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Non-mitochondrial calcium uptake and release
by chondrocyte: Role of ATP, phosphate
ions and inositol triphosphate

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Abstract : The objective of this investigation was to evaluate Ca^{2+} binding characteristics of the endoplasmic reticulum of chick chondrocytes. Using a permeabilized cell preparation in which Ca^{2+} uptake into mitochondria was blocked with ruthenium red, we measured cation uptake by a rapid centrifugation procedure. In terms of Ca^{2+} binding, we demonstrated that uptake was mediated by ATP and blocked by treatment with vanadate. To determine whether or not Ca^{2+} binding proteins existed in these cells we isolated endoplasmic reticulum from chondrocytes. Using a ^{45}Ca overlay technique, we demonstrated the presence of Ca^{2+} binding proteins that were of the same size as the reticuloplasmins. We measured Ca^{2+} release characteristics of avian chondrocyte endoplasmic reticulum. The cells were sensitive to 1,4,5-inositol triphosphate (IP_3)—the rate and extent of Ca^{2+} release from this pool was similar in magnitude to that of other tissues. To evaluate the effects of metabolic factors on cation transport, we examined the effect of glucose, glucose 6-phosphate and inorganic phosphate (Pi). We noted that there was minimal phosphatase activity and exogenous glucose 6-phosphate did not stimulate Ca^{2+} transport. However, Pi markedly increased ATP supported Ca^{2+} uptake. This finding suggests that any mechanism that could serve to elevate the intracellular Pi concentration, whether by hydrolysis of organic phosphate esters or by supplementing the cytosolic Pi concentration, would serve to maintain Ca^{2+} within the endoplasmic reticulum. The results of these studies indicate a central role of intracellular Pi in chondrocyte Ca^{2+} transport.

Introduction

The mechanism by which chondrocytes control intracellular ion transport prior to, and during, mineral deposition is not understood.

Results of studies with other cell types indicate that when ion transients occur there is a dramatic alteration in cell function. For example, it is known that the intracellular inorganic phosphate (Pi) level regulates carbohydrate metabolism and activates oxidative

phosphorylation. In terms of mineralization related activities, Pi modulates the rate of Ca^{2+} transport across lipid membranes¹¹. In addition, in an earlier study, we showed that a low molecular weight phosphorylated compounds may provide Pi for mineral formation²¹.

The intracellular Ca^{2+} concentration has a profound effect on cellular activity. This ion is an important cofactor for membrane ATPase as well as the enzymes associated with the tricarboxylic acid cycle⁸¹. Mitochondria have a high affinity for Ca^{2+} . Indeed, as this cation is taken up avidly by isolated mitochondria, the presence of Ca^{2+} deposit in this organelle has been thought to be linked to mineralization of the extracellular matrix^{4,51}. Recent studies, however, indicate that mitochondrial calcium transport may not be linked to mineral deposition, since, in both excitable as well as some nonexcitable tissues the endoplasmic (sarcoplasmic) reticulum serves as a major Ca^{2+} buffer^{6,71}.

Calsequestrin, a protein present in the terminal cisternae of the endoplasmic reticulum, is a Ca^{2+} binding protein that has a high capacity and moderate affinity for Ca^{2+} ⁸¹. In some tissues, Ca^{2+} has been located in specific cytoplasmic structures that are designated as "calciosomes"⁹¹. It is thought that Ca^{2+} located in the calciosome is in equilibrium with cytosolic Ca^{2+} . Calcium can be released from the endoplasmic reticulum by the hydrolysis of phosphatidylinositol and the formation of 1,4,5-inositol triphosphate (IP_3). When this compound is bound to a specific receptor protein in the endoplasmic reticulum, there is a rapid release of bound Ca^{2+} . Another pathway for release of bound Ca^{2+} is by the synthesis of factors such as those associated with carbohydrate metabolism¹⁰⁻¹²¹. Irrespective of the release mechanism, a transient elevation in cytosolic Ca^{2+} would change the activity of a number of Ca^{2+} dependent enzymes¹³¹.

