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Calcium and neurulation in mammalian embryos

II. Effects of cytoskeletal inhibitors and calcium antagonists on the neural folds of rat embryos

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SUMMARY

The role of calcium in neurulation in mammalian embryos has been studied by culturing rat embryos at 10·4 days of gestation, when the cephalic neural folds have elevated but not fused, in serum containing cytoskeletal inhibitors or calcium antagonists. The effects of these antagonists on the morphology of the cephalic neural folds have been examined by scanning electron microscopy. The different agents caused the cephalic neural folds to part to varying degrees. The neural folds were classified as intact (normal), open (folds parted up to 90° with each other), flattened (folds parted from 90° to 180°) or collapsed (folds parted more than 180°).

The microtubule inhibitors colchicine and nocodazole at 10^{-4} M and $6{\cdot}6\times10^{-4}$ M respectively cause the cephalic neural folds of 10·4-day embryos to collapse after 60 min. At $5{\cdot}2\times10^{-6}$ M the microfilament inhibitor cytochalasin B causes the folds to open after 60 min. Longer term culture of 9·5-day embryos for 24 h in diazepam, which is reported to inhibit myosin synthesis, causes general developmental retardation including a delay in the closure of the neural tube.

Culture of 10·4-day rat embryos for 60 min in papaverine at $2{\cdot}4\times10^{-4}$ M or gallapomil (D-600) at $5{\cdot}0\times10^{-4}$ M, agents which reduce the entry of calcium into cells, causes opening of the elevated cephalic neural folds. In contrast TMB-8, which is purported to perturb some intracellular calcium-dependent functions, does not cause opening of the elevated cephalic neural folds, even at high concentrations.

The results suggest that both microtubules and microfilaments are essential to the maintenance of the elevated cephalic neural folds in rat embryos. The results are also compatible with the idea that calcium ion flux across the membranes of the neuroepithelial cells might be important for the elevation of the neural folds, and thus for successful neurulation.

INTRODUCTION

In vertebrate embryos the elevation of the neural folds, which are destined to fuse and form the neural tube, is probably effected mainly by coordinated changes in the shapes of the cells of the neural ectoderm as they become elongated and tapered (Karfunkel, 1974). Microfilaments appear at the apices of the cells of the neural ectoderm at a time when they are changing shape (Schroeder, 1970; Burnside, 1971; Perry, 1975). In addition, agents such as cytochalasins, which disrupt microfilament function, inhibit neurulation in amphibian (Karfunkel, 1974) and mammalian (O'Shea, 1981; Morriss-Kay, 1983) embryos. Thus it appears that the changes in cell shape that effect neurulation are at least partly generated by contraction of microfilaments in the cells of the neural epithelium.

Key words: calcium, neurulation, rat embryo, colchicine, nocodazole, diazepam, cytochalasin B, papaverine.

In non-embryonic systems the contraction of microfilaments is initiated by changes in the concentration of intracellular free calcium (Huxley, 1973; Hitchcock, 1977), and so it is likely that calcium is important in the control of neurulation. Measurements of calcium in the medium in which amphibian embryos are developing show that calcium is relocated during neurulation (Moran, 1976) and studies using radiolabelled calcium have suggested that calcium is incorporated at different rates in different regions along the neural tube (Barth & Barth, 1972). In addition papaverine, which is thought to alter calcium fluxes (Imai & Takeda, 1967), can prevent neurulation in amphibian (Moran & Rice, 1976) and chick (Lee & Nagele, 1979) embryos, and its effect can be ameliorated or reversed by addition of the ionophore A23187, which increases the permeability of biological membranes to calcium (Reed & Lardy, 1972). Recently it has been shown that papaverine inhibits neurulation in mouse embryos (O'Shea, 1982), suggesting that calcium is important in neurulation in mammalian embryos too. More directly, it has been shown that culture of rat embryos at the elevated neural fold stage in medium without divalent cations causes an opening and then folding back ('collapse') of the neural folds. Restoration of calcium, but not magnesium, to the medium prevents this collapse of the neural folds (Stanisstreet, Smedley & Moore, 1984; Smedley & Stranisstreet, 1985). Lanthanum, which competes for calcium sites (Weiss, 1974), causes the elevated neural folds to open even in the presence of calcium (Stanisstreet *et al.* 1984; Smedley & Stanisstreet, 1985). Thus it appears that extracellular calcium is required for the maintenance of the elevated neural folds, and thus for normal neurulation.

