Palate morphogenesis

V. Effects of cholinergic agonists and antagonists on rotation in embryo culture

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SUMMARY

Morphological studies have shown that the pterygopalatine ganglion in the day-14.5 mouse palatal shelf lies adjacent to the putative contractile system of region-2 cells in the posterior palate. It is of interest to learn whether the ganglion could influence rotation of the palate. Results of embryo culture experiments showed that acetylcholine appeared to play a role in posterior shelf rotation since cholinergic agents (pyridostigmine, bethanechol and carbachol) stimulated elevation of that end of the palate. To characterize the putative receptors mediating the effects of the cholinergic agonists on palate shelf elevation, muscarinic or nicotinic antagonists were introduced into the embryo culture system. Atropine, a muscarinic blocking agent, did not produce any significant effect on palate shelf rotation when tested in overnight and 2 h embryo cultures at $3 \times 10^{-6}$ M and $10^{-4}$ M, respectively. Neither did atropine inhibit significantly the stimulation produced by $10^{-8}$ M bethanechol. Thus, the cholinergic effect was probably not on muscarinic receptors of the contractile system. However, hexamethonium, a nicotinic antagonist, at $10^{-6}$ M and $10^{-4}$ M profoundly inhibited posterior shelf rotation to about 35% of the control value in a 2 h incubation. In addition, $10^{-4}$ M hexamethonium inhibited posterior palate rotation to 11% of the control value after overnight culture. Furthermore, hexamethonium was able to reverse the stimulation of posterior rotation produced by carbachol. Partial inhibition of palate rotation by hexamethonium was also demonstrated when pregnant dams were injected with drug at doses approximately corresponding to $10^{-6}$ and $10^{-4}$ M. Hexamethonium treatment resulted in approximately 30% of the palates not completely rotated at day 15-5 compared to only 9.3% in the control. Hexamethonium also produced a significant increase in the palate gap and a comparable decrease in palate fusion. These effects were slightly greater at the posterior end of the palate. Thus, the cholinergic ganglion in the posterior palate may play a role in regulating shelf rotation at that end through a nicotinic pathway.

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INTRODUCTION

Elevation of the two secondary palatal shelves from a position lateral to the tongue to one horizontal above the tongue occurs between day 14.5 and 15.5 of gestation in mice and shortly thereafter the shelves fuse to complete palate development (Walker & Fraser, 1956). A previous report (Wee, Babiarz, Zimmerman & Zimmerman, 1979) has described the various mechanisms proposed to be responsible for elevation of the palate. Studies in our laboratory have shown that one possible candidate for an 'intrinsic shelf force' is the contractile components present in the palate. Biochemical (Lessard, Wee & Zimmerman, 1974) and morphological (Babiarz, Allenspach & Zimmerman 1975; Kuhn, Babiarz, Lessard & Zimmerman, 1977) studies showed the presence of two putative non-muscle contractile systems: one on the tongue side extending along the top of the shelf from mid-palate to the posterior limit (region 2) and another along the oral epithelium from the mid-posterior palate extending into the anterior end (region 3). In addition, a skeletal muscle system has also been shown to be present on the oral side of the far posterior palate (region 1).

In order to determine whether these putative contractile systems functioned to rotate the palate, agonists and antagonists of neurotransmitters have been tested in embryo culture. It was shown that serotonin stimulated palate rotation, predominantly at the anterior end (Wee et al. 1979). Conversely, acetylcholine appeared to play a role in posterior shelf rotation, since the cholinergic agents, pyridostigmine and bethanechol, stimulated elevation of that end of the palate (Wee, Wolfson & Zimmerman, 1976). Since the pterygopalatine ganglion lies adjacent to the putative contractile system of region-2 cells in the posterior palate (Babiarz et al. 1975; Babiarz, Wee & Zimmerman, 1979) it was of interest to learn whether the ganglion could influence the rotation of the palate. For some time evidence has been accumulating that cholinergic impulse transmission in autonomic ganglia may occur not only through a nicotinic pathway (hexamethonium-sensitive) but also through a muscarinic pathway, sensitive to a block by atropine (Flacke & Gillis, 1968). Therefore, effects on the contractile responses of the palatal shelves to agonists such as bethanechol and carbachol and to antagonists such as atropine and hexamethonium were examined in the present study.

