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CHANGES IN INTRACELLULAR pH AND pH REGULATING MECHANISMS IN SOMITIC CELLS OF THE EARLY CHICK EMBRYO: A STUDY USING FLUORESCENT pH-SENSITIVE DYE

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SUMMARY

1. Measurements of intracellular pH (pH_i) have been made using the fluorescent dye dicyano-hydroquinone (DCH) in somites isolated from 2-day-old chick embryos. Measurements of pH_i, from freshly dissected somites, gave values of $7{\cdot}18 \pm 0{\cdot}02$ (mean $\pm$ S.D., $n = 12$) while in somites kept in media containing 10% fetal calf serum for 2–5 h pH_i was $7{\cdot}36 \pm 0{\cdot}02$ ($n = 11$).

2. In freshly dissected control tissue recovery from an acid load (NH_4Cl pre-pulse), in the nominal absence of HCO_3^-, was $0{\cdot}007 \pm 0{\cdot}003$ pH units/min ($n = 11$). In the presence of 5 mM-HCO_3^- the rate of recovery was increased to $0{\cdot}132 \pm 0{\cdot}003$ pH units/min ($n = 11$). This HCO_3^--dependent recovery was inhibited by pre-treatment with DIDS (5×10^{-4} M) and stopped when Na^+ was replaced in the bathing medium with K^+ or *N*-methylglucamine. Amiloride (10^{-3} M) had no effect.

3. Replacement of Cl^- with gluconate had little effect on pH_i in control somites suggesting that absence of Cl^-–HCO_3^- exchange.

4. These observations are consistent with the presence of a coupled Na^+–HCO_3^- entry and the absence of Na^+–H^+ exchange.

5. In serum-treated cells recovery from an acid load was $0{\cdot}101 \pm 0{\cdot}01$ pH units/min ($n = 11$) in the nominal absence of HCO_3^-. Increasing the HCO_3^- concentration (5–10 mM) or pre-treatment with DIDS had no effect on the rate of acid extrusion. Recovery is Na^+ dependent and inhibitable with amiloride, indicating the presence of Na^+–H^+ exchange.

6. These results suggest that during somitic development the mechanisms regulating pH_i and recovery from acid loading are modified from a Na^+–HCO_3^- influx to a Na^+–H^+ exchanger. This transition allows the cells to increase pH_i by up to 0·2 pH units and may have a role in somitic cell development.

INTRODUCTION

Changes in intracellular pH (pH_i) accompany a wide variety of cellular events and, in many instances, are important in altering cell activity, metabolism and cell proliferation (Nuccitelli & Heiple, 1982; Moolenaar, Tertoolen & deLaat, 1984; Moolenaar, 1986). In cultured cells pH_i and cell behaviour can be modulated by

hormones, growth factors and other mitogens (Moolenaar, 1986). These agents act indirectly by phosphorylating the Na^+–H^+ exchanger and raising its 'set point'; H^+ is extruded and pH_i rises (Grinstein & Rothstein, 1986; Moolenaar, 1986). This alkalinization has been shown to be important in altering the kinetics of regulatory enzymes, protein synthesis and DNA synthesis (Busa, 1986).

During embryogenesis the control of cell proliferation is essential for normal development. However, the mechanisms involved in this control are poorly understood. If pH_i can influence the proliferative behaviour of cultured cells it is possible that alterations in pH_i may modulate embryonic cell growth.

The present experiments examine this hypothesis using cells in isolated somites from 2-day-old chick embryos. Somites are transient structures which form in pairs on either side of the neural tube. They are roughly spherical and are comprised of a simple pseudostratified epithelium. In 3-day-old embryos the somites lose their epithelial integrity and separate into two distinct cell types (dermomyotome and sclerotome) each of which undergoes a period of cell proliferation (Langman & Nelson, 1968). Somites maintained *in vitro* in serum-free media remain epithelial and cell proliferation is depressed (J. I. Gillespie, unpublished observations). If serum is present in the culture medium the somites spread on a fibronectin substrate and the cells increase in number (Bellairs, Sanders & Portch, 1980; Newgreen, 1984). Using this *in vitro* system it is possible to study pH_i during embryonic cell activation.

pH_i was measured by using the fluorescent pH-sensitive dye dicyano-hydroquinone (DCH; Valet, Raffael, Moroder, Wunsch & Rubenstroth-Bauer, 1981; Gillespie & Greenwell, 1987*a*). The emission spectrum of DCH is altered by pH and thus it is different from the more commonly used 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (for example see Paradiso, Negulescu & Machen, 1986). The spectral properties and pH sensitivity of DCH and BCECF have been compared (Musgrove, Rugg & Hedley, 1986). DCH appears to be more sensitive to pH than BCECF but, because it is a smaller molecule, it is more rapidly lost from cells. A preliminary account of this work has been published (Gillespie & Greenwell, 1987*b*).

