

An increase in intracellular pH during neural induction in *Xenopus*

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SUMMARY

In this paper, we show that an intracellular alkalinization of the dorsal ectoderm cells is among the earliest responses to neural induction in *Xenopus*. Planar explants of the dorsal marginal zone were prepared from embryos that had been microinjected during cleavage stages with the fluorescent pH indicator bis-carboxyethyl-carboxyfluorescein-dextran (BCECF-dextran), and intracellular pH (pH_i) was monitored continuously by emission ratio microfluorimetry. During stage 10.5, the dorsal ectoderm cells undergo a sustained intracellular alkalinization of approximately 0.1 pH units in response to neural induction; in the absence of the inductive signal, the pH of the dorsal ectoderm cells decreases slightly. Ectoderm cells within planar explants of the ventral marginal zone show little change in pH during a similar period. This increase in intracellular pH is inhibited by 4, 4'-dihydrodiisothio-

cyanatostilbene-2, 2'-disulfonate (H2DIDS) or a low Na^+ /high Cl^- medium, treatments that presumably affect anion transport. Under these conditions, expression of the anterior neural-specific homeobox gene *engrailed* is not detected, while the notochord-specific epitope recognized by the Tor-70 antibody is expressed in the presence of H2DIDS. This characteristic alkalinization is not evoked by pharmacological agents that reportedly alter ectodermal developmental pathways in *Xenopus* embryos, such as NH_4Cl , phorbol esters, or cAMP-dependent protein kinase agonists. Our results suggest that an ionic regulatory event may participate in the regulation of gene expression in response to neural induction.

Key words: neural induction, intracellular pH, planar signals, *Xenopus*

INTRODUCTION

Neural induction initiates alterations in a variety of cellular activities that lead to the differentiation of neuronal and glial subtypes, the morphogenesis of the brain and spinal cord, and the establishment of positional identity along the anteroposterior and dorsoventral axes of the neural tube. The earliest responses to neural induction begin during gastrulation: the future Rohon-Beard sensory neurons begin to withdraw from the cell cycle (Lamborghini, 1980), the presumptive hindbrain and spinal cord start to converge and extend (Keller et al., 1992a) and the neural-specific patterns of gene expression become established (e.g., NCAM, Jacobson and Rutishauser, 1986, Kintner and Melton, 1987; epi-1, Savage and Phillips, 1989). Many of these changes occur in a characteristic spatiotemporal pattern, suggesting that induction and regionalization of the presumptive neural plate arise through a complex interplay between cell-cell signalling and morphogenetic movements (Keller et al., 1992a,b).

The intracellular regulatory processes that govern and coordinate these cellular aspects of the response to neural induction have largely resisted analysis. The studies of Barth and Barth (Barth, 1966; Barth and Barth, 1974) on 'artificial neural induction' in isolated *Rana* ectoderm indicated that the response to neural induction in vivo might be mediated by ionic signals; their work, together with studies on *Xenopus* and

Ambystoma by Warner and colleagues (Blackshaw and Warner, 1976; Breckenridge and Warner, 1982), specifically implicated Na^+ -dependent regulatory processes.

Although *Xenopus* ectoderm is generally considered relatively resistant to nonspecific 'artificial' treatments that elicit neural-specific responses in the ectoderm of *Rana* and many urodeles, more recent studies using *Xenopus* embryos have suggested that pharmacological activation of protein kinase C leads to neural-specific responses in a limited fraction of ectodermal explants (Davids et al., 1987; Otte et al., 1988). The activities of both protein kinase C (Otte et al., 1988) and cAMP-dependent protein kinase (Otte et al., 1989) increase during neural induction, leading to the speculation that they contribute to the response to neural induction.

Until recently, direct examination of ionic signalling during neural induction was precluded by the difficulty of making ionic measurements for extended periods in whole embryos. We have used an explant system to examine intracellular pH (pH_i) during the response to planar inductive signals during neural induction in *Xenopus* embryos (Fig. 1A). This planar signal alone will induce many aspects of the response to neural induction, including neural-specific gene expression (Dixon and Kintner, 1989; Ruiz i Altaba, 1992), neuronal differentiation (Sater et al., 1993), the establishment of anterior positional identity (Doniach et al., 1992), and the convergence and extension of the posterior neural plate (Keller and Danilchik,

1988; Keller et al., 1992b). We have found that dorsal ectoderm cells undergo an increase in pH_i in response to the planar neural-inducing signal during the first half of gastrulation. Inhibition of the increase in pH_i prevents the expression of the anterior neural-specific gene *en-2*, the *Xenopus* homologue of the homeobox gene *engrailed*. Thus, this intracellular alkalinization may participate in changes in gene expression associated with the induced differentiative pathway.

MATERIALS AND METHODS

Embryos

Embryos were obtained, dejellied and maintained in 33% modified Barth's solution (MBS) as described earlier (Keller et al., 1992a,b). All stages refer to those of Nieuwkoop and Faber (1967). Explants were cultured in modified Danilchik's medium (DFA: 53 mM NaCl, 32 mM sodium gluconate, 4.5 mM potassium gluconate, 1 mM CaCl₂, 1 mM MgSO₄, 5 mM Na₂CO₃, buffered to pH 8.3 with bicine; modified from Keller et al., 1985). Other media included Δ NaCl medium: 10 mM NaCl, 75 mM N-methylglucamine (Sigma), 4.5 mM KCl, 10 mM NaHCO₃, 65 mM HCl, 1 mM CaCl₂, 1 mM MgSO₄, buffered to pH 8.3 with bicine (final Na⁺ concentration: 20 mM; final Cl⁻ concentration: 81.5 mM); and Flickinger solution: 58 mM NaCl, 1 mM KCl, 1 mM Na₂HPO₄, 0.2 mM KH₂PO₄, 0.5 mM CaCl₂, 0.24 mM NaHCO₃, 1 mM MgSO₄, pH 7.5).