The aim of the present experiments is to explore further the role of calcium and calcium-activated microfilaments in neurulation in mammalian embryos. The effects of inhibitors on the elevated neural folds of 10·4-day rat embryos have been determined by short-term *in vitro* culture. Cytochalasin B, which inhibits microfilament function, and colchicine and nocodazole, which prevent microtubule assembly, have been used. In addition the effects on the development of rat embryos *in vitro* of diazepam have been assessed. Diazepam is a benzodiazepam derivative which inhibits the synthesis of myosin (Bandman, Walker & Strohman, 1978; Walker, Bandman & Strohman, 1979), a component of microfilaments, and has been shown to disturb neurulation in avian embryos (Nagele, Pietrolungo, Lee & Roisen, 1981; Lee, Koscuik, Nagele & Roisen, 1983). Attempts have been made to gain clues about the mechanisms of action of calcium during neurulation in mammalian embryos by determining the effects of calcium antagonists (Fleckenstein, 1977) on the elevated neural folds of rat embryos. Papaverine and D-600 (Gallopamil) are thought to reduce calcium entry into the cell by blocking calcium channels (Janis & Schriabine, 1983). In contrast TMB-8 (8-(Diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride) is purported to antagonize certain intracellular calcium-dependent functions (Malagodi & Chiou, 1974; Charo, Feinman & Detwiler, 1976) and has been employed to study the possible role of calcium in other morphogenetic systems (Saunders & Helper, 1983).

MATERIALS AND METHODS

Embryo culture

Rat embryos at the elevated-neural-fold stage were obtained from random-bred white Wistar rats at 10·4 days of gestation, timed from midnight preceding the morning on which vaginal plugs were observed (New, 1978). Embryos were dissected from the uterus in Hank's balanced saline containing $4{\cdot}2\times10^{-3}$ M-sodium bicarbonate (Flow Laboratories, UK). Embryos were cultured in immediately centrifuged rat serum (below) at 37°C in rotating bottles according to the method of New, Coppola & Terry (1973). The cultures were equilibrated with a 20 % O_2, 5 % CO_2, 75 % N_2 gas mixture to provide the oxygen tension appropriate for embryos of this stage (New, Coppola & Cockroft, 1976). Before culture the yolk sac and amnion of the embryos were opened with watchmakers' forceps to facilitate penetration of the culture medium. The effects of different concentrations of the various inhibitors on the elevated neural folds were assessed by culturing embryos in rat serum with or without the inhibitors for 60 min, since previous experiments had shown that the neural folds respond to removal or displacement of calcium from the medium within this time; within 60 min the neural folds lose their characteristic curvature and curl back ('collapse') (Smedley & Stanisstreet, 1985).

Since diazepam is reported to inhibit the synthesis of myosin, longer-term experiments were performed with this inhibitor. Embryos were explanted at 9·5 days of gestation and cultured for 24 h. Embryos were cultured as above except that the cultures were gassed with a 5 % O_2, 5 % CO_2, 90 % N_2 gas mixture to provide the appropriate oxygen tension for earlier embryos (New *et al.* 1976) and the embryonic membranes were left intact. At the end of the culture the crown–rump lengths of the embryos and the diameters of the yolk sacs were measured, and any embryonic abnormalities were noted. The embryos were then scored according to the method of Brown & Fabro (1981). This system uses morphological criteria to assess embryonic development and to ascribe an 'apparent embryonic age' to the embryo.

At the end of the short-term (60 min) or long-term (24 h) cultures embryos were fixed for scanning electron microscopy.