METHODS

Tissue preparation

Embryos were obtained from domestic fowl eggs incubated for 40–50 h at 37 °C at 80% humidity. The embryos, 10–22 somites (stages 10–13; Hamburger & Hamilton, 1951) were removed from the yolk and washed in a balanced salt solution (BSS) containing 140 mM-NaCl, 10 mM-KCl, 2 mM-$CaCl_2$, 2 mM-$MgCl_2$, 10 mM-Na_2HPO_4, 5 mM-$NaHCO_3$, 10 mM-glucose, 1 mg/ml BSA and 10 mM-HEPES, and buffered to pH 7·4. Somites were dissected using fine needles and stored either in BSS alone or in BSS containing 10% fetal calf serum (Northumbria Biological Laboratories).

Ion-substituted salt solutions were made as followers. Na^+-free NMG solution: 140 mM-*N*-methylglucamine chloride, 10 mM-KCl, 2 mM-$CaCl_2$, 2 mM-$MgCl_2$, 10 mM-K_2HPO_4, 5 mM-$KHCO_3$, 1 mg/ml BSA, 20 mM-HEPES buffer and 10 mM-glucose (pH 7·4). Na^+-free K^+ solution was similar but used a total of 150 mM-KCl. Cl^--free solution: 140 mM-sodium gluconate. 10 mM-potassium gluconate, 15 mM-calcium gluconate, 1 mM-$MgSO_4$, 5 mM-Na_2HPO_4, 5 mM-$NaHCO_3$, 1 mg/ml BSA, 20 mM-HEPES buffer and 10 mM-glucose (pH 7·4). Nominally HCO_3^--free solutions were made by omitting the HCO_3^- salt. Solutions were not gassed with CO_2. Analysis of these solutions using a blood-gas analyser (ABL3: Radiometer, Copenhagen) gave values of 1 mM-HCO_3^- and a P_{CO_2} of 3 mmHg for the nominally HCO_3^--free solution and 6 mM-HCO_3^- and 16 mmHg for the solutions with 5 mM-added HCO_3^-.