The objective of this study are four fold : first, to ascertain whether chondrocytes accumulate Ca^{2+} at the level of the endoplasmic reticulum ; second, to determine whether calsequestrin-like proteins are expressed in these cells ; third, to characterize nonmitochondrial

energy supported Ca^{2+} uptake and release by cartilage ; cells and fourth, using a plasma membrane permeabilizing procedure, to examine the uptake of IP_3 and glucose 6-phosphate as modulators of Ca^{2+} flux.

Materials and Methods

Cells

Chondrocytes were isolated from the proximal tibial growth cartilage of the 8 week old White Rock chick using a collagenase digestion procedure. The cells were maintained in the primary culture of DMEM supplemented with 10% fetal calf serum and antibiotics in 95% air and 5% CO_2 for 24 hours in microbiological tissue culture dishes. As red blood cells attach to the surface of the dish, the procedure permitted separation of blood cells from chondrocytes. The chondrocytes were collected by centrifugation and washed. The cells were over 90% vital as judged by trypan blue exclusion and they exhibited a high energy charge ratio (above 8.9). Liver cells were also collected in the same manner.

For evaluation of Ca^{2+} binding proteins in chondrocytes, cells were isolated from the tibial epiphysis of 19 day chick embryos. The cartilage was digested with collagenase and the cells were collected and maintained in culture using procedures that we described previously^{14,1}. Secondary cultures were harvested at 7 days.

Permeabilization

The used procedure was very similar to that described by other workers¹⁵¹. Briefly, cells were washed in a basic buffer containing 20 mM NaCl, 100 mM KCl, 5 mM MgCl, and 10 mM HEPES at pH 7.2. Chondrocytes (30×10^6) were permeabilized by treatment with digitonin in basic buffer (80 $\mu\text{g}/\text{ml}$) for 2 minutes at 37°C. This treatment resulted in permeabilization of the cell membrane as indicated by trypan blue exclusion. Subsequent evaluation by electron microscopy revealed few changes in the morphology of the endoplasmic reticulum. The permeabilized cells were then washed and the pellet was suspended in a buffer containing 1 mM EDTA and 3 μM ruthenium red for 3 minutes at room temperature. The presence of the ruthenium red

resulted in a 30–40 fold decrease in calcium uptake by the permeabilized cells.

Calcium Uptake by Permeabilized Cells

The method used was similar to that described by Wolf, et al¹⁵⁾. The procedure was modified by collecting the cells by centrifugation. Following permeabilization, the cells were isolated by centrifugation at 2,000 g, washed and resuspended in the basic buffer containing 10 μ Ci 45 CaCl₂ (0.6 M). For those experiments, designed to measure Ca²⁺ uptake in the presence of ATP, the concentrations of ATP, Mg²⁺, Ca²⁺, and EGTA were determined using the computer program generated from the dissociation constants of the various ionized chemical species. Chondrocyte calcium uptake values obtained using the computer program were not substantially different from those obtained using the basic buffer system with the final Ca²⁺ concentration adjusted to 64 μ M with CaCl₂.

Electrophoretic Separation of Ca²⁺ Binding Proteins

Following 7 days in culture, cells were washed first with calcium and magnesium-free Hank's balanced salt solution to remove serum and then with phosphate buffered saline (PBS) at pH 7.4. The cells were homogenized by 3 freeze-thawing cycles and completeness of homogenization was checked by microscopy. The homogenate was centrifuged at 14,000 g for 20 minutes to sediment nuclei, membranes, mitochondria, and lysosomes. The supernate was stored on ice. The sediment was washed with PBS and recentrifuged at 14,000 g. The two supernatants were combined and centrifuged at 105,000 g for 3 hours. The sediment was subjected to SDS-PAGE in 10% acrylamide mini gel. The proteins were transferred to nitrocellulose and the membrane was incubated in a buffer (60 mM KCl, 5 mM MgCl₂, and 10 mM imidazole-HCl at pH 6.8) containing 1 mCi 45 Ca for 10 minutes using the method described by Maruyama, et al¹⁶⁾. Binding was assessed by autoradiography.