MATERIALS AND METHODS

Materials. Dulbecco’s Modified Eagles medium (DMEM) was purchased from Grand Island Biological Company. Gentamicin (40 μg/ml) was added to DMEM. The following pharmacological agents were used: atropine sulfate (Elkins-Sinn, Inc.); bethanechol chloride (Merck Sharp & Dohme); carbachol (Sigma Chemical Co.); hexamethonium chloride (Nutritional Biochemicals
Cholinergic palate rotation

CORP.); pyridostigmine bromide (Roche Laboratories); D-tubocurarine chloride (Eli Lilly & Co.); dimethyl sulfoxide (DMSO) (Fisher Scientific Co.). Fresh drug solutions were used for each experiment.

Animals. Female A/J mice (Jackson Laboratory) were mated overnight and checked for pregnancy the following morning. The presence of a vaginal plug was taken as evidence of pregnancy and designated day 0-5 of gestation. At day 14-75 of gestation, the time just prior to palate shelf rotation (Andrew, Bowen & Zimmerman, 1973), pregnant mice were sacrificed by cervical dislocation. The gravid uterus was removed immediately and placed in a sterile dish containing cold DMEM pregassed with 95% O₂-5% CO₂.

Embryo culture. Explantation and culture of the embryos were carried out under sterile conditions as previously reported (Wee et al. 1976; Wee et al. 1979). Culture medium contained 25% fresh human serum (immediately centrifuged; Steele & New, 1974) and DMEM, unless otherwise specified. Individual day-14-75 concepti were removed from the uterine horn and placed in a Petri dish with cold DMEM. Reichert's membrane was removed and a slit was cut in the yolk sac to slip out the embryo. The tongue was carefully excised to allow palate rotation in vitro. Only single embryos with beating hearts were placed into 2 ml of culture medium in glass vials (19 × 48 mm, Kimble Opticlear). Each vial was gassed with a mixture of 95% O₂-5% CO₂, tightly closed with a silicone rubber stopper, and sealed with tape. The explanted embryos were cultured at 37 °C for the time specified with rotation of the vials at 60 rev/min in a Bellco variable speed roller drum.

A morphological rating (MR) of the embryo was based on numbers arbitrarily assigned for development of each of five features: forefeet, hindfeet, ears, hair follicles and eyes (Walker & Crain, 1960). Only embryos with a morphological rating of 5-7 were scored for palate shelf elevation since the posterior end rotated in culture without having appreciably moved in vivo before culture (Wee et al. 1979).

Cultured embryos were fixed in Bouin’s solution for 24 h at which time heads were cut off and mandibles removed to assess palate rotation and fusion. Blocks of fixed heads, cut coronally halfway through the anterior and posterior ends, were placed on end. Values of 1-5 (palate shelf index, PSI) were arbitrarily assigned to express the degree of palate shelf rotation (Wee et al. 1976). A number 1 was assigned for a completely vertical and 5 for a completely horizontal palate; 2, 3 and 4 for intermediate rotation to one quarter, one half, and three quarters of the way to the horizontal position, respectively. Experimental results are presented as % control movement where:

\[
\text{% control movement} = \frac{\text{PSI of treatment group} - \text{PSI of fixed group}}{\text{PSI of control group} - \text{PSI of fixed group}} \times 100, \\
\text{fixed group} = \text{embryos fixed in Bouin's solution immediately after tongues were excised},
\]
control group = embryos fixed after culturing without pharmacologic agents,
treatment group = embryos fixed after culturing with pharmacologic agents.

In vivo administration of hexamethonium was performed by injecting mice intraperitoneally on days 13-5, 13-75, 14-5 and 14-75 of gestation at doses of 0.27 and 27.3 mg/kg. Control mice were injected with solvent which was water. Pregnant mice were sacrificed on day 15-5 of gestation by cervical dislocation. Embryos were removed, fixed in Bouin's solution for 24 h, and palate shelf rotation and fusion were assessed as above. Palate gap, another quantitative measure of palate rotation and fusion, was also determined using an ocular micrometer.

