Acetylcholinesterase (AChE) and pseudocholinesterase (BuChE) activity distribution pattern in early developing chick limbs

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SUMMARY
The distribution of acetylcholinesterase (AChE) and pseudocholinesterase (BuChE) activities was studied by histochemical, quantitative and electrophoretical methods during the early development of chick limbs, from stage 16 to stage 32 H.H. (Hamburger & Hamilton, 1951).

By quantitative methods, true AChE activity was found, and increased about threefold during the developmental period, together with a smaller amount of BuChE which increased more rapidly in comparison with the AChE activity from stage 25 to 32 H.H.

Cholinesterase activity was histochemically localized mainly in interacting tissues, such as the ectoderm (including the apical ectodermal ridge) and the underlying mesenchyme. True AChE was histochemically localized around the nuclei and on the plasma membrane of ectodermal (including AER) and mesenchymal cells, and at the plasma membrane of mesenchymal cell processes reaching the basal lamina between the ectoderm and the mesenchyme. AChE together with BuChE activity was found in the basal lamina between the ectoderm and the mesenchyme, in underlying mesenchymal cells and in deeper mesenchymal cells, especially during their transformation into unexpressed chondrocytes.

During limb morphogenesis, the cellular and regional localization of the enzyme activities showed variations depending on the stage of development and on the occurrence of interactions.

The possibility of morphogenetic functions of the enzyme is discussed.

INTRODUCTION
Ectoderm–mesenchyme interactions are known to play an important role during chick limb morphogenesis (Gumpel-Pinot, 1972, 1973, 1980, 1981a,b,c). An important part of these interactions is taken over by the apical ectodermal ridge and underlying mesenchyme (Saunders & Gasseling, 1968; Zwilling, 1956a,b; Solursh et al. 1981; Murphy, Gasseling & Saunders, 1983). Apical ectodermal ridge (AER) is the name of a particular structure of ectodermal cells which increase in thickness and become pseudostratified on the tip of the limb bud.

Generally, cell and tissue interactions may be mediated by chemical messengers (McMahon, 1974). Among the chemical transmitters acting during early morphogenesis, neurotransmitters and correlated enzymes have been studied in particular detail, and the hypothesis has been advanced that they may act as cell and tissue organizers before taking on their mature neuromuscular function. The presence of

Key words: limb bud, acetylcholinesterase, pseudocholinesterase, cholinergic system.

During chick limb morphogenesis, cholinesterase activity was detected by histochemical methods in the ectodermal hull, in the AER and also in the pre-cartilaginous mesenchymal cells during their aggregation (Drews, 1975). This cholinesterase activity, independent of the neuromuscular system, was called 'embryonic' by the author, suggesting that it may be involved in the inductive processes and/or correlated with cellular movements of morphogenesis.

The name 'cholinesterase' includes two distinct enzyme activities: acetylcholinesterase (AChE, E.C. 3.1.1.7.) and pseudocholinesterase (BuChE, E.C. 3.1.1.8.), whose presence and function has been studied mainly in the neuromuscular system.

Recently, a specific AChE activity was found in the plasma membrane of blastomeres during the embryonic development of an ascidian by the use of a modified histochemical method (Minganti & Falugi, 1980). Subsequently, the method was improved and adapted to vertebrate embryo tissues, and the investigation was extended to early chick embryos and developing chick limbs; preliminary reports of these results were briefly communicated (Falugi, 1981; Minganti, Falugi, Raineri & Pesterino, 1981, 1982; Falugi, Guastalla & Faraldi, 1984).

In the present study we have extended the research into cholinesterase activity during the early development of the chick limb by employing histochemical and electrophoretic methods. Particular attention has been paid to the AER and to the other structures interacting with it during differentiation. The aim of this study was to specify: a) the distribution respectively of AChE and BuChE in the different cholinesterase-positive structures, in order to help us to understand their functions; b) the localization of AChE activity associated with cell membrane, because such an AChE localization during cellular interactions may indicate that the enzyme is involved in the regulation of membrane functions correlated with these interactions.