Microinjection

To permit microinjection into dorsal blastomeres at the 32-cell stage, embryos were tipped 90° and marked with Nile Blue during the first cell cycle to establish and identify the dorsoventral axis (Stewart and Gerhart, 1990). Marked embryos were kept in 5% ficoll in MBS until they were microinjected. At the 32-cell stage, one of the dorsal animal blastomeres was pressure-injected with 5 mM of the fluorescent pH indicator bis-carboxyethyl-carboxyfluorescein (BCECF-dextran) (Molecular Probes, Eugene, OR) (BCECF-dextran) in XBG buffer (100 mM potassium gluconate, 1 mM MgCl₂, 10 mM potassium Hepes, 50 mM sucrose, pH 7.7, modified from Murray and Kirschner, 1989). This BCECF-dextran stock was stored in the dark at -20°C. Based on comparisons with droplets containing known concentrations of BCECF-dextran, we estimate the intracellular BCECF-dextran concentration to be at least 5-50 μ M. After healing, microinjected embryos were transferred into 33% MBS. Planar explants of the dorsal marginal zone (DMZ) were prepared from microinjected embryos at the beginning of gastrulation. This protocol is summarized in Fig. 1B-E. Intracellular BCECF-dextran had no discernible toxic effects: embryos injected with BCECF-dextran developed normally through tadpole stages and cells containing BCECF-dextran often underwent mitosis during experiments.

Explant preparation

Microsurgery was performed in MBS on beds of 2% agar, using eyebrow hair knives and sharpened watchmaker's forceps. Planar explants of the DMZ were isolated from microinjected embryos between stages 10+ and 10.25 and stripped of the external epithelium and all head mesodermal cells, as described in Sater et al. (1993). The explants were then transferred to a glass-bottomed Petri dish coated with 7 μ g/cm² Cel-Tak (Collaborative Research, Bedford, MA). To prevent the explants from rounding up and promote their adhesion to the Cel-Tak, explants were completely covered with coverslip fragments supported by clay and flattened by pressing gently on the glass fragments with forceps. Experiments were performed in DFA unless otherwise noted.

Explants of the DMZ were 20-25 cells across, centered on the dorsal midline; they extended 30-35 cells up from the base of the dorsal lip of the blastopore. Since detailed mapping of the DMZ has

shown that the boundary between the mesoderm and the ectoderm is located 12-15 cells from the base of the dorsal blastopore lip (Keller et al., 1991), explants of dorsal ectoderm alone were taken at least 18-20 cell diameters above the dorsal blastopore lip, to prevent inclusion of mesoderm, and extended animalward approximately 15-20 cell diameters. Explants of the ventral marginal zone (VMZ) were also isolated at stage 10+ to 10.25, prior to the formation of the ventral lip of the blastopore; the proportions of these explants are similar to those of the DMZ.

pH measurements

Measurements of intracellular pH were performed by emission ratio microfluorimetry. Labelled explants were viewed on a Zeiss IM35 inverted microscope. Excitation light was passed through alternating interference filters at 490 and 440 nm (bandwidth = 5 nm) rotated on a stepping motor at 0.5 or 1.0 Hz. Neutral density filters of 0.3, 0.6 and 1.0 (Omega, Brattleboro, VT) were used in concert to reduce the intensity of the excitation light. Emitted light was passed through a 535 nm interference filter. Fluorescent emissions resulting from excitation at each wavelength were photon-counted in synchrony with the stepping motor and stored separately on an IBM AT computer using UMANS software (C. M. Regan, University of Illinois, Champaign-Urbana, IL).

Calibration of BCECF-dextran

Intracellular calibration of BCECF-dextran emission ratios was performed by permeabilization of ectoderm cells with 20 μ g/ml nigericin (Sigma Chemicals, St. Louis, MO) in the presence of a high [K⁺] buffer of known pH (130 mM KCl, 1 mM MgCl₂, 30 mM potassium Hepes). The nigericin was maintained as a 10 mg/ml stock in 100% ethanol at -20°C. Dorsal ectoderm cells were dissociated during a 15-minute incubation in Dissociation Medium (DM: 60% Ca²⁺/Mg²⁺-free Hank's Saline, 0.4 mM EDTA, 10 mM Hepes, 20 mM Na₂CO₃, pH 7.2-7.4).

Pharmacology

Stocks of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA, Sigma) and dibutyryl cAMP (dbcAMP, Sigma) were prepared in dimethylsulfoxide (DMSO) and stored at -20°C. The TPA used in these experiments was derived from two separate lots. The anion transport inhibitor 4, 4'-dihydrodiisothiocyanatostilbene-2, 2'-disulfonate (H2DIDS) (Molecular Probes) was added directly to the culture medium for each experiment.

Immunohistochemistry

Explants of the DMZ were isolated at stage 10+ as described earlier and cultured in glass-bottomed Petri dishes under coverslip fragments supported by modeling clay. Explants were maintained in either DFA, DFA + 200 μ M H2DIDS, or Δ NaCl medium until intact sibling control embryos reached stage 21. Fixation and whole-mount immunohistochemistry were performed as described by Hemmati-Brivanlou and Harland (1989). The monoclonal antibody 4D9, which recognizes the *Xenopus* homologue of *engrailed* protein (*en-2*) (Hemmati-Brivanlou and Harland, 1989), was diluted 1:1 before use; the monoclonal antibody Tor-70, which recognizes a notochord-specific epitope (Bolce et al., 1992), was used at a 1:500 dilution. Antibodies were visualized with a horseradish peroxidase (HRP)-conjugated rabbit-anti-mouse IgG (Sigma) used at 1:100 and developed with diaminobenzidine. All antibody incubations were carried out overnight at 4°C.

RESULTS

Intracellular pH of ectoderm cells during neural induction

We used emission ratio microfluorimetry with the fluorescent

pH indicator bis-carboxyethyl-carboxyfluorescein (BCECF) to measure pH_i during neural induction in these planar explants. When BCECF is excited by 490 nm light, the intensity of the resulting fluorescent emission is dependent upon the pH_i ; whereas, excitation with 440 nm light produces a fluorescent emission whose intensity is independent of pH. By alternating the wavelengths used to excite intracellular BCECF, one can obtain a ratio of the pH-dependent and pH-independent emission intensities. This ratio provides a measurement of pH that is relatively free of artifacts resulting from variations in dye distribution, cell thickness or cellular inclusions.

A dextran-conjugated form of BCECF (BCECF-dextran) was used to avoid artifacts resulting from intracellular compartmentalization of the dye. Since the dextran conjugate will not pass through gap junctions, it could be microinjected during cleavage stages, so that the progeny of the microinjected cell retained the dye at concentrations suitable for microfluorimetry. Initial measurements were carried out using explants in which a single cell was microinjected with BCECF-dextran immediately prior to the experiment. Results of these experiments were indistinguishable from those obtained using explants isolated from embryos microinjected at the 32-cell stage. Explants from embryos microinjected during cleavage stages included labelled cells scattered throughout the explant, allowing us to record repeatedly at intervals from several cells within a single explant.

