CALCIUM REGULATION OF SKELETAL MYOGENESIS: IV A DEFINED CULTURE MEDIUM PERMISSIVE FOR MYOTUBE FORMATION AND THE USE OF THE CALCIUM ANTAGONIST LANTHANUM

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SUMMARY

Lanthanum has been used effectively in studies of calcium physiology in experiments of short duration. In experiments of longer duration, we report that solutions, such as cell culture medium, containing lanthanum (La$^{3+}$) undergo a decrease in pH on the time scale of hours. Presumably, the decrease in pH is a consequence of the hydrolysis of water by the solution-active La$^{3+}$ ions. We have devised a defined culture medium without serum and chick embryo extract which is permissive for myotube formation. This defined medium is also useful for studies of La$^{3+}$ as a calcium antagonist. La$^{3+}$ at concentrations of 0.1 mM reversibly inhibits myotube formation when added in conjunction with Ca$^{2+}$ to low-Ca$^{2+}$ fusion-blocked cultures.

Key words: myogenesis; calcium; lanthanum; defined culture medium.

INTRODUCTION

The rare earth element lanthanum (La$^{3+}$) has been used in numerous biologic experiments primarily as a surrogate probing for calcium functions. A detailed review (9) enumerated many of the uses of La$^{3+}$ in a variety of tissues. La$^{3+}$ is considered to be a powerful competitor for extracellular Ca$^{2+}$ binding sites. In a series of experiments on the role of extracellular calcium in myotube formation of chick muscle in culture utilizing La$^{3+}$, we discovered a significant effect of La$^{3+}$ on culture medium pH.

Our experiments tested whether calcium influx/or cell surface calcium, or both, was necessary for myotube formation (3). The experimental paradigm required La$^{3+}$ to be present in culture solution during most of the period of myotube formation, which lasted up to 24 h. This time period was decreased to 6 to 8 h by applying a fusion block with a calcium-deficient medium. Addition of calcium promoted myotube formation during the ensuing 6 to 8 h from the time of release of fusion block. La$^{3+}$ added at this time reversibly inhibited myotube formation. However, it was evident that the pH of the culture medium decreased substantially making the observed effect ambiguous. Consequently, measures were taken to design a culture medium that could maintain myotube formation and maintain pH in the presence of La$^{3+}$.

In this note we report our observation that micromolar concentrations of La$^{3+}$ produce significant pH shifts on the time scales (hours) in medium ordinarily employed in tissue culture experiments. Further, we report the use of a minimal essential culture medium that will support growth and differentiation of skeletal muscle. This medium designated P-S-K Medium (after the authors of this report), also permits the use of La$^{3+}$ at low concentrations for studies of calcium metabolism and myotube formation which are reported in detail elsewhere (3).

MATERIALS AND METHODS

The studies of pH were done with Leibovitz L15 medium supplemented with 5% chick embryo extract and 15% horse serum (4) or with a basal solution of the following composition (derived from 1): KCl: 5.4 mmol, NaCl: 137 mmol, glucose: 6.1 mmol, CaCl$_2$: 2.5 mmol, 5 g/liter serum albumin (Sigma, St. Louis, MO), and various concentrations of buffer and LaCl$_3$. La$^{3+}$ in the form of LaCl$_3$.7H$_2$O (Fisher certified) was used as a 0.1 M stock solution in double distilled, deionized water. HEPES (Sigma, free acid or Na$^+$ salt) dissolved in double distilled deionized water was a 1.0 M stock solution in double distilled, deionized water. Several combinations of buffer and La$^{3+}$ concentrations were made. Ten milliliter aliquots of solutions were incubated in test tubes at 37$^\circ$ C in tissue culture incubators with normal atmospheric gas pressures at saturated humidity for periods from 15 min to 24 h. pH was read at intervals with two pH meters, Orion combination electrode #91-02, and Radiometer Copenhagen, Thomas electrode #4094-L15, which were standardized at pH 4.00, 7.00, and 10.00 with Fisher standards. In another experiment varied concentrations of calcium were used, see Table 1.
A defined minimal culture medium was formulated which maintained cell viability, myotube formation, and pH with normal atmospheres. Pectoral muscle from Day 12 White Leghorn chicken embryos was used to make the cell cultures (refer to (4) for details). The saline-albumin solution described above was supplemented with amino acids (GIBCO; Grand Island, NY) and 0.005 mM Fe(SO,

Viability was assessed by cell proliferation, morphology, and myotube formation as was done initially using L15 cell culture medium (4). The pH of the solutions at the onset of the experiment was 7.50; pH was determined again 24 h after the addition of LaCl3 and storage at 37 °C. The pH values shown are representative of several experiments, n = 3.