**MATERIALS AND METHODS**

Chick embryos were obtained from fertilized eggs obtained from the Ladi's hatchery, Carasco (Genova). Embryos were removed from the eggs, staged according to Hamburger & Hamilton (H.H., 1951) and the somite number determined.

**Histochemical methods**

*Fixation.* Early embryos were fixed in toto prior to stage 19 H.H.

1) ethanol fixation: 10 drops of cold 80% ethanol (0-4 ml) were added directly onto the embryos, placed in 3-5 ml of cold PBS (phosphate-buffered saline, pH 7.2, 7.4) and when the embryos came to the surface, they were washed with two changes of cold PBS.
AChE and BuChE distribution in developing chick limbs

2) two-step fixation: embryos were fixed in 0.1% paraformaldehyde and 0.5% glutaraldehyde in PBS, for 10 min and then washed in 0.1 M cold maleate buffer pH 6, containing 0.168% sucrose. Then they were incubated in the incubation medium at room temperature for 45 min, washed with cold PBS and fixed with ethanol as above, rinsed in maleate buffer–sucrose and again immersed in the incubation medium.

After stage 19 H.H., the limb buds were removed from the embryos, and fixed using one of the above mentioned methods.

Enzyme reaction. The enzyme reaction was carried out at pH 5.9-6 in the incubation medium described by Karnovsky & Roots (1964), with 0.312% sucrose, added to make the mixture iso-osmotic to the chick embryo. The incubation medium was changed each 30 min, to avoid precipitation during the incubation period (from 3 to 8 h). Part of the material was incubated in the medium described by Tsuji (1974). Both methods allow visualization of the enzyme reaction product; the first method is more sensitive, while the second allows longer incubation times without non-specific precipitation.

Specificity of the enzyme reaction. To check the specificity of enzyme activity, incubation was carried out using different substrates: acetylthiocholine iodide (AcTChI), which is hydrolysed mainly by AChE; butyrylthiocholine iodide (BuTChI), which is hydrolysed mainly by BuChE; acetyl-β-methylthiocholine iodide (AcMTChI), which is specifically hydrolysed by AChE; all of these products were obtained from Sigma Chem. Co., U.S.A.

Determination of enzyme specificity by specific inhibition of the histochemical reaction. Samples were pre-incubated for 30 min in 10⁻³ to 10⁻⁶ M specific inhibitors in Tyrode solution; then they were incubated in the enzyme reaction mixture, containing the same inhibitors at the same concentration: eserine (physostigmine, BDH, England), specific for AChE and BuChE (useful for the evaluation of the presence of non-specific esterases); BW 284c51 (anticholinesterase, Burroughs-Wellcome Labs., U.S.A.), specific for AChE; iso-OMPA (tetraisopropylpyrophosphoramide, ICN Pharm., Canada), specific for BuChE.

Preparation for light microscopy. After the enzyme reaction, samples were washed in PBS, dehydrated slowly in up to 95% ethanol and then embedded in JB4 preparation (Polyscience, U.S.A.). 4 µm sections were obtained with a glass knife, using a Pyramitome (LKB, Sweden).

Quantitative assay

The cholinesterase activity in developing limb buds was determined colorimetrically by the method of Rappaport, Fischl & Pinto (1959), modified by the Sigma Chem. Co. (Techn. Bull. No. 420, 1969). Crude homogenates or Triton X-100 extracts were incubated at 25°C for 30 min in 0.1 M-phosphate buffer, pH 7.8, containing acetylcholine chloride as substrate and m-nitrophenol as indicator. The absorption was measured by a Bausch and Lomb Spectronic spectrophotometer at 440 nm and compared to a standard acetic acid. Enzyme activity was expressed in Rappaport units (1 RU = 1 µmole of AChE hydrolysed/30 min under the above conditions) per mg of protein N, as determined by the xanthoprotein method of Millon-Nasse (Oser, 1965).