Acetylcholinesterase histochemistry. Day-14-5 embryo heads were frozen in liquid nitrogen and 10 μm serial cryostat sections were obtained. Sections of muscle taken from the abdominal wall of an adult mouse were placed on each slide as an internal control. Sections were processed for acetylcholinesterase according to the histochemical method of El-Badawi & Schenk (1967) with some slight modifications. Sections were incubated with 50 % more substrate at 37 °C for 7 h in order to increase staining. All incubation solutions contained iso-OMPA (tetraisopropylpyrophosphoramide) to inhibit non-specific cholinesterase (butylcholinesterase) unless otherwise indicated. One solution contained substrate, one lacked substrate, one contained substrate plus an inhibitor of specific cholinesterase (10⁻⁸ M, Burroughs-Wellcome no. CO67), and one contained substrate and no. CO67 and lacked iso-OMPA. Sections were examined by phase contrast, bright field and dark-field microscopy. Sections were photographed by dark-field microscopy to enhance the brown color of the positive reaction, using high-speed Ektachrome type B (ASA 160).

Histology and light microscopy of fixed sections were performed as described in Wee et al. (1979).

RESULTS

Palate rotation in embryo culture

When embryos were cultured overnight the anterior shelf completely rotated while the posterior end moved to only an intermediate position. Stimulation of this end in culture was observed by the addition of cholinergic agents such as pyridostigmine or bethanechol (Wee et al. 1976). Other agents and combinations were now tested for their effect on posterior shelf rotation. Table 1 indicates that DMSO, a common organic solvent, slightly stimulated posterior shelf rotation. Since anterior palates are always completely rotated after overnight culture, their data are omitted from the table. When DMSO, bethanechol (10⁻⁸ M) and pyridostigmine (10⁻⁸ M) were combined, posterior shelf rotation reached a mean PSI of 4.26 which is very close to the horizontal position.
Cholinergic palate rotation

Table 1. Enhancement of posterior palate shelf rotation

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Posterior PSI</th>
<th>Control movement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed</td>
<td>134</td>
<td>1.22 ± 0.04</td>
<td>—</td>
</tr>
<tr>
<td>Control</td>
<td>158</td>
<td>3.14 ± 0.14</td>
<td>(100)</td>
</tr>
<tr>
<td>+1% DMSO</td>
<td>34</td>
<td>3.62 ± 0.30</td>
<td>125</td>
</tr>
<tr>
<td>+1% DMSO, bethanechol (10⁻⁸ M)</td>
<td>34</td>
<td>4.26 ± 0.24</td>
<td>158 (P &lt; 0.001)</td>
</tr>
</tbody>
</table>

* Overnight culture, n equals the total number of palate shelves monitored, PSI values are mean ± s.e. Significance value in parentheses as computed by Student's t test.

Table 2. Effects of cholinergic (muscarinic) agents on palate shelf movement in mouse embryo culture

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>PSI</th>
<th>Control movement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed</td>
<td>134</td>
<td>1.22 ± 0.04</td>
<td>—</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>2.33 ± 0.33</td>
<td>(100)</td>
</tr>
<tr>
<td>Atropine (3 × 10⁻⁶ M)</td>
<td>28</td>
<td>2.32 ± 0.32</td>
<td>99</td>
</tr>
<tr>
<td>Bethanechol (10⁻⁸ M)</td>
<td>20</td>
<td>3.70 ± 0.38</td>
<td>233 (P &lt; 0.01)</td>
</tr>
<tr>
<td>Atropine (3 × 10⁻⁶ M) ± bethanechol (10⁻⁸ M)</td>
<td>18</td>
<td>3.17 ± 0.44</td>
<td>176</td>
</tr>
</tbody>
</table>

* Overnight culture.

When the effect of atropine (3 × 10⁻⁵ M) on palate shelf rotation was tested in overnight embryo culture as shown in Table 2, no appreciable effect on the posterior shelf could be measured. Bethanechol, as shown previously, profoundly stimulated posterior shelf rotation. Although atropine inhibited slightly the bethanechol-stimulated posterior rotation, the effect is not statistically significant.