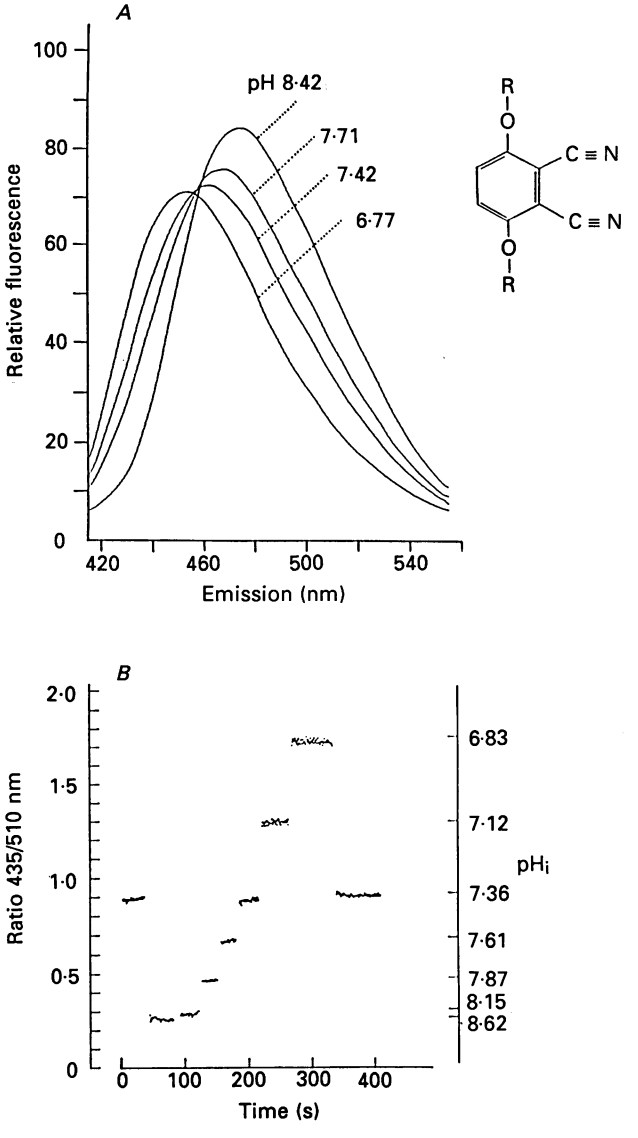

Fig. 1. *A* shows the emission spectra for the pH-sensitive dye dicyano-hydroquinone (DCH; 1 μM) obtained using a Perkin Elmer LS-5 spectrofluorimeter. The dye was dissolved in a solution containing 150 mM-KCl and 10 mM buffer (HEPES or TAPS) and set to different pH values. The excitation wavelength was 405 nm and the emission spectra from 410 to 550 nm were obtained at room temperature (24 °C). The ordinate shows relative fluorescence. As the pH decreases from 8·42 to 6·77 the peak emission decreases and shifts to smaller wavelengths. The inset shows the structure of the dye. When R represents OH this is dicyano-hydroquinone (DCH), the active form of the dye. When R represents acetate this is the membrane-permeable esterified form of the dye 1,4-diacetoxy-2,3-dicyano-benzol (ADB). *B* shows the output of the microspectrofluorimeter as solutions containing DCH (20 μM) at different pH values are perfused through the experimental chamber. The ordinates show the ratio of the intensities of the emitted light at 435 and 510 nm and the corresponding pH of the solution. Temperature, 23 °C.

Dye loading

Cells were loaded with the fluorescent pH-sensitive dye dicyano-hydroquinone using the esterified form 1,4-diacetoxy-dicyano-benzene (ADB; Paesel, F.R.G.). DCH is also known as 1,4-dihydroxyphthalonitrile and its esterified form 1,4-diacetoxyphthalonitrile (Molecular Probes, OR, U.S.A.). Tissue was incubated with 5 μg/ml ADB in BSS, without BSA or serum, for 2–5 min (20–22 °C). No obvious intracellular compartmentalization of the dye was observed. Dye-loaded somites were transferred to a bath (150 μl) on the stage of the microspectrofluorimeter and held by a suction pipette (10 μm diameter). Solutions were pre-heated (37 °C) and passed through the chamber at a rate of 1–2 ml/min.

The emission spectrum of DCH is altered by pH (Valet *et al.* 1981). Figure 1*A* shows the emission spectra at pH 6·77, 7·42, 7·71 and 8·42 (excitation wavelength of 405 nm). Progressive acidification shifts the spectrum to shorter wavelengths. Thus by determining the position of the emission spectrum it is possible to derive the pH in the environment of the dye. This is done by measuring the intensity of the emission at two wavelengths, 435 nm (±5 nm) and 510 nm (±5 nm), and calculating the ratio of intensities at these wavelengths.

Calibration of the dye

The dye was calibrated by perfusing the experimental chamber with solutions of 20–100 μM-DCH (in 150 mM-KCl and 10 mM-HEPES or -TAPS (tris (hydroxymethyl) methylaminopropane-sulphonic acid) buffer) at different pH values (Fig. 1*B*). The dye can, in principle, be calibrated in cells using the K^+–H^+ ionophore Nigericin (Sigma). When the extracellular potassium concentration equals the intracellular concentration the ionophore will equilibrate the intracellular pH with the extracellular pH. In the present experiments intracellular calibration curves of different sensitivity were observed if the K^+ concentration of the medium was 110 or 150 mM. This could be accounted for by the ionophore translocating K^+, in or out of the cell, in preference to H^+ thus making it difficult to equilibrate H^+. The precise form of the calibration curve thus depends on matching the intracellular and extracellular potassium concentrations.

It is possible to estimate independently pH_i from changes in pH_i induced by application of a weak acid and weak base (Szatkowski & Thomas, 1986). In somitic cells, pH_i determined by the weak acid and base method was 0·05–0·1 pH units more acid than estimated by the calibration curve shown in Fig. 1*A*. The reason for this is not clear but it may be akin to the 'red shift' reported for BCECF (see Paradiso *et al.* 1986). Such deviations in the calibration curves for intracellular DCH and DCH in solution have been reported previously (Valet & Raffael, 1984).