Disc electrophoresis

Cholinesterase subunits were separated on 7.5% polyacrylamide gel following the procedure of Ornstein & Davis (1962). Crude homogenates, diluted from 1/1 to 1/10 (v/v) with distilled water and Triton X-100 homogenates were centrifuged at 12000 g; 150–200 µl of the supernatants, diluted 1/1 with 40% sucrose were layered on the top of the gels, without a spacer. Current (2.5–4 mA) was applied to each tube for 2–3 h at 4°C, until the tracking dye (bromophenol blue) reached the end of the gels. 50 µg of AChE purified from Electrophorus electricus, type 6 (Sigma Chem. Co., U.S.A.), in 40% sucrose was employed as the control.

After electrophoresis, gels were fixed (10 min in 80% ethanol at 4°C) and stained by the direct colouring thiocholine method of Karnovsky & Roots (1964). The gels were pre-incubated for 0.5 h at 4°C in medium without substrate and incubated for 24 h at room temperature in the histochemical mixture as above.
RESULTS

Histochemical localization of cholinesterase activity in developing limb

From stage-16 H.H. embryos, cholinesterase activity is localized in the whole ectodermal layer, mainly in the dorsal side of the limb (Fig. 1). The positive reaction is found in most of the ectodermal cells, either in the outer flat cells or in the inner isodiametric ones, which are in contact to the basal lamina.

The enzyme staining of the ectodermal cells is localized around the nuclear envelope, in the cytoplasm, and sometimes also in portions of the cell membrane (Fig. 1B,C). A strong enzyme reaction is localized also in the cells of the AER (Fig. 2, 3), in the basal lamina under the ectoderm and in mesenchymal cells near to the positive ectoderm (Fig. 1, 2, 3). In the early limb, from stage 16 to 18 H.H., the cholinesterase activity of ectoderm and AER cells is mainly localized on the nuclear envelope (Fig. 1, 2). Then, from stage 20–21 H.H., the main localization is at the plasma membrane (Fig. 3). Positive mesenchymal cells are distributed in different localizations; under the AER (Fig. 2A,B,C, 3D), under the dorsal ectoderm (Fig. 1), at the posterior margin of the limb (Fig. 4), and, in the wing bud, also in a small portion of the ventral side (Fig. 1F, 4A, 10). In every one of these localizations, mesenchymal staining is present in well-defined areas. The exact distribution depends on the section level of the limb, on the stage of development, and it is different in leg and wing buds. However, in each of these cases, positive mesenchymal cells have positive processes which reach the positive basal lamina (Fig. 1B arrow, D,E arrows, F). In particular, the distribution of the enzyme activity in the basal lamina and in mesenchymal cells situated under the AER is different at different section levels and/or developmental stages: in the postaxial sections

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Fig. 1. Histochemical localization of cholinesterase activity, obtained by the use of AcTChI as substrate, in the dorsal and ventral side of limb buds. The enzyme activity is revealed by dark staining.

(A) General view of a stage-19 H.H. leg bud. Bar equals 20 µm

(B),(C) Stage-19 limb bud dorsal margin: perinuclear and cytoplasmatic staining is present, the basal lamina is not continuous; enzyme activity localized around mesenchymal cell processes reaching the basal lamina may be associated with plasma membrane or localized in nearby extracellular matrix. Bar equals 8 µm

(D) Stage-21 H.H. leg bud, general view; at this stage the positive mesenchyme localized in the dorsal margin of the bud is deeper than in earlier stages. Bar equals 8 µm

(E) Stage-23 H.H. dorsal side of the leg bud: the enzymic staining is localized on ectodermal cell membranes, in the basal lamina and in mesenchymal cells. Mesenchymal cells have positive processes extending orthogonally to the basal lamina. Positive processes to the basal lamina also come from deeper mesenchymal cells (arrows). Bar equals 40 µm

(F) Stage-21 H.H. wing bud, ventral side. In the wing bud a little portion of positive mesenchyme is also present in the ventral side and over it the ectoderm is thickened and strongly positive to the enzymic reaction. Bar equals 40 µm

a, AER; d, dorsal side; e, ectoderm; l, basal lamina; m, mesenchyme; o, outer layer of ectoderm; p, mesenchymal process; v, ventral side.
Fig. 2. Localization of cholinesterase activity in the apical zone of the early limb bud, obtained by the use of AcTChI as substrate. The enzyme positivity is revealed by dark staining.

