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PROSTAGLANDIN E_1 RECEPTORS ON CHICK EMBRYO MYOBLASTS

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SUMMARY: Differentiation of chick embryo myoblasts in vitro requires both cell-cell recognition and cell-cell fusion. Prostaglandin E_1 is known to play a role in controlling fusion, and a specific receptor has been postulated. We demonstrate two peaks of specific binding activity for prostaglandin E_1 during myoblast differentiation in vitro: one at 36 hours and one at 44 hours of culture. The prostaglandin binding activity of both peaks is sensitive to the inhibitors of prostaglandin synthesis, indomethacin and aspirin, and to the antibiotic tunicamycin. The 36 hour peak of binding activity occurs at the same time as the process of cell-cell recognition (24-36 hours) and recognition and prostaglandin binding exhibit similar sensitivity to indomethacin, aspirin and tunicamycin.

The differentiation of post-mitotic skeletal myoblasts consists of at least two discrete events (1), a cell-cell recognition process, termed alignment, and the subsequent disappearance of plasma membranes between individual nuclei, termed fusion. Using specifically designed in vitro assays, Knudsen and Horwitz (2, 3) have recently been able to separate and study the first events cell-cell recognition. Zalin (4, 5) noted the involvement of prostaglandins in fusion and thereby provided a possible biological basis for the two-step process described by Knudsen and Horwitz (2, 6). These results will make it possible to investigate the relationship between cell-cell recognition and cell-cell fusion. However, we believe that greater knowledge of the molecular events in each process is first required. David and associates (7, 8) have begun to analyze the events which lead up to fusion. They have attributed the timing of fusion in vitro to the prior appearance of receptors for prostaglandin E_1 (PGE_1) on the surface of myoblasts.

Abbreviations: CMF - calcium and magnesium-free Tyrode's salt solution
DME - Dulbecco's modified Eagle's solution (Gibco)
PGE_1 - prostaglandin E_1
TCA - trichloroacetic acid

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In this report we present evidence of two peaks of binding activity for PGE_1 during myoblast differentiation in vitro and provide initial characterization of that receptor activity. We suggest that the peak of binding activity which appears closest in time to cell-cell fusion is that predicted by David and associates (7, 8). The initial peak, which appears 6 to 8 hours earlier, may play a role in staging the second receptor peak and may be directly involved in the process of histotypic cell-cell recognition between myoblasts.

METHODS: Pectoral muscle was obtained from 11 day chick embryos. Obvious vascular, connective and nervous tissue was removed by dissection and the remaining tissue minced. After incubation in calcium and magnesium-free Tyrode's salt solution (CMF) for 10 minutes, and 0.25% trypsin in CMF at 37° for 20 minutes, the tissue was dissociated by pipetting. Trypsin was removed by washing and residual trypsin inactivated by 20 ug/ml soybean trypsin inhibitor (9). Cells were suspended in Dulbecco's modified Eagle's medium (DME) with 60 ug/ml DNase, filtered through a 52 u Nitex filter (to remove remaining pieces of tissue) and either placed in rotation culture according to standard techniques (9) or seeded into multiwell plates. Linbro 24-well plates were seeded with 10^6 cells/well in DME & 10% fetal bovine serum. Determination of fusion (only groups of 3 or more nuclei with no apparent separate membranes were counted) was done by phase contrast microscopy on orcein-stained cultures (2, 3). Our classification was periodically checked by transmission electron microscopy. PGE_1 was added as described in the figure legends. After harvesting by rubber policeman, the cells were centrifuged, washed and the cellular pellet counted for radioactivity. Cells and aggregates in rotation culture were assayed for accumulation of [^{3}H] amino acids or [^{3}H] glucosamine labeled materials into a trichloroacetic acid (TCA) precipitable fraction according to Hausman and Moscona (9). The recognition assay for fusion competent cells was done by harvesting cells cultured for 44 hours by the Ca^{++} removal method of Knudsen and Horwitz (2) and putting them in a standard rotation culture (9).

