QUANTITATIVE INVESTIGATION ON Ca^{++}-AND pH-DEPENDENCE OF MUSCLE CELL FUSION IN VITRO

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SUMMARY: Muscle cell fusion can be triggered by Ca^{++}. The curve of fusion percentage vs. $[Ca^{++}]$ is much steeper than the Langmuir isotherme. The mid point of this curve (representing 50 % fusion) can be shifted from 95 µM to 1100 µM Ca^{++} by lowering the pH from 7.6 to 6.3. In the same pH-range the steepness of the curves decreases by a factor of 3. The curves are discussed in terms of a cooperative Ca^{++}-binding process.

During the embryonic development of cross-striated muscles, multinucleated myotubes originate by cell-fusion from mononucleated myoblasts. Myotubes develop the contractile apparatus and represent the cellular units of adult muscle. The essential steps of this process of cellular differentiation can be followed in in vitro cultures of muscle cells (e.g. 1,2,3).

Shainberg et al. (4) have shown that the fusion reaction depends on Ca^{++}-ions being inhibited by Ca^{++}-deficient media. The aim of the present work was to investigate quantitatively Ca^{++}- and pH-dependence of the fusion process to get a first set of data for a more detailed description of muscle cell fusion.

METHODS:

Seeding and culturing of the cells: Breast muscles of 11 day-old chick embryos were minced in saline G (5) and were subsequently trypsinized for 10 min. at 37 °C in 10 - 20 vol. 0.25 % trypsin in saline G. After centrifugation (2.5 min. at 200 g) the tissue pellet was disintegrated by gentle pipetting in culture medium (CM) containing only 30 µM Ca^{++}. CM consisted of Eagle's MEM with vitamines and amino acids according to Dulbecco's modification + 5 % calf serum (CS) + 5 % embryo extract (EE). The cell suspension was filtered through gauze and centrifuged for 2.5 min. at 200 g. After resuspension in CM (30 µM Ca^{++}, pH 7.4) the cells were counted and diluted to 3×10^5/ml. Portions of

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1.5 ml of this suspension were seeded in gelatinated tissue culture dishes (3.5 cm) and incubated at 37 °C and 90 % rel. humidity. After 16 hours the medium was changed (1.5 ml/dish). After a further 34 hours the medium was changed again (3 ml/dish) and pH and Ca^{++} were adjusted to the values given in Fig. 4. After a further 12 hours the cultures were fixed and stained with Giemsa's stain as described by Paul (6).

pH- and [Ca^{++}]-adjustment: To avoid the effects of changing buffer compositions and to give reasonably stable pH-values, no bicarbonate and CO_2 were used, and during the time of fusion and also the 50 hours before Ca^{++}-induction of fusion, all culture media were buffered with the following buffer mixture: TRICIN(*) (pK 8.15), HEPES (*) (pK 7.55), MOPS(*) (pK 7.2), MES(*) (pK 6.15) each 5 mM. The pH-values were adjusted with the aid of a pH-glass-electrode by titrating with 1n NaOH or 1n HCl starting from the normal pH of 7.4 of the CM. CS and EE were dialysed 4 x 30 min. against the 10fold volume of Ca^{++}-free Earle's BSS before being added to the CM. In order to obtain the final concentrations given in Fig. 4, Ca^{++} was added to the medium as $CaCl_2$ solution prepared from $CaCO_3$ + HCl. No corrections were made for Ca^{++} remaining in CS and EE after dialysis and for possible adsorption of Ca^{++} to protein components.

Counting of the nuclei was performed as described by Shainberg et al. (7). The percentage of fusion equals

$$\frac{\text{number of nuclei in myotubes} \times 100}{\text{total number of nuclei.}}$$

MATERIALS: Trypsin (Flow Lab.); Eagle's MEM Dulbecco's Mod. (Flow Lab.); Giemsa stain (Merck); TRICIN, HEPES, MOPS, MES (Serva, Heidelberg); calf serum: prepared by centrifugation of fresh calfblood (60 min. 1400 g) and subsequent sterile filtration of the supernatant through 3 layers of Sartorius membrane filters (pore sizes 1.2 µ, 0.6 µ, 0.2 µ).
Embryo extract: prepared as described by Paul (6).

RESULTS:

Growth and fusion of myoblasts in THMM buffered media: Freshly

(*) TRICIN: N-tris-(hydroxymethyl)-methylglycine
HEPES: N-2-hydroxyethylpiperazine-N-ethanesulfonic acid
MOPS: Morpholinopropanesulfonic acid
MES: Morpholinoethanesulfonic acid