To examine the pH_i of ectoderm cells during neural induction, we isolated planar explants of the DMZ, which includes both dorsal ectoderm and dorsal mesoderm, from embryos that had been microinjected with BCECF-dextran at the 32- or 64-cell stage (Fig. 1B-E). The external epithelium was removed to improve optical conditions for microfluorimetry and increase the accessibility of the ectoderm cells to the medium. Explants were maintained in DFA, which represents the ionic conditions of the blastocoel as described by Gillespie (1983). The emission ratio of the BCECF-dextran within the dorsal ectoderm cells was monitored continuously or at regular intervals during the first half of gastrulation (stage 10-11). In later experiments, the duration was reduced to stage 10.5-11. No significant loss of emission intensity was observed even during continuous monitoring throughout this period. Negative controls included ectoderm cells within explants of the dorsal ectoderm alone and within explants of the VMZ.

The pH_i of the dorsal ectoderm cells remained constant between stages 10 and 10.5. During stage 10.5, which lasts approximately 90 minutes at 20°C, the pH_i of the responding dorsal ectoderm cells increased by approximately 0.1 pH units over a 20-40 minute period (Fig. 2A,B,H; $n=17$ cells, from 11 experiments). The pH_i remained elevated throughout stage 11. An increase in pH_i greater than 0.05 pH units occurred in over 80% of dorsal ectoderm cells during neural induction (Fig. 2B), resulting in an

average alkalization of 0.103 ± 0.015 pH units. On average, no change in pH_i was observed in ectoderm cells within explants of the VMZ (-0.0004 ± 0.018 s.e.m.) (Fig. 2C,D,H; $n=14$ cells, from 5 experiments). Dorsal ectoderm cells underwent acidification in the absence of contact with the dorsal mesoderm, exhibiting an average change in pH_i of -0.088 ± 0.022 pH units (Fig. 2E,F,H; $n=12$ cells, from 3 experiments). The differences in BCECF-dextran emission ratio are not artifactual, as indicated by the distinct pH_i profiles of each type of explant during this stage. We conclude that the increase in pH_i occurs in response to an inductive signal produced by the dorsal mesoderm.

The sample sizes for all three cases (ectoderm cells in explants of the DMZ, ectoderm cells in explants of the VMZ and cells in explants of the dorsal ectoderm alone) include experiments in which a single cell is monitored continuously and experiments in which each of several cells is sampled at regular intervals. For most experiments, between 3 and 5 cells are sampled initially, and one is selected for relatively continuous monitoring. The other cells are sampled at regular intervals, usually 4-6 times throughout an experiment. This allows us to observe the alkalization in real time in a single cell in each experiment, while regularly monitoring pH_i in other cells.

The emission ratios of intracellular BCECF-dextran were calibrated using a modification of the methods of Thomas et al. (1979). Briefly, cells are depolarized by exposure to a high $[K^+]$ buffer of known pH and then permeabilized with the K^+/H^+ ionophore nigericin. This treatment results in the equilibration of pH_i with the pH of the buffer. Dorsal ectoderm cells containing BCECF-dextran were dissociated and transferred to poly-lysine-coated glass-bottomed Petri dishes containing

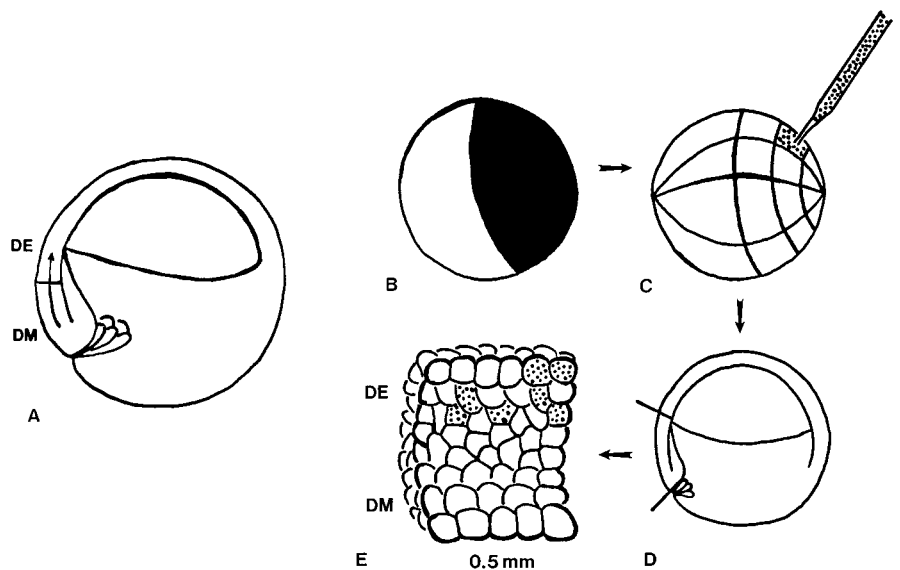


Fig. 1. (A) Neural induction in *Xenopus*. At the beginning of gastrulation, the inductive signal moves from the dorsal mesoderm (DM) up through the plane of the tissue to the responding dorsal ectoderm (DE). (B-E) Introduction of BCECF-dextran into cells within the dorsal ectoderm. (B) Embryos were manually rotated 90° during the first cell cycle to establish the dorsoventral axis in a specific plane, and the future dorsal side was marked with Nile Blue. (C) Microinjection of BCECF-dextran into a dorsal animal blastomere at the 32-cell stage. (D) Isolation of a planar explant of the DMZ from a microinjected embryo. (E) The ectodermal region of the explant includes cells containing BCECF-dextran.