Apparatus

The dual emission microspectrofluorimeter is constructed from a Nikon Diaphot inverted microscope fitted with epifluorescence using a 75 W Xenon lamp source (Gillespie & Greenwell, 1987*a*). The fluorescence signal from the stage is directed to the sideport to which is attached a variable aperture, a shutter and a beam splitter containing a dichroic mirror (455 nm). Transmitted light through the dichroic mirror is filtered at 510 nm (±5 nm), reflected light filtered at 435 nm (±5 nm) and the intensities recorded by separate photomultiplier tubes (PM9924B; EMI). Single photon currents in each tube were coverted to TTL pulses and counted by a dual photon counter (Newcastle Photonic Systems). Photon counts/s in each channel were recorded in a BBC microcomputer, the ratio of intensities (435/510 nm) calculated and displayed. Results are expressed as means ± standard deviations (n = the number of observations). The significance of differences between means was determined using Student's t test. The level of significance was set at 0·05.

RESULTS

Measurement of resting pH_i

In BSS, in the nominal absence of HCO_3^- pH_i is 7·18 ± 0·02 (n = 12). In BSS containing 5 mM-HCO_3^- pH_i is more acid, 7·07 ± 0·09 (n = 5), due to the higher P_{CO_2}. This difference is not significant. In somites kept in BSS, nominally HCO_3^--free, with fetal calf serum pH_i is 7·36 ± 0·02 (n = 11), significantly higher than control somites ($P < 0·005$).

A similar intracellular alkalinization has been observed in other cell types where it has been attributed to the activation of Na^+–H^+ exchange (see Moolenaar, 1986). To determine if this explanation could account for the change in pH_i in serum-treated

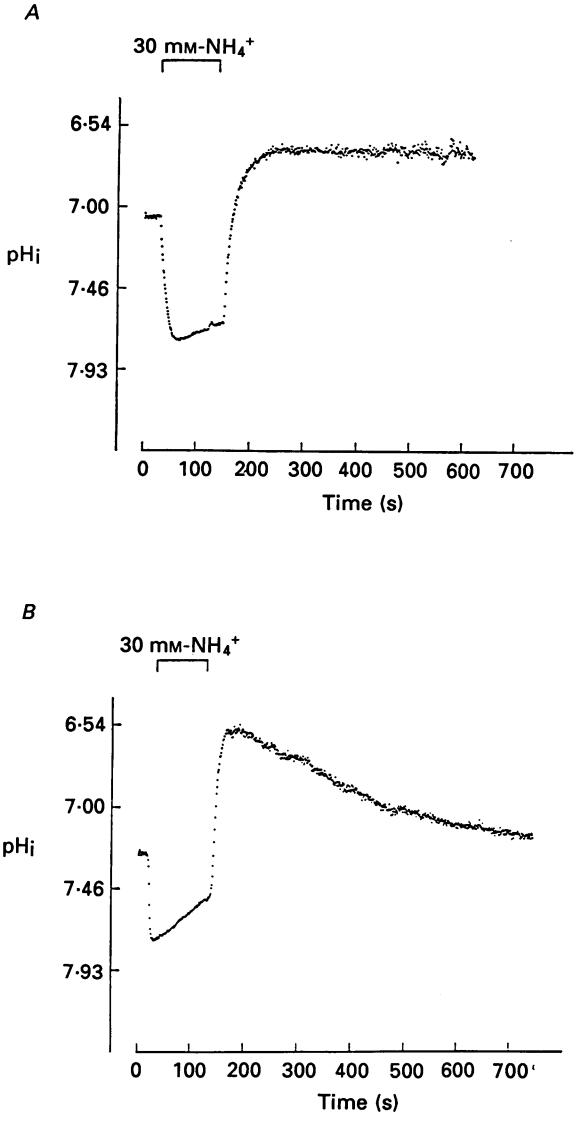

Fig. 2. Records of intracellular pH from somites dissected and maintained in balanced salt solution (BSS) nominally HCO_3^--free (*A*) or maintained for 3 h in BSS + 10% fetal calf serum nominally HCO_3^--free (*B*). The chamber was constantly perfused with BSS, nominally HCO_3^--free, at approximately 1 ml/min. Temperature, 35 °C in *A*; 34 °C in *B*. In each record, for the period indicated, the bath was perfused with BSS containing 30 mM-NH_4Cl.

cells experiments were done to assess the activity of any Na^+–H^+ exchange by imposing an acid load on the cells. This was done by a brief exposure to 30 mM-NH_4Cl.