Since the anterior part of the palate completely rotates after overnight culture, 2 h incubation experiments were performed to observe if these cholinergic agents could affect elevation at the anterior end. First it can be seen that atropine again did not produce any effect on posterior palate rotation (Table 3). Although not significant, atropine seemed to enhance slightly palate rotation at the anterior end. Bethanechol stimulated rotation both at the anterior and posterior ends, and as can be seen, the stimulation is concentration dependent. Atropine did not inhibit significantly the stimulation produced by 10⁻⁸ M bethanechol.

Thus, since atropine showed minimal effects on palate rotation, muscarinic receptors probably are not playing a role in regulating palate shelf rotation.
Table 3. Effects of cholinergic (muscarinic) agents on palate shelf movement in mouse embryo culture

<table>
<thead>
<tr>
<th>Condition*</th>
<th>$n$</th>
<th>PSI Anterior</th>
<th>PSI Posterior</th>
<th>Control movement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed</td>
<td>134</td>
<td>2.61 ± 0.05</td>
<td>1.22 ± 0.04</td>
<td>—</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>2.95 ± 0.17</td>
<td>1.65 ± 0.21</td>
<td>(100)</td>
</tr>
<tr>
<td>Atropine ($10^{-4}$ M)</td>
<td>18</td>
<td>3.17 ± 0.18</td>
<td>1.67 ± 0.26</td>
<td>165</td>
</tr>
<tr>
<td>Bethanechol ($10^{-8}$ M)</td>
<td>20</td>
<td>3.50 ± 0.14</td>
<td>2.00 ± 0.25</td>
<td>262 ($P &lt; 0.01$)</td>
</tr>
<tr>
<td>Bethanechol ($10^{-6}$ M)</td>
<td>30</td>
<td>3.67 ± 0.09</td>
<td>2.67 ± 0.19</td>
<td>312 ($P &lt; 10^{-4}$)</td>
</tr>
<tr>
<td>Atropine ($10^{-4}$ M) + bethanechol ($10^{-8}$ M)</td>
<td>32</td>
<td>3.38 ± 0.11</td>
<td>1.94 ± 0.17</td>
<td>226 ($P &lt; 0.02$)</td>
</tr>
</tbody>
</table>

* 2 h incubation.
To test whether nicotinic receptors could be involved in the morphogenetic rotation of the palate, the effect of hexamethonium was tested.

As indicated in Table 4, hexamethonium at $10^{-4}$ M produced a profound inhibition of posterior palate rotation after overnight culture (11% of control). On the other hand, carbachol, which can activate both muscarinic and nicotinic receptors, at $10^{-5}$ M stimulated posterior palate rotation to 157% of the control value. Stimulation at a lower concentration was less and not significant. Since the major pharmacologic activity of carbachol is through a nicotinic pathway (Chiou & Long, 1969a, b), the effect of hexamethonium on carbachol stimulation of posterior rotation was tested: hexamethonium reversed the stimulation produced by carbachol.

Curare, a nicotinic agent which blocks neuromuscular transmission, when added to the embryo culture medium as D-tubocurarine did not impair posterior movement. In fact, posterior shelf rotation was stimulated in embryos cultured both overnight (Table 4) and for 6 h (Table 5).

Next, the effect of nicotinic agents on rotation of the anterior palate were tested in a short-term incubation. Fig. 1 shows the dose response to hexamethonium in a 2 h culture. At $10^{-6}$ and $10^{-4}$ M hexamethonium inhibited posterior shelf rotation to about 35% of the control value. Conversely, rotation of the anterior palate was stimulated by the antagonist at lower concentrations ($10^{-10}$ and $10^{-8}$ M). The agonist carbachol, at $10^{-8}$ and $10^{-5}$ M, did not stimulate posterior shelf rotation in 2 h, whereas the anterior end was stimulated significantly (Table 5).