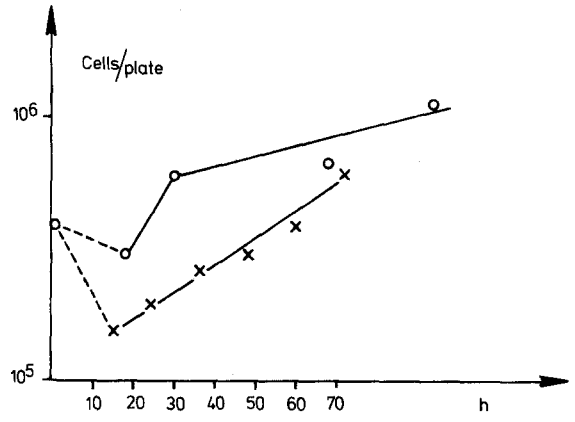

Fig. 1 Growth of myoblasts in bicarbonate-(o——o) and THMM-(x——x) buffered medium at 30 µM Ca^{++}. The cells from 3.5 cm dishes were trypsinized with 1 ml 0.25 % trypsin and counted in a hemocytometer.

explanted myoblasts do grow in media with moderate concentrations of Good's buffers (8) as shown previously for some mammalian cell strains by Eagle et al. (9,10). It can be seen from Fig.1 that myoblasts grow approximately 3 x slower under these conditions than in bicarbonate buffered media. The fusion capacity is not impaired. Twelve hours after Ca^{++} is increased to 1400 µM, fusion reaches 70 - 80 %, under optimum conditions. As can be seen from the steepness of the curves in Fig. 2, the rate of fusion reaches a maximum if induction by Ca^{++} is performed 50 - 56 hours after seeding. Fig. 3 shows muscle cell cultures grown in THMM-medium before (30 µMCa^{++}) and after (1400 µMCa^{++}) fusion at pH 7.4.

Dependence of fusion on [Ca^{++}] and pH: A remarkable shift of the curve of fusion percentage vs. [Ca^{++}] is produced by changing the medium pH (Fig. 4). The midpoint of the fusion curve is shifted from 95 µM Ca^{++} (curve F) at pH 7.6 to 1100 µM Ca^{++} at pH 6.3 (curve A).

The fact that the Ca^{++}-curves (Fig. 4) at pH-values between 8.5 and 7.6 were shifted in the opposite direction (curves H,G,F) may be accounted for by the possibly higher Ca^{++}-binding capacity of the protein components of the CM at higher pH. But other possibilities concerning the dependence on pH of the fusion reaction cannot be excluded at this state.

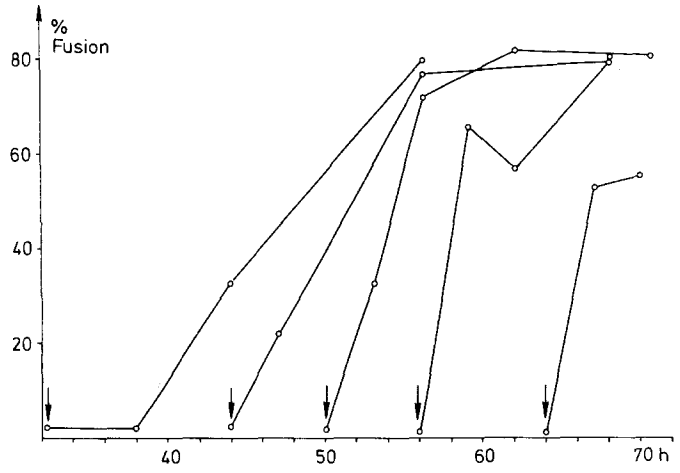

Fig. 2 Induction of fusion at various times of growth. Arrows mark times at which $[Ca^{++}]$ was increased from 30 µM to 1400 µM. Fusion is given as percentage of nuclei in fused cells.

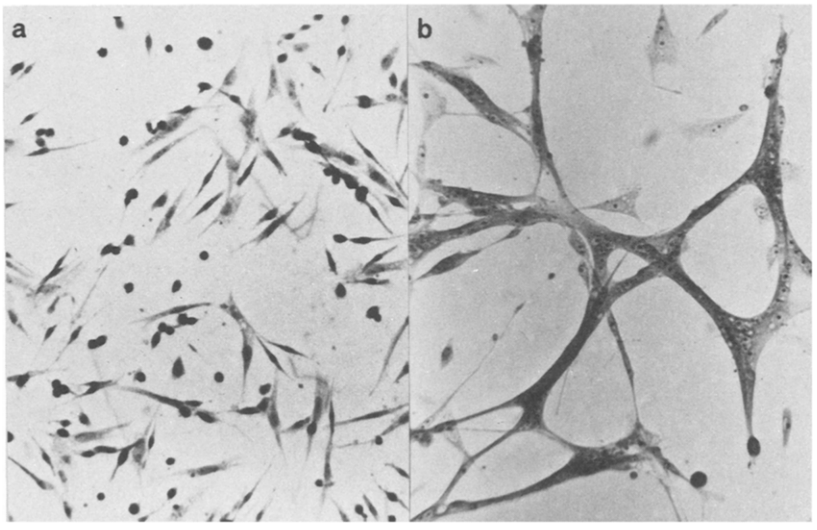

Fig. 3 Muscle cells under low (30 µM) and high (1400 µM) Ca^{++} conditions in THMM buffered medium at pH 7.4.
a) 62 h old myoblast culture (30 µM Ca^{++}).
b) Myotubes developped from a myoblast culture by adding 1400 µM Ca^{++} 50 hours after seeding. Age of culture 62 hours. Fusion time 12 hours.