Chemical Assays

Total calcium was determined by atomic absorption spectrophotometry; protein was assayed using the method of Lowry, et al¹⁷⁾; glucose 6-phosphatase activity was assessed using the technique described by Watts, et al¹⁸⁾.

Data were expressed as Mean $\pm$ S.E. of three or four experiments.

Results

The first series of experiments were directed at characterizing conditions that were optimum for non-mitochondrial Ca²⁺ uptake into the permeabilized cells. Calcium uptake into the permeabilized cells measured over a 15 minute time period was 49.84 ± 5.8 nmol/mg protein/min. When energy supported mitochondrial Ca²⁺ uptake was inhibited by treating the cells with ruthenium red, the mean Ca²⁺ uptake was reduced to 3.2 ± 0.5 nmole/mg protein/min (Fig. 1). Hence, treatment of the cells with the inhibitor resulted in a 10 fold decrease in Ca²⁺ uptake. The study also showed that non-mitochondrial Ca²⁺ uptake was small in comparison with uptake into the mitochondria.

Fig. 1 The effect of inhibitors on Ca²⁺ uptake by chondrocytes. Chondrocytes were permeabilized by exposure to digitonin for 2 minutes and then treated with vanadate (10 and 100 μ M) or ruthenium red (3 μ M) for 3 minutes. Control cells were not exposed to an inhibitor. Chondrocytes were washed and suspended in a buffer that contained 2 mM ATP, 20 mM NaCl, 100 mM MgCl₂, 10 mM HEPES, and 10 μ Ci 45 CaCl₂ (adjusted with CaCl₂ to give a final concentration of 64 μ M) at pH 7.2, for 15 minutes. Calcium uptake was determined by scintillation counting.

Fig. 2 Time dependent uptake of Ca^{2+} by chondrocytes. Permeabilized ruthenium red-treated chondrocytes were incubated with $10 \mu\text{Ci } ^{45}\text{CaCl}_2$ ($64 \mu\text{M}$) at 37°C in the presence (closed circles) and absence (open circles) of 2 mM ATP. Calcium uptake was determined by removing cells and measuring their ^{45}Ca content following rapid centrifugation.

Fig. 3 ATP-supported Ca^{2+} uptake by permeabilized chondrocytes. Permeabilized cells were treated with ruthenium red and Ca^{2+} uptake was determined over a 15 minute time period in the presence of ATP ($0\text{--}10 \text{ mM}$).

In a separate series of experiments the effect of vanadate on Ca^{2+} uptake was examined. Figure 1 shows that treatment of chondrocytes with vanadate resulted in a dose dependent decrease in Ca^{2+} levels in chondrocytes. As vanadate is an inhibitor of endoplasmic reticulum ATPase activity, it is probable that Ca^{2+} uptake into the endoplasmic reticulum is de-

Fig. 4 Nucleotide-supported Ca^{2+} uptake by permeabilized chondrocytes. Permeabilized cells were treated with ruthenium red and Ca^{2+} uptake was determined over a 15 minute time period in the presence of 2 mM ATP, ADP, GTP, and UTP.

Fig. 5 Effect of IP_3 on Ca^{2+} release from chondrocytes. Cells were permeabilized, treated with ruthenium red and loaded with Ca^{2+} in the presence of ATP. IP_3 ($2 \mu\text{M}$) was added to the cell suspension (closed circles). At 0, 10, and 20 minutes, aliquots of the supernatant were removed and counted. Loss of calcium from permeabilized chondrocytes that were not exposed to IP_3 are also shown (open circles).

pendent on the presence of an active ATPase system.

Figure 2 shows that chondrocytes accumulated Ca^{2+} in a linear fashion over a 30 minute time period. In the presence of 2 mM ATP,

Fig. 6 Effect of Pi on Ca^{2+} uptake by chondrocytes. Permeabilized cells were treated with ruthenium red and Ca^{2+} accumulation in the presence of Pi (0–5 mM) was determined over a 15-minute time period.

there was a significant increase in Ca^{2+} accumulation and after 30 minutes, ATP increased the cell Ca^{2+} content two-fold. The rate of Ca^{2+} accumulation was $0.1 \mu\text{mole/mg protein/min}$. This value is comparable to rates for permeabilized liver cells ($0.3 \mu\text{mol/mg protein/min}$) under similar conditions.