Two typical experiments from a control and a serum-treated somite, in the nominal absence of HCO_3^-, are shown in Fig. 2*A* and *B* respectively. The mean rate of recovery from the acid load in the control somites is $0{\cdot}007 \pm 0{\cdot}003$ pH units/min ($n = 11$). In contrast the rate of recovery in the serum-treated cells was $0{\cdot}101 \pm 0{\cdot}01$ pH units/min ($n = 11$), significantly faster than the control ($P < 0{\cdot}005$). These data point to a considerable difference in the ability of the cells to regulate pH_i. The serum-induced changes in pH_i regulation were slow to appear, in that addition of serum (10% fetal calf serum) to the media bathing control somites did not produce any significant change in pH_i over the period of a few minutes. Somites had to be incubated for more than 2 h to observe the elevation of pH_i. Tissue kept in culture for 24–36 h maintained this elevated pH_i and activity of the Na^+–H^+ exchange mechanism.

Intracellular buffering power

The intracellular buffering power (B) was estimated from the initial alkalinization produced on exposure to NH_4Cl using the following expression:

$$B = d[NH_4^+]_i/dpH_i,$$

where $d[NH_4^+]$ is the amount of base added to the interior of the cells and dpH_i is the resulting change in pH_i (see Boron & DeWeer, 1976; Boron, 1986). The values obtained show a significant increase in B from $20{\cdot}9 \pm 2{\cdot}1$ mM/pH unit ($n = 12$) in control somites to $34{\cdot}1 \pm 2{\cdot}3$ mM/pH unit ($n = 5$) in the serum-treated cells. In control somites bathed in 5 mM-HCO_3^- B was $29{\cdot}4 \pm 2{\cdot}0$ mM/pH unit.

The alkalinization on application of NH_4Cl is transient. In control somites in the absence of HCO_3^- the rate of change of pH_i is $0{\cdot}06 \pm 0{\cdot}009$ pH units/min ($n = 13$) and in serum-treated cells is $0{\cdot}21 \pm 0{\cdot}009$ pH units/min ($n = 11$), significantly faster ($P < 0{\cdot}005$). The change in rate of acidification may reflect an increase in the entry of NH_4^+ or the activation of mechanisms in the serum-treated cells to cope with alkali loads.

Recovery from acid loading in control somites

If HCO_3^- is added during the slow recovery from the acid load in control cells the recovery is accelerated. In the example shown in Fig. 3*A* the initial rate of recovery increased from 0·01 to 0·12 pH units/min. In ten other experiments the mean rate of recovery in control cells in 5 mM-HCO_3^- was $0{\cdot}132 \pm 0{\cdot}016$ pH units/min, significantly faster than in the absence of HCO_3^-.

This increased rate of alkalinization is probably due to an influx of HCO_3^-. Several mechanisms have been identified which transport HCO_3^-. In general terms there are three types (Boron, 1986), (a) Cl^-–HCO_3^- exchange, (b) Na^+–HCO_3^-/Cl^-–H^+ exchange and (c) Na^+–HCO_3^- co-transport. Each of these mechanisms can be inhibited by the stilbene derivative DIDS (4,4-diisothiocyanatostilbene-2,2-disulphonic acid). A series of experiments was done to determine whether or not the recovery mechanism is sensitive to DIDS. Pre-incubation of control somites in BSS containing DIDS (5×10^{-4} M) abolished the HCO_3^--stimulated recovery (Fig. 3*B*). The mean rate of recovery in the DIDS-pre-treated cells in 5 mM-HCO_3^- was $0{\cdot}011 \pm 0{\cdot}005$ pH units/min ($n = 4$), not significantly different from the initial rate in the absence of HCO_3^-.

The Na^+ dependence of the recovery process was examined in experiments in which the extracellular Na^+ was replaced with either *N*-methylglucamine or potassium. Figure 3*C* shows that in control cells bathed in 10 mM-HCO_3^- recovery