Preparation of rat serum

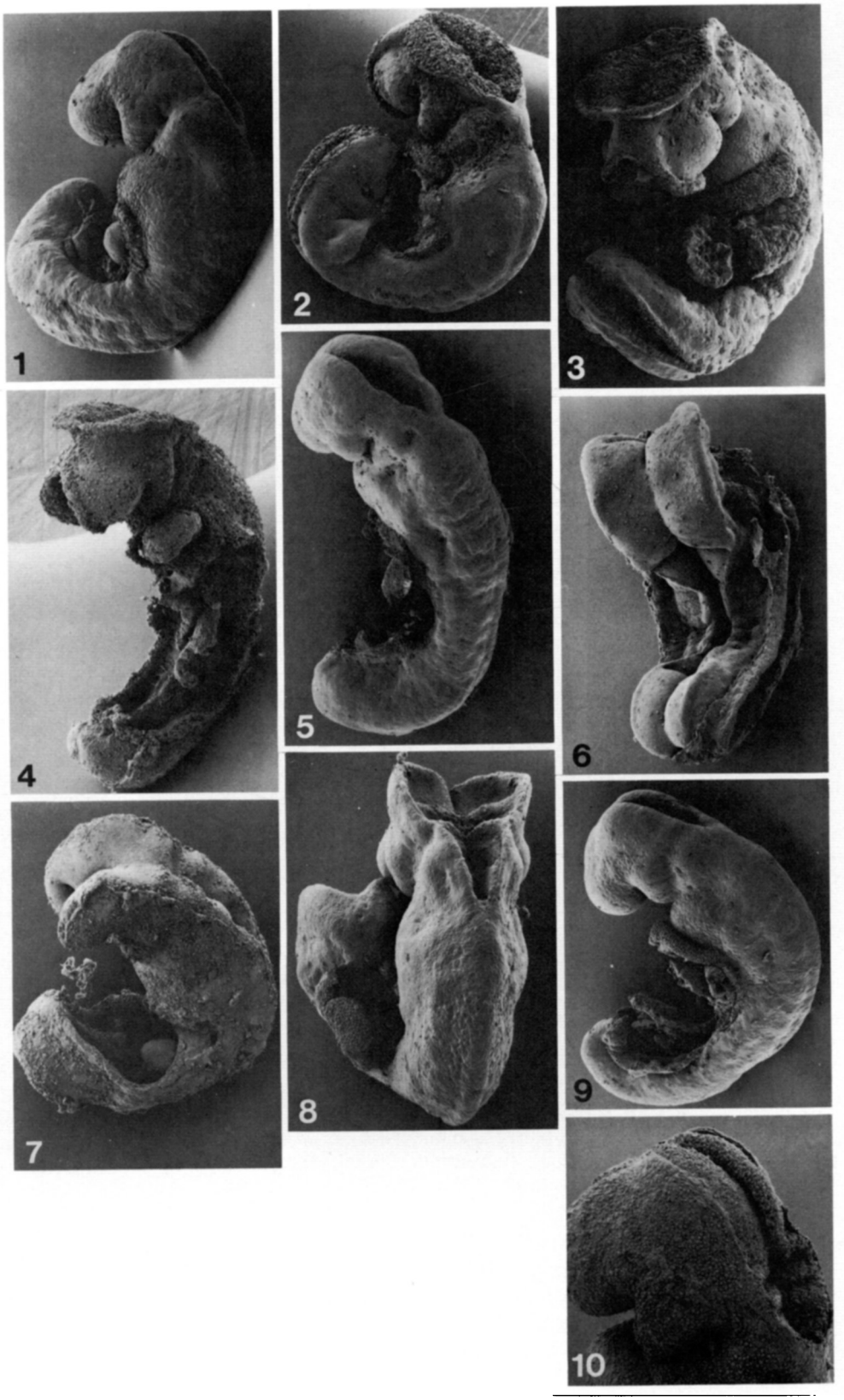
Rat serum was prepared as for long-term embryo culture (New, 1978). The blood was centrifuged immediately following withdrawal from the dorsal aorta and allowed to stand until the plasma clot had formed. The clot was removed and the serum was collected after recentrifugation. Streptomycin and penicillin (Flow Laboratories, UK) were added to final concentrations of 100 $\mu g\,ml^{-1}$ and 100 i.u. ml^{-1} respectively. The serum was stored at −20°C for a maximum of 28 days. Immediately before use the thawed serum was inactivated at 56°C for 30 min.