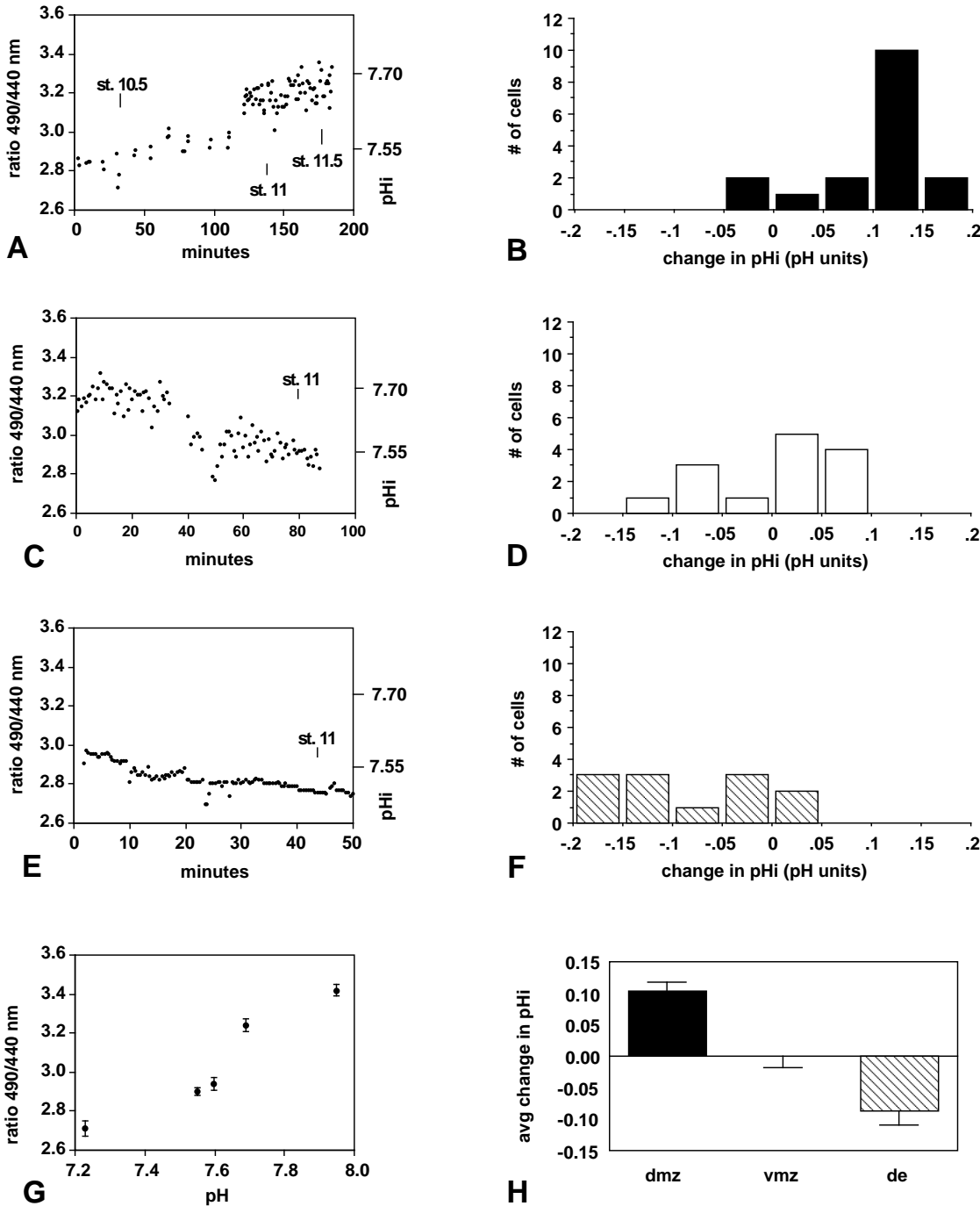


Fig. 2. (A) Intracellular pH of a single dorsal ectoderm cell during neural induction. Dorsal ectoderm is located within a planar explant of the DMZ, contiguous with the dorsal mesoderm. The pH_i increases during the latter half of stage 10.5. (B,D,F) Frequency distribution of the magnitude of changes in pH_i during neural induction. (B) Results for responding dorsal ectoderm within the DMZ: $n=17$ cells, from 11 experiments. (C) Intracellular pH of a single cell within a planar explant including ventral ectoderm and ventral mesoderm. (D) Ectoderm within the VMZ: $n=14$ cells, from 5 experiments. (E) Intracellular pH of a cell within an explant containing isolated dorsal ectoderm; none of the inductively active mesodermal cells are present. (F) Dorsal ectoderm alone: $n=12$ cells, from 3 experiments. (G) Calibration of response of intracellular BCECF-dextran. Points represent emission ratio values (mean \pm s.e.m.) based on a minimum of 9 cells from at least two experiments for each pH value tested. (H) Average change in intracellular pH during neural induction (mean \pm s.e.m.). dorsal marginal zone (dmz):

$+0.103 \pm 0.015$; ventral marginal zone (vmz): -0.0004 ± 0.018 ; dorsal ectoderm only (de): -0.088 ± 0.022 . Change in pH_i is defined as the difference between the pH_i at beginning of stage 10.5 and the pH_i at the beginning of stage 11. Note that only the ectoderm in contact with the dorsal mesoderm undergoes an increase in pH_i .

DFA. After the initial emission ratio was monitored, the DFA medium was replaced with a calibration buffer and 20 $\mu\text{g/ml}$ nigericin was added. Addition of nigericin caused a drop in ratio over approximately 20 minutes, which presumably could be attributed to proton release from intracellular acidic compartments. The ratio then increased, equilibrating completely (i. e., no change in ratio for 10 minutes) after 30-45 minutes. Emission ratios of several cells were sampled after the ratio had equilibrated. The buffer was then replaced with an

identical buffer at a different pH_i ; equilibration with the second buffer occurred within 10 minutes. Generally, two different pH levels were tested in a given experiment. The results of the intracellular calibration of BCECF-dextran are shown in Fig. 2G. In addition, emission ratios for BCECF-dextran in solution were calibrated at two pH values at the beginning of each experiment.

Individual cells display a variation in initial pH_i that ranges between 7.6 and 7.75. Our estimates of the initial pH_i of

