measurements of pH_i were also performed on cells which were reactivated by incubation in media containing 100 nM bombesin + insulin for 24 h and the steady state and the pattern of pH regulation compared with quiescent vesicles. As mentioned above, no clear shift of the resting pH could be detected after reactivation with bombesin despite a clear effect on vesicular growth. The pattern of pH_i regulation in bombesin treated vesicles is illustrated in fig.2. The recovery of an induced NH_4Cl acid load was again incomplete in nominally HCO_3 free solution (fig.2A), full recovery occurred in the presence of HCO_3 and was insensitive to amiloride (fig.2B). The latter experiment is of special relevance because it shows that, in vesicles stimulated to grow by bombesin, the full capacity to regulate pH_i, in the absence of the Na^+/H^+ antiport remains intact. Although the resting pH_i appeared not to be dependent on the Na^+/H^+ antiport, the possibility still remained that a stimulation of the Na^+/H^+ antiport was masked by the HCO_3 dependent mechanism. This is, however, unlikely since the steady state pH_i in bombesin stimulated vesicles on recovery from an acid load and in the absence of HCO_3 was 7.03 ± 0.021 (n = 3) and not significantly different from fresh or restricted tissue (6.88 ± 0.09; n = 5). This would suggest that there is no alteration in the set point of the Na^+/H^+ exchanger after long-term stimulation with bombesin. Furthermore, there was no apparent change in the value to which pH_i relaxed in the presence of HCO_3 suggesting that the set point for this mechanism was also unaltered. Values for the steady state pH recovered in the presence of 10 mM HCO_3 were 7.25 ± 0.17 (n = 4) and 7.20 ± 0.19 (n = 5) for quiescent and bombesin treated vesicles, respectively.

In summary, the results show that bombesin did not produce an immediate or long-term stimulation of the Na^+/H^+ exchanger judged by its effect on the resting pH_i and the rate of recovery from an acid load. Similarly, we could not detect any change in the functions of the Na^+:HCO_3 influx mechanism by bombesin. In our view, these observations limit the model of a pH shift as a universal signal for DNA proliferative replication.

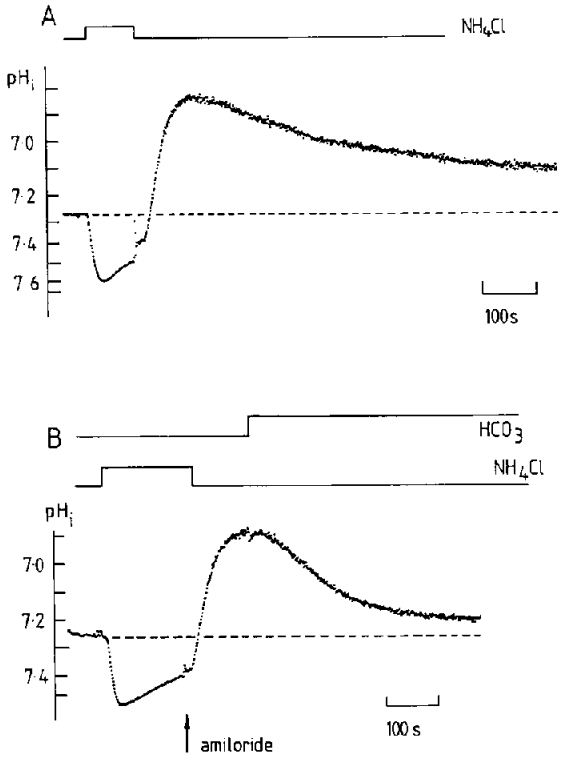

Fig.2. Recovery from an acid load in cells from an otic vesicle reactivated by incubation with bombesin for 22 h. (A) The tissue was acidified by a pulse of 30 mM NH_4Cl for the time indicated. The cells were then allowed to recover in standard solution in the nominal absence of HCO_3. (B) A similar experiment as in A but the cells were exposed to amiloride (10^{-3} M) during the recovery. Where shown 10 mM HCO_3 was added to the standard solution.

Acknowledgements: We gratefully acknowledge the support of The British Council, DGICYT (grant no.PB86/0326), Action Research for the Crippled Child and The Wellcome Trust.

REFERENCES

[1] Rozengurt, E. (1986) Science 234, 161–166.
[2] Moolenaar, W.H. (1986) Annu. Rev. Physiol. 48, 363–379.

[3] Moolenaar, W.H., Tertoolen, L.G.J. and De Laat, S.W. (1984) Nature 312, 371–374.
[4] Bright, G.R., Fisher, G.W., Rogowska, J. and Lansing Taylor, D. (1987) J. Cell Biol. 104, 1019–1033.
[5] Nuccitelli, R. and Heiple, J. (1982) in: Intracellular pH: Its Measurement and Utilisation in Cell Functions, pp.567–586, Alan R. Liss, New York.
[6] Busa, W.D. (1986) Annu. Rev. Physiol. 48, 389–402.
[7] Whitaker, M.J. and Steinhardt, R.A. (1982) Q. Rev. Biophys. 15, 593–666.
[8] Represa, J.J., Miner, C., Barbosa, E. and Giraldez, F. (1988) Development 102, 87–96.
[9] Miner, C., Represa, J.J., Barbosa, E. and Giraldez, F. (1988) Roux's Arch. Dev. Biol. 197, 294–297.
[10] Hamburger, V. and Hamilton, H.L. (1951) J. Morphol. 88, 49–92.
[11] Van de Water, T.R. and Rubin, R.J. (1974) Ann. Otol. Rhinol. Laryngol. 83, suppl.14, 1–16.
[12] Gillespie, J.I. and Greenwell, J.R. (1987) J. Physiol. 391, 10P.
[13] Valet, G., Raffael, A., Moroder, E., Wunsch, E. and Rubenstroth-Bauer, G. (1981) Naturwissenschaften 68, 265–266.
[14] Boron, W. and Boulpeap, E.L. (1983) J. Gen. Physiol. 81, 53–94.
[15] Gillespie, J.I. and Greenwell, J.R. (1988) J. Physiol. 405, 385–395.
[16] Dickens, C.J., Gillespie, J.I. and Russell, E. (1988) in preparation.