In vivo administration of hexamethonium

To obtain further evidence that the effect of the nicotinic antagonist on palate shelf rotation was not due to an artifact of the embryo culture system,
Table 5. Effects of cholinergic (nicotinic) agents on palate shelf movement in mouse embryo culture

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Anterior</th>
<th>Posterior</th>
<th>Control movement (%)</th>
<th>Anterior</th>
<th>Posterior</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed</td>
<td>134</td>
<td>2.61 ± 0.05</td>
<td>1.22 ± 0.04</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2 h incubation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>32</td>
<td>3.12 ± 0.11</td>
<td>2.28 ± 0.19</td>
<td>(100)</td>
<td>(100)</td>
<td></td>
</tr>
<tr>
<td>Carbachol (10^{-8} M)</td>
<td>26</td>
<td>3.50 ± 0.11</td>
<td>2.54 ± 0.24</td>
<td>174 (P &lt; 0.02)</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>Carbachol (10^{-5} M)</td>
<td>24</td>
<td>3.71 ± 0.11</td>
<td>2.04 ± 0.25</td>
<td>216 (P &lt; 0.001)</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>6 h incubation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control – serum</td>
<td>8</td>
<td>4.62 ± 0.26</td>
<td>1.50 ± 0.33</td>
<td>(100)</td>
<td>(100)</td>
<td></td>
</tr>
<tr>
<td>Curare (2 × 10^{-3} M) – serum</td>
<td>36</td>
<td>4.75 ± 0.11</td>
<td>3.08 ± 0.26</td>
<td>106</td>
<td>664 (P &lt; 0.01)</td>
<td></td>
</tr>
</tbody>
</table>
Cholinergic palate rotation

Fig. 1. Palate shelf rotation in embryo culture with various concentrations of hexamethonium. Day-14-75 embryos whose morphological rating were between 5 and 7 were cultured for 2 h. Values represent percentage of control movement by hexamethonium-treated groups as described in Materials and Methods. Number of palates employed are indicated in parentheses for the indicated concentrations of hexamethonium. P values for statistical significance using Student's t test are also indicated.

Hexamethonium was injected intraperitoneally into pregnant dams and palate closure was monitored at day 15.5 of gestation (Table 6). A manifestation of the inhibition of palate shelf rotation by hexamethonium was the significant increase in the palate gap observed in treated embryos. This effect was slightly greater at the posterior end of the palate. Hexamethonium at the lower dose administered (0.27 mg/kg) appeared to be as effective as the higher dose (27.3 mg/kg) in blocking palate rotation. Hexamethonium produced about 30% of the palates not completely rotated compared to only 9.3% in the control. Furthermore, hexamethonium caused a comparable decrease in palate fusion at the anterior and posterior ends. Hexamethonium at 0.27 mg/kg produced a higher percentage of resorption (16%) compared to the control (7.4%) and the 27.3 mg/kg administered dose (6.7%). The higher resorption rate with
### Table 6. Effect of fetal palate by administration of hexamethonium to pregnant mice

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>Palate gap (mm)</th>
<th>Fused palate (%)</th>
<th>Plate not completely rotated (%)</th>
<th>Resorption (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Anterior</td>
<td>Posterior</td>
<td>Anterior</td>
<td>Posterior</td>
</tr>
<tr>
<td>Control</td>
<td>140</td>
<td>0.06 ± 0.03</td>
<td>0.08 ± 0.02</td>
<td>89</td>
<td>73</td>
</tr>
<tr>
<td>Hexamethonium (0.27 mg/kg)</td>
<td>132</td>
<td>0.21 ± 0.04</td>
<td>0.25 ± 0.04</td>
<td>64</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(P &lt; 0.00025)</td>
<td>(P &lt; 0.0001)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexamethonium (27.3 mg/kg)</td>
<td>104</td>
<td>0.18 ± 0.04</td>
<td>0.21 ± 0.04</td>
<td>73</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(P &lt; 0.005)</td>
<td>(P &lt; 0.0025)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 2. Histology of palate shelves after 4 h in embryo culture. (A) Posterior palate of control embryo. PSI = 4. × 170. (B) Posterior palate of embryo cultured in presence of $10^{-4} \text{M}$ hexamethonium. PSI = 2. × 140. Region 2 at the tongue side of the posterior palate is indicated. TE, Tongue side epithelium. Bars, 50 μm.
the lower dose also occurred when the drug was injected intramuscularly (data not presented).

**Histologic analysis of palate**

Histology of the posterior palates from hexamethonium-treated embryos in 4 h incubations showed no apparent morphological abnormalities (Fig. 2), even though a statistical analysis had showed palate rotation to be significantly inhibited.