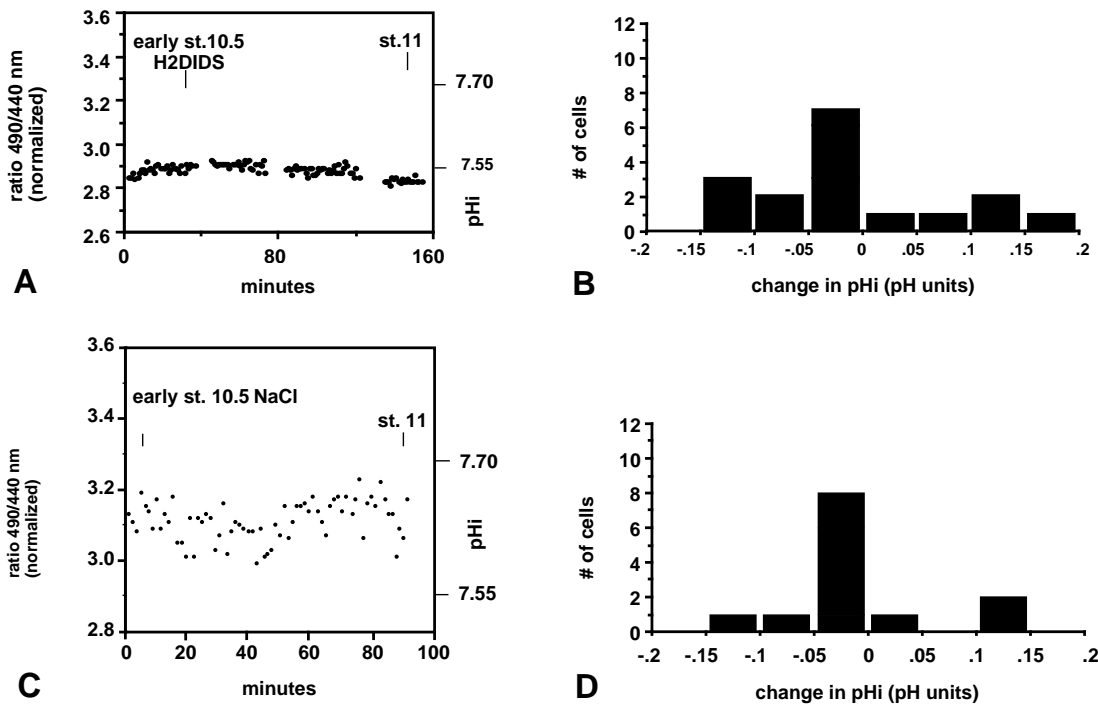


Fig. 3. Inhibition of anion transport and intracellular pH. (A) Effects of H2DIDS on intracellular pH of a single dorsal ectoderm cell during neural induction in a planar explant. Explants were prepared between stages 10+ and 10.25 and transferred directly into DFA containing 200 μ M H2DIDS. (B) Frequency distribution of the magnitude of changes in pH_i in ectoderm cells treated with H2DIDS during neural induction. In B and D, cells showing no change are represented in the -0.05 to 0 pH unit group. This treatment prevented the alkalization in 13 of 17 cells in 3 experiments. (C) Effects of Δ NaCl medium on pH_i of a single

ectoderm cell during neural induction. Explants from stage 10+-10.25 embryos were transferred directly into Δ NaCl medium. (D) Frequency distribution of changes in pH_i in ectoderm cells treated with Δ NaCl during neural induction. Under these conditions, the alkalization was inhibited in 11 of 13 cells from 3 experiments.

ectoderm cells are similar to electrophysiological measurements of pH_i in *Xenopus* blastomeres from earlier stages (4-cell to early blastula: pH 7.62-7.9, Turin and Warner, 1980) (stage 6: pH 7.67-7.94, Guthrie et al., 1988) (stage 7-8: pH 7.73-7.8, Lee and Steinhardt, 1981).

Ion-sensitive fluorescent indicators can be subject to artifacts resulting from dye compartmentalization, binding to intracellular components, viscosity, ionic strength, etc. (Chaillet and Boron, 1985). To determine the amount of BCECF-dextran bound or compartmentalized within *Xenopus* embryonic cells, we exposed dissociated gastrula ectoderm cells containing BCECF-dextran to 20 μ M digitonin (Fisher Scientific, Fairlawn, NJ). Greater than 99% of the intracellular emission intensity was lost within 2 minutes (data not shown), indicating that compartmentalization or binding of BCECF-dextran was negligible in these cells. In addition, we used laser scanning confocal microscopy to examine dissociated gastrula cells labelled with BCECF-dextran. In these cells, the cytoplasm shows a uniform level of fluorescence devoid of punctate highly fluorescent areas that would indicate dye compartmentalization (data not shown).

Inhibition of intracellular alkalization

We sought to identify conditions that prevent the increase in pH_i in order to test whether the alkalization is necessary for the expression of neural-specific genes. Transport systems responsible for intracellular acid extrusion include the Na⁺/H⁺ antiporter and Na⁺-dependent anion transport systems such as Na⁺-dependent Cl⁻/HCO₃²⁻ exchange. Preliminary treatments designed to disrupt the Na⁺/H⁺ antiporter had undesirable effects on cell viability or explant cohesiveness; therefore, we concentrated on conditions that would inhibit Na⁺-dependent

anion transport. First, we examined the effects of the anion transport inhibitor 4, 4'-dihydrodiisothiocyanatostilbene-2, 2'-disulfonate (H2DIDS) on pH_i during neural induction (Fig. 3A). In the presence of 200 μ M H2DIDS, no net increase in pH_i was observed in 13 of 17 cells over 3 experiments. In half of these cases, a slight, transient increase in pH_i occurred, but the emission ratio always returned to baseline before the onset of stage 11. Thus in 82% of cells exposed to H2DIDS, the intracellular alkalization normally elicited by the neural inducing signal was either greatly attenuated or absent. Explants cultured in the presence of H2DIDS remain intact and viable for extended periods, and cell-cell contacts within H2DIDS-treated explants are morphologically indistinguishable from those of control explants.