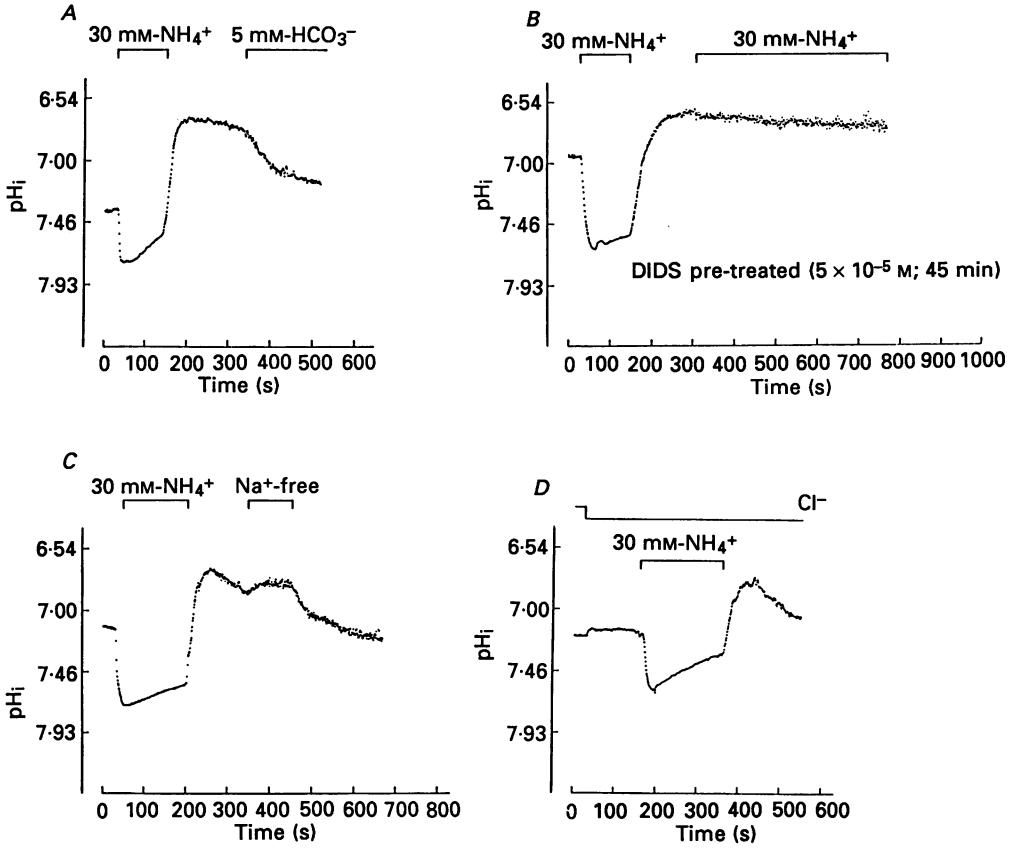

Fig. 3. Measurement of intracellular pH in control somites dissected and maintained in BSS. *A*, the effect of increasing HCO_3^-. Initially the somite was bathed in nominally HCO_3^--free solution. For the periods indicated 30 mM-NH_4Cl and 5 mM-HCO_3^- were added to the bathing solution. Temperature, 36 °C. *B*, the effect of pre-incubation with DIDS for 45 min on the HCO_3^--dependent recovery from an acid load. For the times indicated 30 mM-NH_4Cl and 5 mM-HCO_3^- were added to the perfusion solution. Temperature, 34 °C. *C*, Na^+ dependence of recovery. During the recovery from an acid load, the bathing solution was changed to one containing *N*-methylglucamine (Na^+-free). HCO_3^- concentration, 5 mM. Temperature, 36 °C. *D* the effect of Cl^- removal on pH_i and the recovery from acid load. The somite was bathed in solution containing 5 mM-HCO_3^-. At the time indicated the bathing medium was changed to Cl^--free solution (Cl^- replaced with gluconate). 30 mM-NH_4Cl was added to the bathing solution to acid load the cells. Temperature, 35 °C.

is stopped when Na^+ is replaced with *N*-methylglucamine (four experiments). A similar result was obtained with K^+ substituting for Na^+ (two experiments). This indicates that the recovery is a Na^+-dependent process.