Chondrocyte Ca^{2+} uptake was dependent on the ATP concentration. Figure 3 shows that maximum Ca^{2+} accumulation occurred when the ATP concentration was 5 mM. We also examined the ability of nucleotides other than ATP to support cell Ca^{2+} uptake. We found that UTP and to a lesser extent ADP promoted Ca^{2+} uptake by permeabilized chondrocytes (Fig. 4). GTP had no discernible effect on Ca^{2+} accumulation.

The aim of the second experiment series was to determine whether or not a Ca^{2+} pool, which was sensitive to metabolic signals, existed in the chondrocyte endoplasmic reticulum. Figure 5 indicated that IP_3 caused a dramatic loss of Ca^{2+} from permeabilized cells that had been preloaded with Ca^{2+} . The rate of release appeared to be very rapid in the first 10 minutes; exposure to the agent for an other 10 minutes resulted in a slower, although significant decrease in the cell Ca^{2+} concentration. A concentration of $2 \mu\text{M}$ IP_3 provided maximum Ca^{2+} release from permeabilized cells.

The effect of the metabolic intermediates

Fig. 7 Electrophoretic separation of Ca^{2+} binding proteins of chondrocyte endoplasmic reticulum obtained from chick embryos. Chondrocytes were homogenized and an endoplasmic reticulum fraction isolated by differential centrifugation. This protein fraction was subjected to SDS-PAGE in 10% acrylamide. The proteins were transferred to nitrocellulose and incubated with $^{45}\text{Ca}^{2+}$. The presence of Ca^{2+} binding proteins were visualized by autoradiography. An arrow indicates a major Ca^{2+} binding band.

glucose 6-phosphate and glucose as well as phosphate ions on Ca^{2+} uptake and release by the permeabilized chondrocytes was determined. Neither glucose nor glucose 6-phosphate influenced Ca^{2+} transport by chondrocytes (data not shown). In contrast, Pi modified Ca^{2+} uptake characteristics of the permeabilized cells in the presence of 2 mM ATP. Figure 6 clearly shows that an increase in the medium Pi concentration from 0 to 5 mM resulted in 3 fold increase in the rate of cell Ca^{2+} uptake. This result clearly indicated that any mechanism that could serve to elevate the Pi concentration would serve to maintain Ca^{2+} within the endoplasmic reticulum; alternatively, Pi depletion in the membrane would facilitate Ca^{2+} release.

Finally, proteins of the endoplasmic reticulum of chondrocytes were examined to ascertain whether they contained Ca^{2+} binding proteins (Fig. 7). Following electrophoresis and blotting onto nitrocellulose, a prominent Ca^{2+} binding band (molecular weight of 65,000)

was observed. A number of minor bands with molecular weights of 80,000 and 100,000 daltons were also present.

Discussion

While it is recognized that the endoplasmic reticulum serves to control calcium flux, little was known of the characteristics of this system in chondrocytes. Using a permeabilized cell preparation in which mitochondrial calcium uptake had been blocked, we showed that calcium was taken up by a chondrocyte membrane system; that Ca^{2+} binding proteins existed in the endoplasmic reticulum that were of the same size as reticuloplasmins⁸; that ATP was required for cation accumulation; that Ca^{2+} uptake was blocked when the cells were treated with an ATPase inhibitor; and that the Ca^{2+} pool was responsive to a known modulator of reticulum bound Ca^{2+} . Together these findings indicate that there exists a Ca^{2+} binding system within the chondrocyte, probably associated with the endoplasmic reticulum, that serves to regulate ion transport. Considerable similarities in Ca^{2+} transport characteristics were seen between chondrocyte and other cell types^{6,7}. We showed, for the first time, that an IP_3 sensitive Ca^{2+} pool was present in the endoplasmic reticulum. The rate and extent of Ca^{2+} release was similar in magnitude to that of other tissues⁹. Measurement of the time course of Ca^{2+} release showed that there was an initial rapid release followed by a slower phase—this was probably due to Ca^{2+} reuptake by the endoplasmic reticulum. A similar course is seen with pancreatic islet cells¹⁵. The observation that Ca^{2+} uptake required ATP or other high energy donors and was blocked by vanadate suggested that nucleotide hydrolysis was a requirement for cation binding. That GTP had no measurable effect on Ca^{2+} accumulation was not surprising, as a recent study showed that this nucleotide might modulate IP_3 dependent cation release¹⁹.