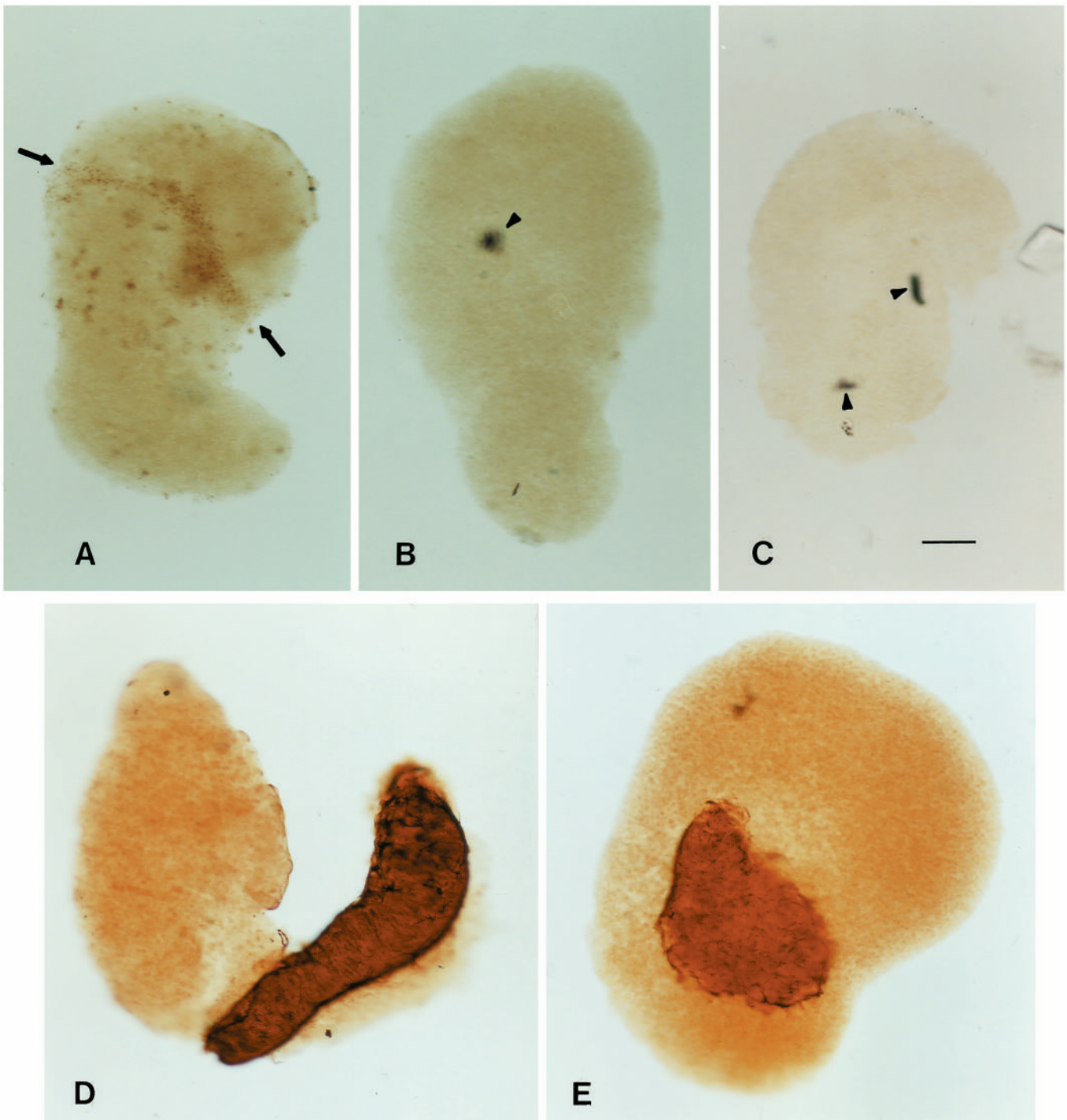
Second, we tested the effects of medium in which the [Na⁺] is reduced from 95 mM to 20 mM, and the [Cl⁻] is increased from 55 mM to 81.5 mM (Δ NaCl medium) (Fig. 3B). Under these conditions, the ion concentration gradients necessary for Na⁺-dependent Cl⁻/HCO₃²⁻ exchange should be disrupted. In this medium, the increase in pH_i was blocked in 11 of 13 cells over 3 experiments. One of the two cells that underwent an alkalization was located in the interior of the explant, at least 10 cell diameters from the edge. At such an interior position, the interstitial ion concentrations may be unable to equilibrate rapidly with those of the external medium because of unstirred layer effects. Thus the ion concentrations in the interior of the explant may differ from those of the medium. In Δ NaCl medium, as in the presence of H2DIDS, the explants survive for at least 24 hours and cell-cell contacts appear normal.

We have examined the effects of H2DIDS and Δ NaCl medium on the expression of the *Xenopus* homologue of the homeobox gene *engrailed* (*en-2*). The *en-2* gene product is first

detected at stage 14 (early neurula) in both the deep and superficial layers of neural tissue at the boundary between the presumptive midbrain and hindbrain regions (Hemmati-Brivanlou and Harland, 1989). Recent studies have shown that expression of en-2 is induced by planar inductive signals (Doniach et al., 1992). Explants of the DMZ were prepared at the beginning of gastrulation and cultured in the presence or absence of H2DIDS until stage 21. Additional explants were cultured during a similar interval in Δ NaCl medium. Explants were then assayed for the presence of en-2 by whole-mount immunohis-

tochemistry, using the monoclonal antibody 4D9 (Hemmati-Brivanlou and Harland, 1989).

In control explants, en-2 is expressed in a band or patch extending partially or completely across the width of the explant in the neurectodermal region (Fig. 4A). The en-2 protein is localized to the nucleus, resulting in a distinctive punctate staining pattern. The staining in these explants appears less intense than it does in whole embryos (Hemmati-Brivanlou and Harland, 1989) or in DMZ explants that include the epithelial layer (Doniach et al., 1992), because the epithe-



lial cells that express en-2 are not present. The en-2 protein is not detected in explants cultured in the presence of H2DIDS (Fig. 4B). Similarly, en-2 expression is inhibited in explants cultured in ΔNaCl medium (Fig. 4C).

To determine whether the absence of detectable en-2 expression by H2DIDS reflects a nonspecific inhibitory effect, control and H2DIDS-treated explants were also immunostained with the notochord-specific monoclonal antibody Tor-70 (Bolce et al., 1992). In control explants, the Tor-70 epitope is expressed in an elongated region of cells that have converged and extended, forming the presumptive notochord (Fig. 4D). In H2DIDS-treated explants, the Tor-70 epitope is expressed in a compact cell mass. Although convergence and extension are apparently inhibited by H2DIDS, notochordal differentiation evidently proceeds in the absence of these morphogenetic movements (Fig. 4E). Thus, treatment with H2DIDS does not prevent notochord-specific gene expression.

Effects of NH_4Cl

Sive et al. (1989) have proposed that amphibian neural induction occurs in two steps: a transition from an epidermal to a nonepidermal state of specification, followed by a second step initiating neural development. Ectoderm that does not progress beyond the first step differentiates as cement gland, an epithelial secretory organ that lies ventral to the anterior edge of the neural plate. Cement gland formation, including the initiation of cement gland-specific gene expression, can be elicited by prolonged treatment with 10 mM NH_4Cl (Picard, 1975; Sive et al., 1989), suggesting that alterations in pH_i might mediate the earliest response to the neural-inducing signal, a switch from epidermal to nonepidermal development.

We examined the effects of NH_4Cl on the pH_i of gastrula ectoderm cells to determine whether it mimics the increase in pH_i that we observe during neural induction (Fig. 5). Treatment with 10 mM NH_4Cl produced a temporary alkalization of approximately 0.1 pH units. After 90 minutes, however, the pH_i exhibited a net decrease of approximately 0.05 pH units. This sustained acidification in response to NH_4Cl treatment has been observed in *Xenopus* oocytes (Lee and Steinhardt, 1981). Apparently, the *Xenopus* embryonic plasma membrane is permeable to NH_4^+ , which equilibrates across the membrane more slowly than does the freely permeant NH_3 . Intracellular acidification presumably results from entry of the NH_4^+ ion

into the cell and its subsequent dissociation into NH_3 and H^+ . Thus, while it does trigger a transient alkalization, treatment with NH_4Cl does not elicit the sustained increase in pH_i that occurs in response to the endogenous neural inducing signal.