Experiments were done in which Cl^- was replaced with gluconate (Fig. 3*D*). On removing Cl^- pH_i fell by $0{\cdot}009 \pm 0{\cdot}001$ pH units ($n = 4$). After a few minutes in Cl^--

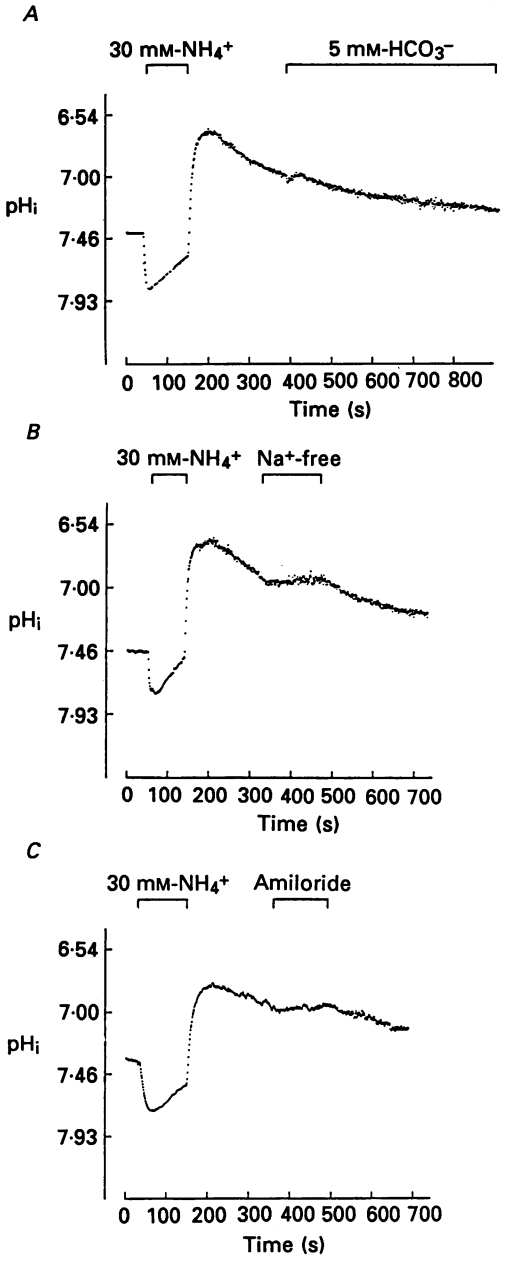

Fig. 4. pH_i recovery in serum-treated somites. *A*, the effect of 5 mM-HCO_3^- on the time course of recovery from an acid load in somite cells bathed in nominally HCO_3^--free solution and pre-treated for 3 h in BSS + 10 % fetal calf serum. For the period indicated 30 mM-NH_4Cl was added to the perfusion fluid to acid load the cells. 5 mM-HCO_3^- was added to the perfusion fluid at the time shown. Temperature, 34 °C. *B* and *C*, the effect of Na^+ removal (*B*) and amiloride (*C*) on the time course of recovery from an acid load. Somites were pre-incubated in BSS + 10 % fetal calf serum for 3 h in *B* and 2 h in *C* prior to dye loading and pH_i recording. Acid loading was achieved during a brief exposure to NH_4Cl. In *B*, during the recovery phase the bathing solution was changed to one containing *N*-methylglucamine (Na^+-free). In *C*, amiloride (10^{-3} M) was added to the perfusion media. Temperature in *C*, 35 °C.

free solution acid loading and recovery were no different from controls. These results show that there is little dependence of pH_i on Cl^- suggesting that there is little or no Cl^-–HCO_3^- exchange activity in these cells. Furthermore if intracellular Cl^- falls as a consequence of removing extracellular Cl^- this should interfere with any Na^+–HCO_3^-/Cl^-–H^+ exchange. If the membrane permeability to Cl^- is low then a brief exposure to Cl^--free solutions might not reduce intracellular Cl^- sufficiently to affect Na^+–HCO_3^-/Cl^-–H^+ exchange. Somites bathed in Cl^- solutions for up to 1 h did not differ in resting pH or recovery from an acid load compared to control cells (three experiments). These observations would indicate that Na^+–HCO_3^-/Cl^-–H^+ exchange is not present.

In five experiments amiloride (10^{-3} M) was added to the bathing solution during the recovery from acid load in control cells bathed in 10 mM-HCO_3^- BSS but no deviation in the time course was noted (data not shown). This suggests that Na^+–H^+ exchange, via an amiloride-sensitive mechanism, is not functional. The absence of recovery in HCO_3^--free solution supports this idea.

Recovery from acid loading in serum-treated somites

The observation that the resting pH_i in serum-treated cells is different from controls and that recovery from an acid load proceeds in the absence of HCO_3^- points to a basic change in the cellular mechanisms regulating pH_i. Figure 4*A* demonstrates absence of any effect of HCO_3^- on recovery in a somite, in the nominal absence of HCO_3^-, acid loaded in the same way as in the previous experiments. Raising the HCO_3^- concentration to 5 mM has no effect on the time course of recovery (four experiments). Incubation of serum-treated somites with DIDS (40 min) also had no effect on the time course of recovery either in the presence or absence of HCO_3^-.