(A), (B), (C) Stage-18 H.H., postaxial section. At this early stage the staining is localized mainly at the nuclei and cytoplasm of AER cells, in the basal lamina and in underlying mesenchymal cells. Bar equals 40 μm

(D) Perinuclear localization of the enzyme. Bar equals 15 μm

(E) Stage-23 H.H. BuChE activity, obtained by the use of AcTChI as substrate and BW 284c51 as inhibitor (the same result was obtained by the use of BuTChI as substrate). Bar equals 50 μm

a, AER; l, basal lamina; m mesenchyme.
Fig. 3. Localization of cholinesterase activity in the AER cells, obtained by using AcTChI as substrate; the enzyme staining is dark.

(A), (B) Stage-21 H.H. The AER is prominent, the staining is localized around the nuclei and on plasma membrane of AER cells. The staining of basal lamina is strong in the dorsal side of the limb, fainter under the AER (pre-axial section); due to magnification of the picture, the dorsal mesenchyme positive to the histochemical reaction is cut off. Bar equals 20 μm (A); 8 μm (B)

(C) Postaxial section of the same limb, showing cholinesterase activity under the AER. Bar equals 8 μm

(D) Stage-24 H.H., the staining is localized on the plasma membrane of AER cells. The AER is low, due to flattening of the ectodermal cells. Bar equals 20 μm

a, AER; d, dorsal side; l, basal lamina; m, mesenchyme; o, outer layer of ectoderm.
and/or young stages the reaction is positive in these sites (Fig. 2, 3C); in pre-axial sections and/or later stages the reaction is less strong (Fig. 3A,B) and sometimes it is not apparent. In later stages (24 H.H.) AER cells are still positive to the reaction, although they are flattened, and the AER appears less prominent (Fig. 3D).

From stage 21 H.H., cholinesterase activity is present also in deeper mesenchymal cells (Fig. 1D, E, F, Fig. 4, Fig. 5A). This positive enzyme activity involves also chondrogenic mesenchymal cells (Fig. 5, 6). The cholinesterase activity of the first chondrogenic cells is spatially connected with the ectodermal activity: the enzyme-positive chondrogenic cells are localized almost at the apex of a wide triangle of mesenchymal positive cells (defined anteriorly by a sharp boundary), whose base is contiguous with the basal lamina of the dorsal ectoderm (Fig. 5A, B).

When chondrogenic aggregation has begun (stage 23–25 H.H.), the enzyme activity is present only in the chondrogenic blastema, surrounded by mesenchyme which does not display 'embryonic' cholinesterase activity (Fig. 5C, D, 6A, B, C). During cartilage differentiation a little enzymic reaction is present only in cells, while it is not detectable in the matrix by the histochemical methods employed (Fig. 6D).

Fig. 4. Cholinesterase staining obtained by the use of AcTChI as substrate. The enzyme positivity is revealed by dark staining. Posterior margin of a stage-23 H.H. wing bud. In the middle of the positive area a blood vessel is visible. In the wing a little portion of ventral mesenchyme is also positive (arrow). (A) Bar equals 200 μm. (B) Bar equals 40 μm

e, ectoderm; d, dorsal side; l, basal lamina; m, mesenchyme.
Fig. 5. Cholinesterase staining obtained by the use of AcTChI as substrate. The enzyme positivity is revealed by dark staining.

(A), (B) Stage-24 H.H. wing bud: the triangle of positive mesenchyme is in contact with positive ectoderm and with precartilaginous cells; a sharply defined boundary is present between the positive and negative mesenchyme. Bar equals 200 μm (A); 40 μm (B).