RESULTS AND DISCUSSION: Both indomethacin (8-20 uM) and aspirin (0.5-2 mM), thought to block the synthesis of prostaglandins and thereby interfere with fusion, blocked the in vitro fusion of chick embryo myoblasts into myotubes (Fig. 1). However, we were unable to obtain inhibition of fusion at 1 uM indomethacin, as reported by Zalin (4), possibly due to differences in our method of classifying nuclei into myotubes. The same concentrations of both drugs had no effect on myoblast cell-cell recognition when measured over a one hour period by Knudsen and Horwitz (2) or by us; however, studies of aggregation over a period of 6 hours using standard techniques (9) demonstrated an effect of both indomethacin and aspirin (Fig. 2). While the nature of this interference is not yet clear it is not likely due to inhibition of protein synthesis; treatment with 20 uM indomethacin

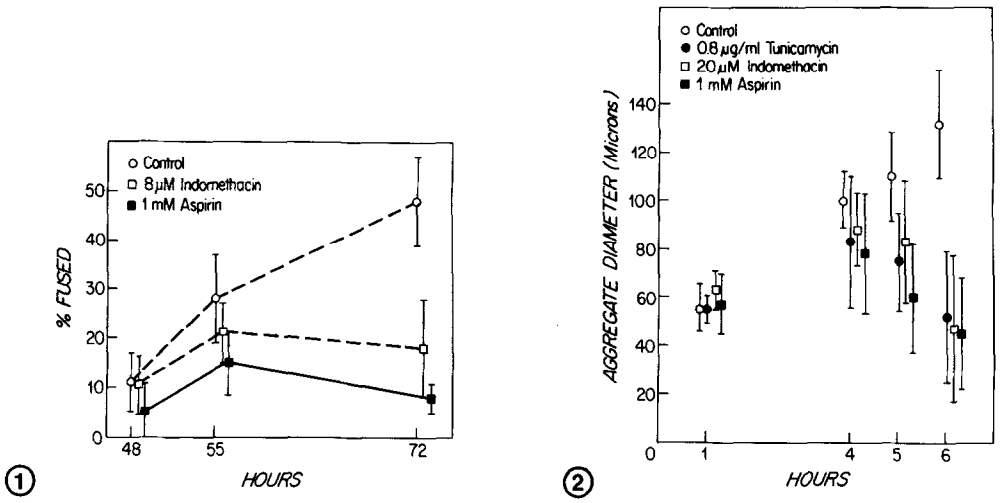

Figure 1. **Fusion of myoblasts in vitro. The percentage of nuclei in myoblasts (fused) was determined on orcein-stained cultures (2, 3). All cultures were maintained in and provided with new DME & 10% fetal bovine serum alone or with indomethacin or aspirin at 44 hrs. The inhibition of fusion by indomethacin was dose-dependent; 20 uM was necessary to block 95% of fusion (not shown). This represents one of three identical experiments; the mean and standard deviation are shown.**

Figure 2. **Rotation aggregation (recognition) of myoblasts. Suspensions of single cells were prepared from 44 hr cultures as described in methods. Cell and aggregate suspensions were photographed at each time point and the diameter of 50-100 aggregates was measured from the photographs (15). This represents one of three identical experiments; the mean and standard deviation are shown.**

resulted in only a 7% reduction in the incorporation of [^{3}H] amino acids during 5 hours of aggregation. Aspirin gave similar results. The sensitivity of cell aggregation to these drugs raised the possibility that prostaglandins, especially PGE_1, demonstrated by Zalin (4) to play a role in the control of myoblast fusion, might play a role in the earlier process of cell-cell recognition as well.

David and associates (7, 8) have suggested a model for the role of PGE_1 in myoblast fusion which involves a cell surface PGE_1 receptor, thought to become functional on the surface of myoblasts in vitro at least 6-8 hours before fusion. This corresponds to 42-44 hrs of culture in our system. Since this was postulated to be a cell surface material (present well before fusion) and clearly was involved in PGE_1 metabolism, we investigated the appearance of this binding activity in relation to the time of myoblast cell-cell recognition and subsequent fusion in vitro.