Experiments were done to determine the means by which these treated cells now regulate pH_i, in particular its dependence on Na^+ and sensitivity to amiloride. Somites bathed in the nominal absence of HCO_3^- were acid loaded and, during the recovery phase, Na^+ in the bathing media was replaced with *N*-methylglucamine (Fig. 4*B*). The effect was to retard and even reverse the recovery process. This experiment, and four others, demonstrate that the recovery process is Na^+ dependent. In one experiment the same result was obtained when K^+ was used to replace Na^+.

When high concentrations of amiloride (10^{-3} M) are added to the bathing medium during the recovery phase from an acid load, in the nominal absence of HCO_3^-, recovery stopped (Fig. 4*C*; one of five experiments). These observations on the Na^+ dependence and the amiloride sensitivity of the recovery suggest that a Na^+–H^+ exchange mechanism is now active in these treated cells. The absence of any effect of added HCO_3^- may indicate the loss of the Na^+–HCO_3^- influx mechanism in the treated cells.

DISCUSSION

The data show that the resting pH_i of freshly dissected and serum-treated somitic cells are different by approximately 0·2 pH units. The experiments on acid-loaded cells point (a) to the presence of a Na^+–HCO_3^- co-transport in control cells and (b) to the activation of a Na^+–H exchange mechanism in serum-treated cells. It therefore

seems likely that activation of Na^+–H^+ exchange is responsible for the difference in pH_i.

In other cells serum and identified growth factors (e.g. epidermal growth factor and platelet-derived growth factor) can alter the sensitivity of the internal binding site on the Na^+–H^+ exchanger for H^+ (see Moolenaar, 1986). In these cell systems the Na^+–H^+ exchanger is demonstrably present in acid-loaded unstimulated cells and activation with the growth factors involves a change in the set point of the Na^+–H^+ exchanger to a more alkaline value. In the present experiments there is no indication of Na^+–H^+ exchange in the control cells. This could imply that the intracellular pH cannot be made sufficiently acid to activate the exchanger or that Na^+–H^+ exchange is not present in a functional form in the membrane of early somitic cells. In cultured cells stimulation of Na^+–H^+ by growth factors usually occurs over the period of a few minutes (Moolenaar *et al.* 1984). No such short-term change could be noted in somites and a minimum period of 2 h was necessary before any alteration in pH_i could be detected. This slow time course may suggest that the Na^+–H^+ exchange mechanism is not functionally present in the membrane in the control cells and that serum stimulation may therefore activate the synthesis of the exchanger, its insertion into or its assembly within the membrane.

One of the earliest events in activating cell growth and cell proliferation is an increase in pH_i (see Moolenaar, 1986). The present data lead to the speculation that embryonic somitic cells behave in the same way. There is however no direct evidence that this occurs *in vivo*. If raising pH_i were to play a role in the development of somitic cells, questions arise regarding the means of stimulating this increase. It is possible that the cells autonomously activate the Na^+–H^+ exchange mechanism thereby controlling their own fate, or alternatively an intra-embryonic signal, analogous to a growth factor, may stimulate the cells.

Regulation of pH_i, under the conditions of an acid load, in the control somites appears to be dependent on a process which depends on Na^+ and HCO_3^-. Removal of extracellular Cl^- has no significant effect on pH_i or on the recovery from an acid load in the presence of HCO_3^-. Thus the family of transporters, Na^+–HCO_3^-/Cl^-–H^+ exchange, which have been identified in several invertebrate cells (Thomas, 1977; Boron, 1985, 1986), are unlikely to be present in somitic cells. The present data can be interpreted to suggest that the control somites possess a Na^+–HCO_3^- co-transport mechanism. Such Na^+–HCO_3^- co-transport has been reported in perfused renal tubules (Boron & Boulpaep, 1983; Alpern, 1985; Yoshitomi, Burkhardt & Frompter, 1985) and bovine corneal epithelium (Jentsch, Stahlknecht, Hollwede, Fischer, Keller & Wiederholt, 1985; Jentsch, Janicke, Sorgenfrei, Keller & Wiederholt, 1986; Jentsch, Schwartz, Schill, Langer, Leppel, Keller & Wiederholt, 1986).

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