In the adult, the pterygopalatine ganglion is parasympathetic (Weston, 1970), and in the day-14-5 embryo it is found adjacent to region-2 cells in mid to mid-posterior sections. One measure of its functional state would be the presence of acetylcholinesterase at this time in development. Fig. 3 shows activity of acetylcholinesterase in cryostat sections of palate; the brown color represents activity appearing white in dark-field microscopy. The ganglion
produced a strong reaction, as well as the pterygopalatine nerve which courses alongside of the ganglion. In later development, this nerve serves a sensory function (Weston, 1970). The pterygopalatine nerve as it descends to the oral side of the palate courses through the putative non-muscle contractile system of region-3 cells. Neither region 2 nor 3 showed significant esterase activity at this level of sensitivity.

**DISCUSSION**

The experiments carried out in this study were to determine further whether the putative non-muscle contractile systems (region 2, tongue side, mid-posterior; region 3, oral side, mid-anterior) functioned to rotate the mouse palate. An analysis of the cell morphology during palate rotation indicated that the peripheral mesenchymal cells, including regions 2 and 3 become elongated and are arranged perpendicular to the basement membrane, and thereafter round up as the shelf assumed the horizontal position. Epithelium on the tongue side also undergoes cell shape changes during rotation (Babiarz et al. 1979). Similar observations were made in the hamster palate (Shah, 1979). These studies suggested that contraction of cytoplasmic processes and cell movement were responsible, at least in part, for shelf rotation. Further support for this hypothesis came from an experiment in which fetal heads were glycerinated to allow ATP to penetrate palatal cells; as a consequence the palate rotated. Cytochalasin B blocked the ATP-induced palate elevation. Since cytochalasin B is known to disrupt microfilaments, it was concluded that the non-muscle contractile cells in the palate play a role in palate elevation and that contraction of the actomyosin containing microfilaments supplies the motive force (Wee & Zimmerman, 1979). Finally it has been shown that neurotransmitters influence palate rotation supporting the role of a cell-mediated process (contraction and migration). Serotonin has been shown to regulate rotation of the anterior end of the palate (Wee et al. 1979) and various cholinomimetic agents such as pyridostigmine, bethanechol (Wee et al. 1976) and carbachol have been shown to enhance posterior palate shelf rotation.

During the course of these studies, it was found that DMSO, a common organic solvent, stimulated posterior shelf rotation. Two cholinergic agents, pyridostigmine ($10^{-6}$ M) and bethanechol ($10^{-8}$ M) in the presence of 1% DMSO induced posterior palate rotation close to the horizontal position ($\Psi = 4.26$). This result is of interest because DMSO increases membrane permeability, and nerve excitation is essentially a phenomena produced by an increased ionic permeability of the excitable membranes. There have been also many clinical reports on the analgesic, sedative, antipsychotic and other effects of DMSO on the nervous system (Sawada & Sato, 1975).

To characterize the putative receptors mediating the effects of cholinergic agonists on palate shelf elevation, nicotinic or muscarinic antagonists were
introduced into the embryo culture system. Atropine, an effective muscarinic ganglion blocking agent, did not have any appreciable effect on posterior shelf rotation when added alone to the embryo culture system. On the other hand, hexamethonium, a preganglionic nicotinic blocking agent, profoundly inhibited posterior palate shelf rotation to 11 % (10^{-4} M) and 32-34 % (10^{-6} and 10^{-4} M) of control values after overnight and 2 h incubations, respectively. Hexamethonium also inhibited carbachol stimulation of posterior rotation. Even though the posterior palate is much more sensitive to culture perturbation than the anterior end (Wee et al. 1979), hexamethonium is the only pharmacologic agent tested thus far that inhibits posterior rotation to below 50 % of the control value. Therefore it is concluded that the primary cholinergic effect is mediated by a nicotinic receptor. Since the pterygopalatine ganglion lies adjacent to the non-muscle contractile system of region-2 cells in the posterior palate (Babiarz et al. 1975; see also Fig. 3), these results suggest that the ganglionic fibers play a regulatory role in posterior shelf elevation by influencing region-2 cells.