Table 3 contains the essential information for the effect of La+++ on pH in a basal salt solution containing serum albumin. The pH of these solutions was stable unless La+++ was present in which case pH varied with solution composition and the concentration of La+++. Serum albumin enhanced the buffering capacity of the solution. For example, in a solution of identical salt concentration but without serum albumin (10 mmol HEPES), 1 mM La+++ produced a pH change from 7.5 to 6.8 in 24 h. A solution stored 10 d at 5 °C had a pH of 4.7. Over time the basal salt solution containing HEPES buffer and serum albumin showed a decrease in pH, with the rate and degree of pH decline greater in the lower buffer and higher La+++ concentration solutions. The design of the experiment was suitable for a three-way analysis of variance. Using a commercial program (Hewlett-Packard 9825-15014) the data were analyzed with a Hewlett-Packard 9825A desktop calculator. The results yielded strongly significant effects of buffer concentration (P <0.001), La+++ concentration (P <0.03) and time in solution (P <0.20). In addition there was a significant interaction (P <0.05) between buffer concentration and time.

The other interactions were not significantly different.

Utilizing the above information, we established the best combination of culture medium, buffer, and incubator conditions to maintain myotube formation in a defined minimal medium so that we could use La+++ solutions to study calcium metabolism related to myotube formation. P-S-K medium contains the basal salt solution (Table 3) buffered with 10 mM HEPES and supplemented with GIBCO amino acid mixture (2%), with calcium concentration varied to provide for conditions that were permissive or nonpermissive for myotube formation. P-S-K medium was tested for stability of pH with graded calcium (5) and lanthanum concentrations. Table 1 shows that greater pH stability was obtained at 0.2 and 0.7 mM CaCl2, which fell due to the decrease in pH the observation was not definitive, see Table 2. Consequently, an alternative cell culture medium was devised.

\[ \text{La}^{+++} \text{mM} \quad 1 \text{h} \quad 2 \text{h} \quad 4 \text{h} \quad 8 \text{h} \quad 24 \text{h} \\
0.0 \quad 7.50 \quad 7.50 \quad 7.46 \quad 7.44 \quad 7.5 \\
0.05 \quad 7.53 \quad 7.53 \quad 7.45 \quad 7.35 \quad 7.25 \\
0.1 \quad 7.55 \quad 7.52 \quad 7.45 \quad 7.35 \quad 7.20 \\
0.2 \quad 7.34 \quad 7.32 \quad 7.49 \quad 7.34 \quad 7.20 \\
0.5 \quad 7.49 \quad 7.48 \quad 7.41 \quad 7.33 \quad 6.90 \\
1.0 \quad 7.45 \quad 7.45 \quad 7.25 \quad 7.32 \quad 6.83 \\
2.5 \quad 7.43 \quad 7.38 \quad 7.15 \quad 7.29 \quad 6.80 \\
\]

\[ ^{1} \text{P-S-K medium consisted of KCl: 5.4 mmol, NaCl: 137 mmol, glucose: 6.1 mmol, CaCl2: 0.025 mmol, serum albumin: 5 g/l, HEPES: 10 mmol, and GIBCO amino acid mixture: 20 µl/ml. The medium was sterile during the experiment. The pH of the solutions at the onset of the experiment was 7.50; pH was determined again 24 h after the addition of LaCl3 and storage at 37 °C. The pH values shown are representative of several experiments, n = 3. Comparable values were obtained with the same medium in cell cultures.}^{1}

\[ ^{2} \text{La}^{+++} \text{mM} \quad 1 \text{h} \quad 2 \text{h} \quad 4 \text{h} \quad 8 \text{h} \quad 24 \text{h} \\
0.0 \quad 7.50 \quad 7.50 \quad 7.46 \quad 7.44 \quad 7.5 \\
0.05 \quad 7.53 \quad 7.53 \quad 7.45 \quad 7.35 \quad 7.25 \\
0.1 \quad 7.55 \quad 7.52 \quad 7.45 \quad 7.35 \quad 7.20 \\
0.2 \quad 7.34 \quad 7.32 \quad 7.49 \quad 7.34 \quad 7.20 \\
0.5 \quad 7.49 \quad 7.48 \quad 7.41 \quad 7.33 \quad 6.90 \\
1.0 \quad 7.45 \quad 7.45 \quad 7.25 \quad 7.32 \quad 6.83 \\
2.5 \quad 7.43 \quad 7.38 \quad 7.15 \quad 7.29 \quad 6.80 \\
\]

\[ ^{1} \text{L15 medium contained 10 mM HEPES, 5% chick embryo extract, and 15% horse serum. All solutions were sterile during the experiment. Lanthanum was added from a freshly prepared stock solution of 0.1 M to the desired concentration. The pH was determined with the pH meter and the standardization buffers at 37 °C. The data are representative of several experiments, n = 3. Comparable values were obtained with the same medium in cell cultures.}^{1}

\[ ^{2} \text{L15 medium contained 10 mM HEPES, 5% chick embryo extract, and 15% horse serum. All solutions were sterile during the experiment. Lanthanum was added from a freshly prepared stock solution of 0.1 M to the desired concentration. The pH was determined with the pH meter and the standardization buffers at 37 °C. The data are representative of several experiments, n = 3. Comparable values were obtained with the same medium in cell cultures.}^{1} \]