Effects of some protein kinase agonists

Previous work indicating that protein kinases A and C are activated in response to neural induction (Davids et al., 1987; Otte et al., 1988, 1989) led to the hypothesis that the alkalization might occur via protein kinase C-activated Na^+/H^+ exchange. This possibility prompted us to determine whether the protein kinase C agonist TPA, either alone or sequentially in conjunction with the cAMP-dependent protein kinase agonist dibutyryl cAMP, could elicit the alkalization in explants of dorsal ectoderm. Explants containing only the dorsal ectoderm were prepared as described earlier, and pH_i was monitored during and after the addition of TPA. The culture medium and TPA concentrations were identical to those used by Otte et al. (1988, 1989). Initial experiments were performed in Flickinger's medium using 250 nM TPA; under these conditions, Otte et al. (1988, 1989) obtained a neural-

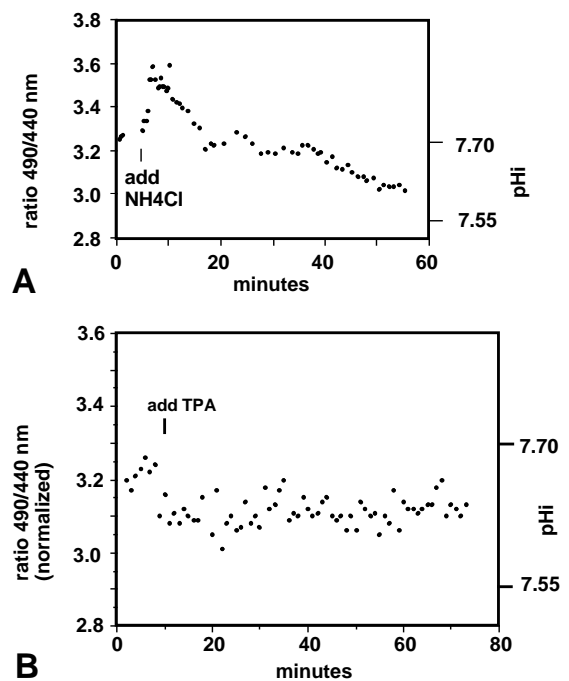


Fig. 5. Effects of NH_4Cl and TPA on intracellular pH of gastrula ectoderm cells. (A) Treatment of ectoderm cells with NH_4Cl . Dissociated ectoderm cells containing BCECF-dextran were transferred to poly-lysine-coated dishes containing DFA medium. After an initial recording of the emission ratio, NH_4Cl was added to a final concentration of 10 mM and remained present throughout the experiment. Immediately following addition of NH_4Cl , the pH_i increased by approximately 0.1 pH units. Within 15 minutes, the pH_i had returned to the initial level; it later decreased, resulting in an acidification of 0.05 pH units after 90 minutes ($n=12$ cells in 3 experiments). (B) Treatment of ectodermal explants with TPA. Addition of 250 nM TPA produced an immediate drop in pH_i , which failed to return to initial levels during the following hour. TPA was present throughout the recording period. ($n=11$ of 12 cells in 4 experiments).

Fig. 4. Effects of H2DIDS and ΔNaCl medium on tissue-specific gene expression. (A) Visualization of en-2 expression in control explants. The en-2 protein can be seen within the nuclei of cells in a band across the ectodermal region of the explants (arrows) (14 of 16 explants from 3 experiments). The en-2 protein is not detected in explants cultured in the presence of 200 μM H2DIDS (B; 0 of 14 explants from 3 experiments) or in ΔNaCl medium (C; 0 of 6 explants from 2 experiments). In B and C, dark areas indicated by arrowheads are artifacts. (D,E) Effects of H2DIDS on expression of the notochord-specific Tor-70 epitope. (D) Control explants show expression of the Tor-70 epitope within the elongated notochord. The degree of convergent extension displays substantial variability in these explants (7 of 7 explants from 2 experiments). (E) The Tor-70 epitope is expressed in H2DIDS-treated explants (10 of 10 explants from 2 experiments). Cells that express the Tor-70 epitope occupy a smaller area within the mesodermal region. Convergent extension is reduced or absent in explants treated with H2DIDS. Scale bar in C = 90 μm , for all panels.

specific response in approximately 35-45% of explants of gastrula ectoderm.

A representative experiment is shown in Fig. 6A. Surprisingly, cells acidify immediately after addition of 250 nM TPA; the pH_i leveled out within 10-20 minutes, although it did not return to the initial level. This decrease in pH_i was accompanied by the rapid dissociation of the explants. Identical results were obtained for TPA at concentrations ranging from 20 to 250 nM (data not shown), and for explants maintained in either DFA or Flickinger solution, the medium used by Otte et al. (1988) (data not shown). Treatment with either 1 mM dibutyryl cAMP or DMSO alone had no effect on pH_i (data not shown).

We also sought to determine whether the sequential pharmacological activation of protein kinase C and protein kinase A would produce the intracellular alkalinization observed during neural induction in explants. Dissociated dorsal ectoderm cells from stage 10⁺ embryos were placed in Flickinger solution, treated first with 250 nM TPA and then with 1 mM dibutyryl cAMP 4 hours later, conditions that reportedly elicit a neural-specific response. Intracellular pH was monitored continuously during and immediately after the addition of TPA and at regular intervals thereafter. The results are similar to those obtained by treatment with TPA alone; no synergistic effect on pH_i is observed (data not shown).

DISCUSSION

We have demonstrated that dorsal ectoderm cells undergo an increase in intracellular pH in response to planar neural-inducing signals. This ionic signal occurs during stage 10.5, nearly halfway through gastrulation; several other responses, including expression of NCAM (Jacobson and Rutishauser, 1986) and convergence and extension of the presumptive hindbrain and spinal cord (Keller et al., 1992b), become specified at stage 11. In addition, some of the cells that will later differentiate as Rohon-Beard spinal neurons begin to withdraw from the cell cycle at or before stage 11 (Lamborghini, 1980). Thus, the intracellular alkalinization is among the earliest known responses to neural induction.

Intracellular alkalinization is a common response to external signals. In many cells, activation of protein kinase C by mitogens stimulates Na^+/H^+ transport, resulting in an intracellular alkalinization in the range of pH 7.1-7.4. This regulatory sequence is often associated with a dramatic increase in metabolic activity and frequently accompanies reentry into the cell cycle (Rozengurt, 1992). In the sea urchin egg, the alkalinization that occurs following fertilization is known to activate a tyrosine kinase (Jiang et al., 1991).