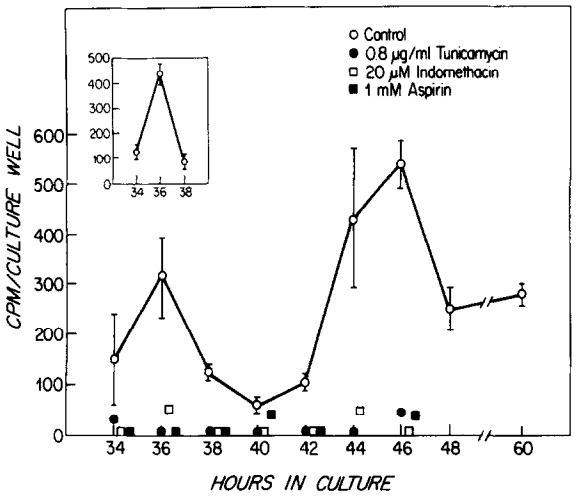

Figure 3. Specific binding of [^{3}H] PGE_1 to myoblasts in vitro. Individual wells of 10^6 cells, maintained as in Fig. 1, were provided with new DME (without serum) and 1 nM tritium-labeled PGE_1 one hour before harvesting (10). Both labeled and a 1000 fold excess of unlabeled PGE_1 were added to the same well (10) one hour before harvesting; paired wells were labeled with PGE_1 alone. All wells were run in triplicate. Specific binding was defined as the difference between total binding (labeled PG only) minus the binding obtained with both labeled and the 1000-fold excess of unlabeled PG. The main figure represents one of three experiments with tunicamycin, indomethacin or aspirin added at 30 hrs. The insert represents the compiled data from two additional experiments for the 36 hr peak only. The mean and standard deviation are shown.

Exposing myoblasts, differentiating in vitro, to [^{3}H] PGE_1 revealed a peak of binding activity at the time predicted by David (8), i.e. 42-44 hours in our system. Specific binding was determined by the method of Rao (12) in the presence of 1000-fold excess of non-labeled PGE_1; this peak was shown to consist largely of specific binding (Fig. 3). This observation suggested a prostaglandin receptor, presumably on the cell surface, but did not necessarily indicate that PGE_1 is the preferred ligand.

As Fig. 3 shows, there was also an earlier appearing peak of specific binding activity for PGE_1 to myoblasts in vitro. This peak consistently appeared at 36 hours of culture, 6 to 8 hours before the activity predicted by David, or 12-16 hours before fusion. The rise of binding activity was of 3-4 hours duration, beginning to rise at 34-35 hours and returning to baseline at 38 hours (not shown). This observation raised two questions: what is the role of the 36 hr peak of PGE_1 binding, and are the 36 hr and 44 hr receptors different molecules? Since indomethacin and aspirin

clearly block myoblast fusion and have some "late" effect on cell aggregation, we asked whether one of both of these peaks of binding activity might be susceptible to these drugs.

Figure 3 demonstrates that at the concentrations of both indomethacin and aspirin necessary to block fusion of myoblasts these PGE_1 receptors were either not present or were unable to bind the ligand in vitro. Therefore, the inhibition of myoblast fusion by these drugs which was observed by Zalin might be due not only to their effects on PGE_1 synthesis but also their interference with the timely appearance of these receptors. This observation also raises the possibility that the 36 hr receptor may be participating in cell-cell recognition between myoblasts; this recognition occurs in vitro from 24 through 36 hours. In this context, we are investigating whether the 36 hr receptor may be necessary for the appearance of the 44 hr, post-alignment, receptor.