Inhibitors and calcium antagonists

Colchicine (Sigma Ltd) was dissolved directly in serum; nocodazole (Sigma Ltd) was prepared as a stock solution (10^{-2} M) in spectroscopic grade ethanol so that the final concentration of ethanol in serum did not exceed 1 %. Cytochalasin B (Sigma, UK) was prepared as a stock solution (1 mg ml^{-1}) in dimethyl sulphoxide (DMSO) so that the final concentration of DMSO in serum did not exceed 1 %. Diazepam (Roche Products Ltd, UK) and papaverine (Sigma, UK) were dissolved directly in serum. D-600 (Gallopamil, Knoll AG, Germany) and TMB-8 (Aldrich Chemical Co., UK) were prepared as stock solutions (5×10^{-2} M and 5×10^{-1} M respectively) in spectroscopic grade ethanol (BDH, UK), so that the final concentration of ethanol in the serum did not exceed 1 %. At the highest concentrations used none of the agents altered the pH of serum measured at 37°C in equilibrium with 5 % CO_2.

Scanning electron microscopy

Embryos were fixed overnight in 2·5 % glutaraldehyde in 10^{-1} M-cacodylate buffer, pH 7·3 (Karnovsky, 1965). They were washed in changes of buffer and the embryonic membranes were removed using watchmakers' forceps. The embryos were dehydrated in a graded ethanol series,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the absolute ethanol was replaced with liquid CO_2 and the embryos were dried by the critical-point method. The embryos were affixed to stubs, coated with gold–palladium and observed and photographed using a Philips 501B scanning electron microscope.

The numbers of embryos observed for each experimental condition are given in the tables. For each condition embryos from at least two different litters were used.

RESULTS

In the majority of these experiments embryos at 10·4 days of gestation were used. At this stage the neural folds were fused medially, the posterior neural folds were still open and the cephalic neural folds were elevated but not yet fused (Fig. 1). The results of control cultures of embryos in serum alone, or in serum containing the solvents at the highest concentrations used in the experimental cultures, showed that after 60 min the cephalic neural folds remained elevated.

The inhibitors and antagonists at different concentrations produced a range of effects on the cephalic neural folds and following observations by scanning electron microscopy neural folds were classified as 'intact', 'open', 'flattened' or 'collapsed'. Intact neural folds were similar to those in the controls where the neural folds appeared not to have been affected (e.g. Fig. 1). Embryos with open

Fig. 1. Scanning electron micrograph of control 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, following 60-min culture in rat serum. Neural folds have remained intact. ×42.

Fig. 2. Scanning electron micrograph of 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, following 60-min culture in rat serum containing 10^{-4} M-colchicine. Cephalic neural folds have collapsed. ×38.

Fig. 3. Scanning electron micrograph of 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, following 60-min culture in rat serum containing $6{\cdot}6\times10^{-4}$ M-nocodazole. Cephalic neural folds have collapsed. ×48.

Fig. 4. Scanning electron micrograph of 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, following 60 min culture in rat serum containing $2{\cdot}1\times10^{-5}$ M ($10\,\mu g\,ml^{-1}$) cytochalasin-B. Cephalic neural folds have collapsed. ×35.

Fig. 5. Scanning electron micrograph of control 9·5-day rat embryo following 24 h culture in rat serum. The neural folds have fused medially and the cephalic neural folds have elevated. ×40.