Gap junctional conductance in amphibian embryonic cells is highly sensitive to alterations in pH_i , decreasing with intracellular acidification (Spray et al., 1981, Turin and Warner, 1980). Gap junctional coupling in ventral blastomeres of *Xenopus* embryos is significantly increased following an alkalinization of approximately 0.15 pH units (Guthrie et al., 1988), an increase similar in range and magnitude to that which we have observed during neural induction. Changes in pH_i may also regulate gap junctional communication indirectly by altering tyrosine kinase activity. The inhibitory action of *v-src* on gap junctional communication is sensitive to pH in 3T3 cells (Hyc

and Rose, 1990), and can be mediated by phosphorylation of connexin 43 (Swenson et al., 1990).

If neural induction by planar signals is dependent upon gap junctional communication, then the intracellular alkalinization could conceivably modulate or enhance their transmission. A role for gap junctional communication during inductive interactions in *Xenopus* embryos has been suggested by Warner and colleagues (Warner et al., 1984), who found that embryos injected with antibodies directed against gap junction proteins during cleavage stages exhibit defects in anterior neural structures. While we lack direct evidence that neural induction is mediated in part by gap junctional communication, the possibility remains that planar inductive signals could be propagated through the ectoderm by an increase in gap junctional communication triggered by the alkalinization. Analysis of the effects of the intracellular alkalinization on gap junctional communication during neural induction will require direct measurement of gap junctional coupling in the newly induced dorsal ectoderm.

Our results suggest that the increase in pH_i participates in the response to neural induction, since inhibition of this increase by either pharmacological agents or ion substitution leads to an inhibition of en-2 expression. Earlier findings showing that treatment of isolated ectoderm with NH_4Cl would elicit cement gland differentiation (Picard, 1975; Sive et al., 1989) are consistent with this hypothesis, although the transient alkalinization produced by this treatment does not represent the ionic changes that occur during neural induction. Treatment with H2DIDS does not prevent the expression of the notochord-specific Tor-70 epitope, although it may interfere with the convergence and extension of the axial mesoderm. The expression of a notochord-specific marker in the presence of H2DIDS, however, suggests that the lack of detectable en-2 protein can be attributed to the inhibition of alkalinization during the response to neural induction, rather than to nonspecific effects of H2DIDS. The effects on en-2 expression do not allow us to distinguish whether the increase in pH_i is part of a primary response to neural induction or mediates secondary aspects of the inductive response that may contribute to positional identity.

While a thorough analysis of pH regulation during neural induction is beyond the scope of this paper, our preliminary observations indicate that the increase in pH_i is mediated primarily by Na^+ -dependent anion transport. First, the alkalinization is attenuated or prevented by both the anion transport inhibitor H2DIDS and by $\Delta NaCl$ medium, in which alterations in Na and Cl content should greatly reduce Na^+ -dependent Cl^-/HCO_3^- exchange. Treatment of ectoderm cells with the protein kinase C agonist TPA does not produce an alkalinization, a finding that argues against a role for protein kinase C-induced Na^+/H^+ exchange. Grandin and Charbonneau (1991) found that TPA or synthetic diacylglycerols do not elicit the intracellular alkalinization that occurs immediately after fertilization in *Xenopus*; In the oocyte, TPA produces an acidification similar to that which we have observed in ectoderm cells, suggesting that it may act as a weak acid. Moreover, Webb and Nuccitelli (1982) were unable to detect a Na^+/H^+ antiporter in amphibian oocytes. These findings suggest that, in amphibian embryonic cells, pH_i regulation in response to external signals may operate primarily through anion transport systems. In many cell types, both the Na^+/H^+ antiporter and the Na^+ -

dependent $\text{Cl}^-/\text{HCO}_3^-$ exchanger are dependent upon pH_i (Moolenaar, 1986); the pH_i sensitivities of these transport systems in relatively alkaline amphibian embryonic cells are unknown.

In the absence of strong candidates for the endogenous neural-inducing signal, several studies have endeavored to investigate the response to neural induction using agents that evoke aspects of the neural-specific response. Most recently, these have included NH_4Cl and TPA. As discussed earlier, the response to NH_4Cl is somewhat similar to the ionic response to the endogenous signals. One interpretation is that a brief increase in pH_i is sufficient to bring about a shift from epidermal to nonepidermal (i.e., cement gland) gene expression; moreover, a sustained increase in pH_i may be required for subsequent responses to neural induction.

No such parallels can be drawn between the ionic changes elicited by TPA, with or without dibutyryl cAMP, and those occurring in vivo. Our results raise the question of whether the neural-specific responses elicited by TPA are due to the activation and translocation of protein kinase C, or to toxic side effects that create a 'sublethal cytolysis' which may itself trigger a neural-specific response (Holtfreter, 1947). Nevertheless, correlations in the spatiotemporal patterns of protein kinase C isoform expression and activity point toward a role for protein kinase C in neural induction (Otte et al., 1988, 1989, 1990) or the maintenance of neural competence (Otte et al., 1991; Otte and Moon, 1992).

Intracellular alkalinization can be used as a dynamic marker of the response to neural induction on a cell-by-cell basis in real time, i. e., as a continuous record during the response itself. Most studies of neural induction compare features of the responding tissue, such as patterns of gene activity, in the presence and absence of the inductive signal. Despite the power of this approach, it fails to acknowledge that the cells fated to give rise to the nervous system undergo a dramatic rearrangement during neural induction, when they move from a primarily mediolateral array to a primarily anteroposterior array. More important, *the establishment of positional identity in the newly induced neural ectoderm is committant with these morphogenetic movements*. In addition, the mounting evidence that planar signals play a significant role in neural induction via distinct long-range and short-range responses (Keller et al., 1992b; Doniach et al, 1992; Sater et al., 1993), indicates that the spatial dynamics of planar, vertical and homeogenetic signals are of critical importance in neural induction. Moreover, Saha and Grainger (1992) suggest that specification of anteroposterior pattern is in progress during the second half of gastrulation. Thus, to understand the establishment of anteroposterior pattern, it is necessary to examine the relationship between the transmission of inductive signals and induced cell behaviors *prior* to the time at which the molecular correlates of positional identity are expressed.

Such an investigation requires the analysis in real time of both inductive signals and morphogenetic responses to neural induction. As a first step, future studies will map the spatiotemporal pattern of signal transmission throughout neural induction by using the alkalinization as an assay for the response to neural induction in living cells. Other studies now in progress will elucidate the role of this ionic signal in the response to neural induction.

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