(C) Stage-25, (D) stage-26 H.H. limb bud: the mesenchymal precartilaginous addensation appears stained, the mesenchyme surrounding the precartilaginous anlagen is not stained. Bar equals 40 μm.

c, precartilage mesenchymal cells.
Fig. 6. Cholinesterase activity of precartilaginous addensation. The enzyme positivity is revealed by dark staining.

(A) Stage-25 H.H. limb bud, section 5 μm thick. Bar equals 400 μm
(B) Stage-25 leg bud, in toto. The staining was obtained by the use of BuTChI as substrate. Bar equals 400 μm
(C) Stage-26 H.H. limb bud: the core of the precartilage anlagen does not display enzyme activity, while the cells around it present nuclear and plasma membrane (arrows) staining. Incubation carried out with AcTChI as substrate. Bar equals 40 μm
(D) Maturing cartilage: the enzyme staining is weak.

cm, chondrogenic mesenchyme; c, determined cartilage.
Histochemical specification of enzyme activity (Table 1)

By the use of specific substrates we had the possibility of distinguishing three different distributions of cholinesterase activity: BuChE alone, AChE alone, AChE and BuChE together.

BuChE activity can be detected by histochemical methods using BuTChI as substrate; AChE alone was detected by comparing the enzyme localizations obtained using respectively AcTChI (which hydrolyses AChE and a small percent of BuChE) and AcMTChI (which hydrolyses merely AChE).

BuChE alone was never found in any of the structures examined; AChE, alone or together with amounts of BuChE not detectable with the histochemical methods employed, was found only in the ectoderm and in the AER cells. AChE together with BuChE was found: (1) in the basal lamina under the ectoderm and in the subjacent mesenchymal cells, with major AChE activity mainly localized in the dorsal side of leg buds; (2) in clustered pre-cartilaginous mesenchymal cells (Fig. 2E, 6).

Selective inhibition of histochemical reaction (Table 2)

By the use of $10^{-4}$ to $10^{-5}$ M-eserine, the inhibition was complete in every one of

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<th>Substrates</th>
<th>Localization of enzyme activity</th>
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<td>cell membrane</td>
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<td>AcTChI</td>
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<td>AcMTChI</td>
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<td>BuTChI</td>
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Revelation of cholinesterase activity in different localizations by specific substrates in early developing limb buds. ++ = strongly positive, + = positive, − = negative.

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<th>Inhibitors</th>
<th>Localization of enzyme activity staining inhibition</th>
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<td>$10^{-5}$ M-eserine</td>
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<td>$10^{-4}$ M-BW 284c51</td>
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<td>$10^{-3}$ M-iso-OMPA</td>
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Effect of cholinesterase activity specific inhibitors in different localizations in limb buds of different stages, incubated with AcTChI as substrate. ++ = complete inhibition; + = partial inhibition; − = no inhibition.
the studied structures. The same result was obtained by the use of $10^{-4}\text{M-DFP}$. By the use of $10^{-4}\text{M-BW 284c51}$, only the reaction localized in the ectoderm and in the AER cells was strongly inhibited: a residual reaction was visible in the basal lamina between ectoderm and mesenchyme, and in the cytoplasm of mesenchymal positive cells. Residual reaction was present throughout the clustered mesenchymal pre-cartilage cells (Fig. 6A,B). The residual reaction was never associated with plasma membranes or localized at the perinuclear envelope; it was often formed by tiny granules of precipitated salts. The histochemical reaction appeared unaffected by the use of $10^{-3}$ to $10^{-6}\text{M-iso-OMPA}$.

**Quantitative analysis of cholinesterase activity during development**

We have shown the results relating to wing and leg buds together, because the differences between them revealed by this method are not significant. The mean cholinesterase activity of the stage 17–18 H.H. limb is 0.15 RU/mgN; it rises to 0.166 RU/mgN at stage 24–25, and to 0.4 RU/mgN at stage 28–30–32. Overall, the cholinesterase activity increases about threefold from stage 17 to 32. Abdominal skin of stage-32 H.H. chick embryos shows a mean cholinesterase activity amounting to 0.05 RU/mgN.