Fig. 6. Scanning electron micrograph of 9·5-day rat embryo following 24 h culture in rat serum containing 10^{-4} M-diazepam. Neural folds have failed to fuse medially. ×62.

Fig. 7. Scanning electron micrograph of 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, following 60-min culture in rat serum containing 2×10^{-3} M-papaverine. Cephalic neural folds have flattened. ×40.

Fig. 8. Scanning electron micrograph of 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, following 60-min culture in rat serum containing 5×10^{-4} M D-600 (Gallopamil). Cephalic neural folds have opened. ×50.

Fig. 9. Scanning electron micrograph of 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, following 60-min culture in rat serum containing 10^{-3} M TMB-8. Cephalic neural folds have remained elevated. ×42.

Fig. 10. Scanning electron micrograph of 10·4-day rat embryo, in which the cephalic neural folds had elevated but not fused, following 60-min culture in rat serum containing 10^{-2} M TMB-8. Cells are dissociating, but cephalic neural folds have remained elevated. ×74.

neural folds were those in which the cephalic neural folds had parted to form an angle of up to 90° with each other (e.g. Fig. 8); embryos with flattened neural folds were those in which the folds had parted from 90° to 180° (e.g. Fig. 7). Embryos with collapsed neural folds were those in which the neural folds had parted to 180° or greater (e.g. Fig. 2). Although different treatments produced a range of effects on the cephalic neural folds, the effects were consistent for each condition.

Effects of microtubule and microfilament inhibitors on neural folds

The effects of 60-min culture in the presence of microtubule or microfilament inhibitors on the cephalic neural folds of 10·4-day rat embryos are summarized in Table 1. Colchicine at 10^{-4} M caused the elevated neural folds to collapse (Fig. 2). Nocodazole at $3{\cdot}3\times10^{-5}$ M caused the neural folds to open, and at $6{\cdot}6\times10^{-4}$ M nocodazole caused the cephalic neural folds to collapse (Fig. 3). Cytochalasin B at $2{\cdot}1\times10^{-6}$ M ($1\,\mu g\,ml^{-1}$) did not appear to affect the neural folds whereas at 5×10^{-5} M ($2{\cdot}5\,\mu g\,ml^{-1}$) cytochalasin B caused the neural folds to open. At $2{\cdot}1\times10^{-5}$ M ($10\,\mu g\,ml^{-1}$) cytochalasin B caused the cephalic neural folds to collapse and the cells of the neural epithelium to become rounded (Fig. 4). Thus it appears that either microtubule or microfilament inhibitors can cause the elevated neural folds of rat embryos to lose their characteristic shape and reopen.

Effects of diazepam on embryos

The effects on rat embryos of 24 h culture in the presence of diazepam are summarized in Table 2. Control embryos explanted at 9·5 days and cultured for 24 h *in vitro* appeared normal and similar to the embryos explanted at 10·4 days. The embryos had an average of 13 somites and estimations by the method of Brown & Fabro (1981) showed that they had an average apparent embryonic age of 10·5 days. The neural folds had fused medially and the cephalic neural folds had elevated but not fused (Fig. 5).

Diazepam at 5×10^{-5} M did not cause abnormal development although it did appear to delay development. The average apparent age of the embryos was 10·3 days and the average somite number was 11·4. At 10^{-4} M-diazepam had a greater inhibitory effect on general embryonic growth. Yolk-sac expansion was less than in controls and the mean apparent embryonic age was 9·8 days. The neural folds of such embryos were not fused medially (Fig. 6). At 5×10^{-4} M-diazepam prevented development of the embryos. Thus diazepam causes a general retardation of development, including a delay in the closure of the neural folds.