Evaluation of the proteins in the endoplasmic reticulum indicated that there existed one major protein and up to 3 minor proteins which bound Ca^{2+} . They had similar molecular weights as the reticuloplasmins, the luminal content of the endoplasmic reticulum⁸. In

terms of molecular weight, the chondrocyte protein is similar in weight to calsequestrin and calreticulin, 55 and 59 kd proteins of plasmacytoma and liver cells respectively^{8,20}.

We conducted experiments to ascertain whether glucose 6-phosphate could modulate Ca^{2+} uptake by the permeabilized chondrocytes. As cation transport has been linked with glucose 6-phosphatase activity in pancreatic islets^{10,15}, we measured the activity of this enzyme in sections of the avian growth cartilage. In contrast to other cell types, glucose 6-phosphate has no significant effect on Ca^{2+} uptake (or release) by chondrocytes. The finding that the activity of this enzyme was very low suggested that a mechanism that linked phosphorylation of glucose esters to calcium accumulation might not be functional in chondrocytes.

While there was minimal phosphatase activity and exogenous glucose 6-phosphate did not stimulate Ca^{2+} transport, the possibility existed that Pi alone might serve as a metabolic trap for the cation. To evaluate this possibility, cells were permeabilized and Ca^{2+} uptake determined in the presence of Pi . Results of the experiment clearly showed that this anion markedly increased Ca^{2+} uptake. This finding suggests that any mechanism that could serve to elevate the intracellular Pi concentration, whether by hydrolysis of organic phosphate esters or by supplementing the cytosolic phosphate concentration, would serve to maintain Ca^{2+} within the endoplasmic reticulum. This dramatic effect of Pi on Ca^{2+} binding to the endoplasmic reticulum lends further importance to our earlier observation concerning Pi control of Ca^{2+} transport. Those studies showed that in model systems, the Pi concentration regulated Ca^{2+} transport across a lipid interphase¹; we also showed that at the cartilage calcification front, the appearance of Pi atoms in the matrix was coincident with mineral deposition^{2,21}. Results of all of these studies point to a more central role of intracellular Pi on chondrocyte Ca^{2+} transport and the mineralization process that has hitherto been reported.

抄録：本研究では、ニワトリ軟骨細胞の小胞体(ER)の Ca^{2+} 結合の特徴を検索した。すなわち、digitonin で透過性を高めた細胞を用い、ミトコンドリアの Ca^{2+} の取り込みを ruthenium red で阻害後、 Ca^{2+} の取り込みおよび放出に対する vanadate, ATP, 1, 4, 5-inositol triphosphate (IP_3), glucose, glucose 6-phosphate および無機リン (Pi) の影響を検討した。

その結果、 Ca^{2+} の取り込みは、vanadate で阻害され ATP 添加で増加すること、Pi は ATP に依存した Ca^{2+} の取り込みを著しく増加させること、 IP_3 は ER に蓄積された Ca^{2+} の放出を促すことが判明した。しかし、glucose 6-phosphate はほとんど影響しなかった。また、ER に reticuloplasmin と同じ分子量の Ca^{2+} 結合蛋白が存在することが示された。以上の所見から、細胞内 Pi 上昇させるような機構が ER の Ca^{2+} 維持に働いており、細胞内 Pi が軟骨の Ca の移動に中心的な役割を演じていることが示された。

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