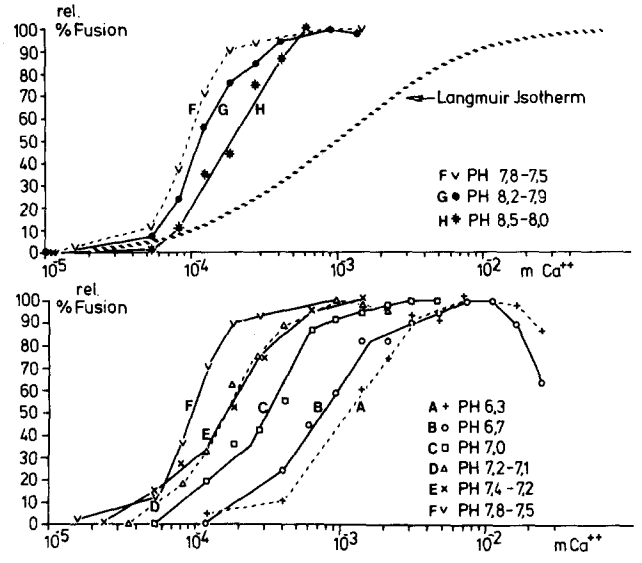

Fig. 4 Relative fusion percentages dependent on Ca^{++}-concentration of the medium at various pH-values. For each point 3 plates (3.5 cm) were evaluated and 1000 - 1300 nuclei counted. Maximum absolute fusion percentages were 45 % (A), 58 % (B), 65 % (C), 70 % (D), 72 % (E), 75 % (F), 70 % (G), 70 % (H). In order to get the relative percentages maximum fusion was set ≙ 100 % for each curve.

As indicated in Fig. 4 the pH of the media could be held constant only at low values (6.3 - 7.2). At higher pH (7.4 - 8.5) considerable changes to more acidic values occurred during the 12 hours of induction.

DISCUSSION: For comparison, a Langmuir adsorption isotherme is given in Fig. 4. It is evident that the fusion curves are much steeper. Thus, any simple interpretation of the fusion curves in terms of a process being dependent on Langmuir adsorption or Michaelis Menten kinetics is not in accord with the data. Fusion proceeds from 10 to 90 % while the Ca^{++}-concentration increases by a factor of 3 (curve F) compared with a factor of 81 for a Langmuir adsorption dependent process. Possible explanations for the steepness of the curves could be found by assuming a cooperative binding of Ca^{++}. For example, binding could occur either to the cell membrane or to the subunits of a Ca^{++}-dependent enzyme which modifies the membrane. On the other hand intra-

cellular processes preceding or accompanying fusion (e.g. movement of the cytoskeleton, transport of vesicles) could be influenced also by external [Ca^{++}]-variations via membrane potential and permeability changes. But assuming that intracellular pH is unaltered by changing medium pH, the observed shift of the fusion vs. [Ca^{++}] curves is probably explained better by suggesting competition of H^+- and Ca^{++}-ions for binding sites on the cell surface. In any case, Ca^{++}-binding would trigger the fusion. The result would be the transition of the cell membrane from an unfusionable to a fusionable state. Under this assumption the decreasing steepness of the curves (F,E,D,C,B,A) with falling pH-values would be consistent with a lowering of interaction energies between the suggested cooperative subunits of an enzyme or membrane system. This energy lowering might result from H^+-binding to electron rich groups (e.g. NH, COO^-, $-O-PO_3^{--}$) involved in stabilizing interactions between the subunits. On the other hand, the shift of the curves to higher Ca^{++}-concentrations with increasing H^+-concentrations (curves F-A) could correspond to a competition between Ca^{++}- and H^+-ions for the same binding sites.

It is to be mentioned that the observed dependence on Ca^{++} of the fusion process is quite specific: all experiments were performed in presence of 8×10^{-4} M Mg^{++}. The steepest curves appear in the range of physiological pH. It is suggested that the observed phenomenon is a biological one in the sense that <u>in vivo</u> (i.e. a higher Ca^{++}-concentration than used <u>in vitro</u> during prefusion period) a still unknown physiological stimulus (possibly a hormone-like substance or even the cell to cell contact) triggers the change of the cell membrane which makes it fusionable. This change would then be accompanied by a strong increase in the Ca^{++}-affinity of the membrane. In this connexion observations made by Träuble (11) on phospholipid suspensions are interesting: Temperature dependent phase changes are accompanied by a cation exchange. The results of Quinn and Dawson (12) point in the same direction: dramatic changes in calcium adsorption on phospholipid monolayers are observed in the range of physiological pH-values. Our results could be interpreted in terms of this hypothesis in the following way: During the prefusion period <u>in vitro</u> evidently all requirements necessary for fusion which originate from cell activity have been fullfilled. And now in inversion of the biologi-

cal sequence of events it is possible to trigger the fusion by Ca^{++}.

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