Surprisingly, curare, which blocks neuromuscular transmission, stimulated posterior palate elevation (Tables 4, 5). Serum was deleted from the culture medium in these experiments because it was previously suspected that curare may have been bound to serum protein during embryo culture. Since developing skeletal muscle (region 1) is present in the far posterior palate (Babiarz et al. 1975; Innes, 1978), it may be that this muscle mass may be playing an antagonist rather than an agonist role in posterior shelf rotation. Further support for this notion comes from the observation that if a surgical cut is made in cross section in the posterior third of the palate, this end rotates in a direction opposite to the remaining palate in embryo culture (Brinkley & Vickerman, 1979).

Results of administering hexamethonium into pregnant dams further showed an inhibiting effect on embryonic palate development. The primary effect of hexamethonium seemed to be on palate shelf rotation, as manifested by a higher number of palates not completely rotated (PSI = 5), and also by a significant increase in the distance between the shelves (palate gap). Similarly, the comparable decrease in percentage of palates fused could also be a consequence of delayed palate rotation. The lower administered dose of hexamethonium (0.27 mg/kg) seemed to be as effective as the higher dose (27.3 mg/kg) in inhibiting palate rotation and showed greater embryotoxicity as indicated by a higher resorption rate. The lower dose of 0.27 mg/kg administered approximately corresponds to a concentration of 10^{-6} M, assuming equal distribution of the antagonist in all body compartments. It is of interest that 10^{-6} M hexamethonium produced an inhibition of posterior shelf rotation comparable to 10^{-4} M antagonist after embryos were cultured for 2 h.

As previously indicated, it is thought that serotonin plays a major role in regulating rotation of the anterior end of the palate by an effect on a cell-
mediated process (Wee et al. 1979). Results of the present study show that cholinomimetic agents such as bethanechol and carbachol also stimulated anterior palate rotation, but none of the cholinergic antagonists tested were able to inhibit rotation at this end. On the other hand, methysergide, a blocking agent for the serotonin receptor, is an antagonist that inhibits anterior rotation to below 50% of control at $10^{-4}$ M (Wee et al. 1979). Cholinergic neurons have been reported to contribute to the regulation of some serotonergic pathways (Hery et al. 1978) by mediating the spontaneous release of 5-HT. Acetylcholine and other cholinomimetic agents have been shown to stimulate serotonin release from invertebrate brain neurones (Ascher, Glowinski, Taur & Taxi, 1968). Bhattacharya & Nayak (1978) have postulated also that physostigmine first raises brain levels of free acetylcholine, which in turn depolarizes serotonergic nerves in the rat brain. Since it has been shown that serotonin is present in the palate (Zimmerman & Roberts, 1977; Zimmerman, unpublished observations), it is possible that cholinomimetic agents such as carbachol and bethanechol act indirectly by enhancing the spontaneous release of serotonin in the anterior palate. Such an effect could thereby stimulate anterior palate rotation. It is not clear why the nicotinic antagonist, hexamethonium, caused a stimulation of the anterior shelf at lower concentrations ($10^{-10}$ to $10^{-8}$ M). This effect is probably not mediated by the action of hexamethonium as a ganglionic blocking agent of nicotinic receptors since a stimulation was produced. The stimulatory effect could be caused by the weak anticholinesterase activity of hexamethonium (Lullmann et al. 1971; Gandiha, Green & Marshall, 1972) thereby increasing palatal acetylcholine and hence rotation. Alternatively, the stimulating effect of hexamethonium could involve other types of receptors as reported by Hilton (1977), whereby (1) certain cholinergic responses are not inhibited by muscarinic or nicotinic antagonists, but (2) are stimulated by nicotinic blocking agents which in turn are inhibited by ouabain or taurine (related to γ-aminobutyric acid).

Results from the present study illustrate further the possible complexity of the regulatory mechanisms involved in palate rotation. The classical 'one neuron–one transmitter' situation may not be applicable to the control of palatal shelf rotation. Arrays of neurotransmitters, neurohormones and second messengers may be functioning directly on palatal cells to affect rotation or indirectly on the ganglion or nerves as presynaptic and postsynaptic neurotransmitters.

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Cholinergic palate rotation


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