Since the cholinesterase activity is made up by AChE and BuChE activity, the use of specific inhibitors has allowed to check the different specific activities. The inhibition with $10^{-4}\text{M-BW 284c51}$ causes a loss of nearly 60 % activity in the early stages (16–18 H.H.), and of nearly 30 % in later development; iso-OMPA on the contrary causes a 20 % decrease in the early stages (17–18 H.H.), and a further decrease (about 50 %) from stage 25 H.H.

The inhibition with $10^{-4}\text{M-eserine}$ causes an activity that is different at different stages: at stage 17–18 H.H. residual activity is 0.03 RU/mgN, then it decreases to nearly zero at successive stages. The developmental changes in AChE and BuChE quantity are referred in the Fig. 7 histogram.

**Electrophoretic analysis of the enzyme activity** (Fig. 8)

By electrophoresis, three forms of cholinesterase were separated in leg and wing buds throughout development from stage 17 to 30 H.H.

At the earliest developmental stages examined (at the sample concentration) band 1 is more heavily stained than the other bands with both AcTChI and AcMTChI as substrates. At later stages, from 20 to 30 H.H., the three bands are equally stained by the reagents. BuTChI gives a reaction only in band 1 at stage 17 H.H. and in band 1 and 3 at later stages.

The stage 32 H.H. abdominal skin presents only the band 3 revealed with BuTChI. The bands are also sensitive to specific inhibitors (see Fig. 7). The AChE from *Electrophorus electricus*, used as control, gives five electrophoretically mobile forms, comprising bands more stained with AcTChI (1 and 2) and bands stained equally by AcTChI and BuTChI.
**AChE and BuChE distribution in developing chick limbs**

Fig. 7. Quantitative changes of cholinesterase activity during the early development of chick limb bud. 1 RU = 1 μmol of ACh hydrolysed/30 min in the assay conditions. (1) Stage-16 to 18 H.H. limb buds; (2) Stage-23 to 25 H.H. limb buds; (3) Stage-28 to 32 H.H. limb buds; (4) Stage-32 H.H. abdominal skin. a, incubation with AcTChI as substrate; b, is obtained from the elaboration of data obtained with either BuTChI as substrate or AcTChI as substrate and specific AChE inhibitors. The difference between a and b at each stage represents the amount of true AChE activity.

**Association of enzyme activity to cell membranes**

There was always specific AChE activity. It was found in the plasma membrane of ectodermal cells (Fig. 1) and in the plasma membrane and around the nuclear envelope of most of the AER cells (Fig. 2, 3). The plasma membrane activity was found on the whole plasma membrane or on portions of the plasma membrane, in contact with other cells or with the basal lamina (Fig. 1, 2, 3). In the mesenchyme of the early limb bud, such activity was present on the whole membrane, or on the membrane of the processes reaching the basal lamina between ectoderm and mesenchyme, mainly in the dorsal side of the leg, and also in the ventral side of the wing bud (Fig. 1F). From stage 20 H.H., membrane AChE specific activity was also found in deeper mesenchymal cells (Fig. 1E, 4). After stage 21 H.H. it was also associated with the plasma membrane of some of the clustering precartilage mesenchymal cells (Fig. 5B, arrow).

**DISCUSSION**

**General localization of the cholinesterase activity**

As expected, the general localization of the enzyme activity was similar to that obtained by Drews (1975). Nevertheless some differences were observed, probably due to the different methods employed. The first difference is the positive enzyme reaction of the cell membrane; the second is the presence of enzymic activity in the basal lamina under the ectoderm; the third is the detection of further enzyme
activity in superficial and deep mesenchymal cells and in processes of the former reaching the basal lamina; the fourth is the presence of some staining in cartilage cells during their maturation. These findings may lead one to think that the enzyme activity detected by us in such localizations could be represented by different molecular forms of the enzyme, less stable at embedding temperatures and/or using some fixation techniques, as Whittaker (1982) points out referring to the
membrane-localized enzyme found in ascidian embryos (Minganti & Falugi, 1980). Actually, it is known that both AChE and BuChE do exist in multiple molecular forms, with different functions (Witzeman & Bonstead, 1982). Some of these molecular forms are heavily inactivated at relatively low temperatures (Edwards & Brimijoin, 1983), such as the temperature required for embedding in polyethylene glycol. 'Embryonic' AChE, both soluble and associated with cell membranes, was found to be present in sea urchin blastomeres in two of these less-stable forms (Ozaki, 1974, 1976). The histochemical method used in the present work is based on a mild fixation procedure, and small pieces were incubated in toto at room temperature in the reaction medium. The fixation method was carefully tested by the detection of AChE activity associated with the human intact erythrocyte membrane (Minganti et al. 1981; Falugi, unpublished data), where the presence of the enzyme is well known, but it has never been detected by histochemical methods.