Effects of calcium antagonists on neural folds

The effects of 60-min culture in the presence of calcium antagonists on the elevated cephalic folds of 10·4-day rat embryos are summarized in Table 3. Papaverine at 2×10^{-4} M caused the cephalic neural folds to open; papaverine at 2×10^{-3} M caused them to flatten (Fig. 7). D-600 (Gallopamil) at 10^{-4} M appeared to have no effect on the neural folds, but at 5×10^{-4} M it too caused the cephalic

Table 1. *Effects of microtubule and microfilament inhibitors on elevated neural folds of 10·4-day rat embryos*

Inhibitor	Concentration (M)	State of neural folds
Colchicine	10^{-4} (n = 16)	Collapsed
Nocodazole	$3{\cdot}3\times10^{-5}$ (n = 15)	Open
	$6{\cdot}6\times10^{-4}$ (n = 16)	Collapsed
Cytochalasin B	$2{\cdot}1\times10^{-6}$ (1 μg ml^{-1}) (n = 3)	Intact
	$5{\cdot}2\times10^{-6}$ (2·5 μg ml^{-1}) (n = 17)	Open
	10^{-5} (5 μg ml^{-1}) (n = 16)	Flattened
	$2{\cdot}1\times10^{-5}$ (10 μg ml^{-1}) (n = 21)	Collapsed

folds to open (Fig. 8). At 10^{-3} M D-600 caused the cephalic neural folds to flatten. TMB-8 at 10^{-3} M did not appear to affect maintenance of the neural folds, which remained intact (Fig. 9). Even at 10^{-2} M TMB-8 did not cause the folds to open, although it did cause some dissociation of the lateral ectoderm and detachment of the lateral ectoderm from the neural ectoderm at the apices of the folds (Fig. 10). Thus it appears that those calcium antagonists which are reported to affect calcium fluxes across the cell membrane caused the neural folds to open whereas TMB-8, which is purported to inhibit certain intracellular calcium-dependent functions,

Table 2. *Effects of diazepam on rat embryos cultured* in vitro *for 24 h from 9·5 days*

Concentration of diazepam (M)	Apparent embryonic age	Number of somites	Yolk sac diameter (mm)
Control (n = 10)	10·47 ± 0·02	13·0 ± 0·04	2·32 ± 0·16
5×10^{-5} (n = 10)	10·30 ± 0·04	11·4 ± 0·04	2·18 ± 0·13
10^{-4} (n = 11)	9·89 ± 0·06	7·7 ± 0·02	1·79 ± 0·15
5×10^{-4} (n = 10)	—	—	0·99 ± 0·17

did not cause the folds to open even at concentrations high enough to produce some cell dissociation.

DISCUSSION

The present results show that inhibitors with a variety of actions on the cell cause opening, and then collapse of the elevated neural folds of 10·4-day rat embryos. The mechanisms of actions of these inhibitors provide indications of the cellular requirements for the maintenance of the elevated neural folds and, by implication, of the cellular machinery employed during normal neurulation.

Microtubules appear in the neural cells of amphibian embryos at the time when they are becoming elongated, and thus it has been suggested that they are responsible for this aspect of the change in cell shape (Waddington & Perry, 1966). Colchicine and nocodazole interfere with microtubule function by binding to the constituent protein tubulin (Beeb, Feagans, Blanchette-Mackie & Nau, 1979). Continuous exposure of avian (Handel & Roth, 1971; Karfunkel, 1972) or amphibian (Burnside, 1971; Lofberg & Jacobson, 1974; Brun & Garson, 1983) embryos to microtubule inhibitors during the period of neurulation results in a failure of closure of the neural tube. Neurulation is inhibited in mouse embryos too by long-term exposure to colchicine (Ferm, 1963; O'Shea, 1981). The present results extend these observations since they show that microtubule inhibitors not only prevent neurulation, but also that short-term exposure to such inhibitors reverses the elevation of the neural folds.