Treatment of myoblasts with 0.8 ug/ml tunicamycin, which blocks dolichol-phosphate mediated glycosylation (10, 11), resulted in a similar "late" interference with cell-cell aggregation; aggregation was reduced 60% at 6 hours (Fig. 2) with a concomitant 90% inhibition of [^{3}H] glucosamine incorporation and a less than 5% inhibition of amino acid incorporation. This observation suggested the involvement of a glycosylated protein at some step in the myoblast cell-cell recognition process. Since this result was similar to that obtained with indomethacin and aspirin we asked whether 36 hr PGE_1 receptor might also be sensitive to tunicamycin. Figure 3 demonstrates that the binding activity at both 36 and 44 hours of culture was blocked by tunicamycin.

None of these drugs directly compete with PGE_1 for the receptor because adding the drugs at the time of PGE_1 addition, one hour before harvasting, does not result in loss of binding activity. The drugs must be present, at least, 3 hours before PGE_1 addition to block binding. These data are consistent with an effect on receptor synthesis but we cannot rule out direct binding of indomethacin, aspirin and tunicamycin to the PGE_1 receptors.

The present data provide no information as to whether the two receptors are different only in time of whether they are actually different molecules. Both are sensitive to tunicamycin, suggesting that they may be glycoproteins or, at least, molecules glycosylated via a tunicamycin-sensitive process. Both receptor peaks are sensitive to fusion-blocking concentrations of indomethacin and aspirin. This may be due to pleiotropic effects of the drugs, independent of their known effect on the synthesis of PGE_1. Alternatively, the synthesis of

prostaglandins, PGE_1 or others, may play a more extensive and earlier role in myogenesis than was previously suggested.

The receptor which appears at 44 hours is clearly that predicted by David and associates (8) and presumably helps to time myoblast fusion by the cAMP dependent modification of Ca^{++}-transport proteins in the myoblast plasma membrane; this is thought to occur after the 44 hour peak (8). The function of the receptor for PGE_1 which appears at 36 hours in culture is less clear. The information presented here is consistent with a role in myoblast cell-cell recognition (alignment); the 36 hr peak and the later stages of myoblast cell-cell recognition occur simultaneously and are sensitive to tunicamycin, indomethacin and aspirin. We are currently investigating this possibility using a partially purified preparation of myoblast plasma membrane glycoproteins (13) which enhance cell-cell aggregation (14, 15).

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REFERENCES

1. Nameroff, M. and Munar, E. (1976) Develop. Biol. 49, 288-293.
2. Knudsen, K.A. and Horwitz, A. (1977) Develop. Biol. 58, 328-338.
3. Knudsen, K.A. and Horwitz, A. (1978) Develop. Biol. 66, 294-307.
4. Zalin, R.J. (1977) Develop. Biol. 59, 241-248.
5. Zalin, R.J. (1979) Develop. Biol. 71, 274-288.
6. Horwitz, A.F., Wight, A., and Knudsen, K.A. (1979) Biochem. Biophys. Res. Comm. 86, 514-521.
7. David, J.D., See, W.M., and Higginbotham, C-A (1981) Develop. Biol. 82, 297-307.
8. David, J.D. and Higginbotham, C-A (1981) Develop. Biol. 82, 308-316.
9. Hausman, R.E. and Moscona, A.A. (1973) Proc. Nat. Acad. Sci. U.S. 70, 3111-3114.
10. Mahoney, W.C. and Duksin, D. (1979) J. Biol. Chem. 254, 6572-6576.
11. Gilfix, B.M. and Sanwal, B.D. (1980) Biochem. Biophys. Res. Comm. 96, 1184-1191.
12. Rao, Ch. V. (1976) Methods in Receptor Research, II, ed. Blecher, M. Marcel Dekker, New York pp. 615-640.
13. Walsh, F.S. and Phillips, E. (1981) Develop. Biol. 81, 229-237.
14. Hausman, R.E. and Moscona, A.A. (1975) Proc. Nat. Acad. Sci. U.S. 72, 916-920.
15. Hausman, R.E. and Moscona, A.A. (1976) Proc. Nat. Acad. Sci. U.S. 73, 3594-3598.