The two-step fixation which we have used with most of the samples is per se a control, because it also makes it possible to detect the cholinesterase activity found with usual fixation procedures. The localization of this cholinesterase activity is quite unaffected by the first step of the method.

**Developmental changes of cholinesterase activity**

Quantitative and electrophoretic analyses of cholinesterase activity show the presence of a true AChE, increasing threefold from early to later stages. Together with AChE there is also BuChE activity. The AChE activity is pre-eminent in early stages; from stage 24 BuChE activity increases more rapidly than the AChE activity and at stage 28 H.H. its amount is equal to that of AChE. The rapid increase in BuChE activity and its appearance in two of the three electrophoretically mobile bands, corresponds to the beginning of cartilage condensation and differentiation.

The specification of histochemically detected cholinesterase activity, obtained either with different incubation substrates, or with specific inhibitors of different cholinesterases, showed the presence of a true AChE activity alone in the ectoderm and AER, but AChE activity accompanied by BuChE activity in the basal lamina, in mesenchymal cells and their processes reaching the basal lamina, and in precartilage mesenchymal cells. These findings are consistent with the results of Schroder (1980). This author, using both colorimetry and disc electrophoresis techniques, found that cholinesterase activity of the limb chondrogenic blastema is made up of AChE and BuChE activities, and concluded that the cholinergic system revealed by the presence of these enzymes may be involved in the regulation of the embryonic development of the limb.

**AChE activity associated with cell membranes**

In this study, we have paid particular attention to the localization of enzyme activity at the plasma membrane of ectodermal and mesenchymal cells and of the mesenchymal cell processes which reach the basal lamina. This was shown to be a specific AChE activity (see Table 1, 2). AChE activity associated with cell membranes is
recognized in the neuromuscular system of vertebrates (Tennyson, Brzyn & Slotwiner, 1971; Tennyson, Brzyn & Kremzner, 1973; Miki & Mizoguti, 1982) and invertebrates (Spielholtz & Van der Kloot, 1973; Booth, Statton & Larsen, 1975; Raineri & Falugi, 1983). AChE activity associated with the plasma membrane of non-neuromuscular cells has been detected by histochemical methods in cultured human tumour cells in contact with each other or confluent (Falugi, Balza & Zardi, 1983a; Falugi, Castellani & Zardi, 1983b), in early chick embryo structures, such as 'area opaca' endoderm (Falugi et al. 1984), in blastomeres of ascidian early embryos (Minganti & Falugi, 1980), of a teleost (Fluck, 1982) and of sea urchins (Ozaki, 1974, 1976). In erythrocyte membranes it has been thoroughly studied by quantitative methods and recently also by histochemical methods (Geyer & Linss, 1980; Halbhüber et al. 1982). In all of the cases mentioned, the enzyme was thought to be involved in the regulation of membrane functions. For erythrocytes, such involvement was shown by recording the variation of transmembrane ion flux after the addition of specific AChE inhibitors (Finin, Volotovskii & Konev, 1979). So, we may suppose that AChE localized on plasma membrane may also be involved in the regulation of membrane functions in limb buds, where it may trigger morphogenetic events.