The fungal metabolite cytochalasin B has been shown to perturb microfilament function, and thus has been used as a probe for microfilament-dependent

Table 3. *Effects of calcium antagonists on elevated neural folds of 10·4-day rat embryos*

Antagonist	Concentration (M)	State of neural folds
Papaverine	$2{\cdot}4\times10^{-4}$ (n = 15)	Open
	$2{\cdot}0\times10^{-3}$ (n = 19)	Flattened
D-600	10^{-4} (n = 15)	Intact
	$5{\cdot}0\times10^{-4}$ (n = 16)	Open
	10^{-3} (n = 15)	Flattened (some dissociation)
TMB-8	10^{-3} (n = 15)	Intact
	10^{-2} (n = 17)	Intact (dissociation)

embryogenetic processes (Schroeder, 1978). For example, treatment of amphibian or avian embryos with cytochalasin B causes the neural folds to flatten (Karfunkel, 1974) and culture of mouse embryos over the period of organogenesis in cytochalasin B prevents the formation of wedge-shaped cells in the neural epithelium and consequently inhibits neurulation (O'Shea, 1981). More directly related to the present observations, it has been shown recently that cytochalasin D causes collapse of the already elevated neural folds in rat embryos (Morriss-Kay, 1981, 1983). The present results confirm that cytochalasin B at 10^{-5} M causes opening of the neural folds; higher concentrations cause their flattening. Thus it appears that functional microtubules and microfilaments are required for the maintenance of the elevated neural folds, and thus for successful neurulation in mammalian embryos.

It appears that neurulation depends on coordinated changes in the shapes of individual cells, wrought by microfilaments and microtubules. Since the contraction of microfilaments in non-embryonic systems is controlled by the level of intracellular free calcium, calcium is implicated as being important in the morphogenesis of the neural tube. Indeed it has been suggested that a change in membrane permeability to calcium, leading to a localized rise in the concentration of intracellular free calcium, might be one trigger for changes in cell shape of the type which effect neurulation (Stanisstreet, 1982). Compatible with this suggestion are the observations that removal of calcium from the medium in which rat embryos are cultured rapidly causes the cells of the neural epithelium to lose their characteristic elongated shape resulting in collapse of the neural folds (Smedley & Stanisstreet, 1985; Morriss-Kay, personal communication), whereas addition of calcium to the culture medium appears to accelerate slightly neural fold closure (O'Shea, 1981). The present results show that papaverine and the related calcium antagonist D-600 (Gallopamil) cause reopening of the elevated neural folds of rat embryos. These antagonists are thought to block active calcium channels (Lee & Tsien, 1983) and treatment of amphibian embryos with papaverine alters the normal calcium fluxes associated with the morphogenesis of the neural tube (Moran & Rice, 1975; Moran, 1976), suggesting that extracellular calcium may indeed be important to the changes in the shapes of the neuroepithelium cells that effect neurulation. TMB-8 is reported to inhibit some calcium-dependent intracellular functions by reducing the release of calcium from intracellular stores (Malagodi & Chiou, 1974; Charo *et al.* 1976) or by binding to membranes which are the sites of certain calcium-dependent processes (Murer, Stewart, Davenport & Siojo, 1981). The present results suggest that TMB-8-sensitive functions are not involved in neurulation in mammalian embryos since TMB-8, unlike papaverine or D-600, did not cause opening of the cephalic neural folds, even at high concentration.

Thus it appears that an alteration in the calcium balance of the neuroepithelial cells is one of the essential steps in the events that lead to the changes in cell shape required for neurulation. A variety of teratogens induce abnormalities of the neural tube in which the neural folds fail to fuse, and such abnormalities occur,

apparently spontaneously, in laboratory animals and humans. Such abnormalities could be caused, not only by a failure of the folds to fuse once they have elevated, but also by a failure of the folds to elevate and appose normally. Significant to the present work is a suggestion that some teratogens, such as the local anaesthetic xylocaine, might derange neurulation by displacing calcium (O'Shea & Kaufman, 1980). The present results confirm that agents which are known to disturb the relocation of calcium reverse the elevation of the neural folds, and thus would perturb neurulation by preventing the apposition of the neural folds. Further experiments are planned in which the movements of calcium during the elevation of the neural folds in rat embryos are traced more directly using radiolabelled calcium, and in which the effects of substances which disturb the formation of the neural folds on calcium distribution are determined.

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