The hypothesis that neurotransmitters and their correlated enzymes may be involved in induction processes during morphogenesis has been advanced by a number of authors (Buznikov et al. 1968; Gustafson & Toneby, 1970, 1971; McMahon, 1974; Drews, 1975; Satoh, 1979; Le Douarin, 1980; Misawa, Doull, Kitos & Uyeki, 1981; Minganti et al. 1981, 1982; Fluck, 1982; Layer, 1983). This kind of intracellular signalling is thought to be a link in the evolutionary progression from the prenervous function of neurotransmitters and correlated enzymes as tissue organizers to their participation in cell-cell interactions occurring during synaptic transmission (Buznikov & Shmukler, 1981; Minganti et al. 1981). Electron microscopy studies are needed to provide evidence of the exact localization of this enzyme activity.

Conclusions

AChE and BuChE activities in the different cholinesterase-positive structures of developing chick limb are localized. The presence of AChE activity associated with cell membrane is evident. This activity is found in structures such as the ectoderm, the AER and the underlying mesenchymal cells. These structures have been demonstrated as interacting with each other during limb differentiation (Gumpel Pinot, 1972, 1973, 1980, 1981a,b,c; Solursh et al. 1981; Murphy et al. 1983). Mesenchymal cell processes that reach the basal lamina have been shown to be necessary for the normal development of the limb (Sawyer, 1982) and for the control of cartilage development. The postaxial margin of the limb, where we have found enzyme activity in the basal lamina and in mesenchymal cells more than in the preaxial zone, is known to play an important role in the guidance of limb development (Rowe & Fallon, 1981). Furthermore, the localization of AChE activity at the
Fig. 9. A possible model for the control exerted by ectoderm and AER on a target tissue (T).

The figures (A),(B) represent leg bud postaxial sections (where the mesenchyme is also positive under the AER).

(A) Stage-19 H.H.; (B) stage-21 H.H.; (C) stage-21 H.H. wing bud, postaxial section; (D) stage-21 H.H. leg bud, pre-axial section. The dotted areas represent the mesenchyme positive to the cholinesterase reaction; the arrows represent the possible directions of control exerted on T cells. The double arrow under the AER of (A) represents the reciprocal interaction between AER and mesenchyme.

plasma membrane of precartilage mesenchymal cells has a spatiotemporal correspondence in cell interactions. In these cells, two processes have been implicated; the first involves the conversion of mesenchyme to unexpressed chondrocytes and the second involves mesenchyme-dependent expression of chondrogenic differentiation (Solursh et al. 1982). At the first step we have detected strong cholinesterase positivity, at the second less enzyme activity.

In early stages (18 to 21 H.H.) the enzymatic reaction of the mesenchyme is strictly defined in small areas, under the AER (in postaxial sections) and in the dorsal side of the leg bud. A possible explanation of this particular feature may be the control exerted by the ectoderm (including AER) on the developmental events inside the mesenchyme (Solursh et al. 1981). Such control may be directed towards particular target cells or territories, e.g. the differentiating chondrocytes. One of the possible models of the action of this control may be that represented in Fig. 9. The regulation mechanism has to be complex, and most probably the cholinergic systems represent only a part of it. Hopefully some in vitro experiments will provide information on the function of the cholinergic system in ectoderm–mesenchyme interactions during limb bud differentiation. In the wing bud, there is also a ventral
site of mesenchymal cholinesterase activity. The ectoderm is higher and more stained with AChE reaction than in the remaining ventral surface (Fig. 1F, 4A arrow, 5A, 9C). This difference may be due to the different symmetry of the future skeletal pattern of the wing.

These findings may give further support to the hypothesis that plasma membrane associated AChE may be part of a cholinergic system involved in the regulation of membrane functions during morphogenesis. BuChE activity, which we have found together with AChE activity in the basal lamina of the ectoderm, the cytoplasm of mesenchymal cells, chondrogenic blastema cells and maturing cartilage, has been studied in the central nervous system (Graybiel & Ragsdale, 1982), but its role in these non-neuromuscular structures is not known, although the possibility of morphogenetic functions of this enzyme has been discussed (Layer, 1983).

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REFERENCES


AChE and BuChE distribution in developing chick limbs


