Note

Effects of Calmodulin and Calmodulin Antagonists on Neutral Ca$^{2+}$-ATPase in the Membrane Fraction of Human Milk

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Intracellular calcium ions at a low level are vital in the control of many important aspects of cellular metabolism.\(^{11}\) (Ca$^{2+}$ + Mg$^{2+}$) -ATPases (EC 3.6.1.3) are responsible for specific ATP-dependent Ca$^{2+}$ transport.\(^{11}\) ATPases stimulated by Ca$^{2+}$ at an optimal value of pH 7.0 have been found in human milk by Kanno et al.,\(^{11}\) concentrated in the membrane fraction. Called milk fat globule membrane (MFGM), this membrane is derived from the apical plasma membrane of mammary secretory cells during the secretion of milk lipid.\(^{6,7}\) Furthermore, the affinity for Ca ions of most Ca$^{2+}$-ATPases in the plasma membrane from a variety of cell types is dependent on calmodulin, which is a multifunctional calcium-binding protein.\(^{6,7}\) Calmodulin has been shown to stimulate the effects of calcium ions on several membrane ATPases such as erythrocyte membrane, synaptic membrane, and sarcoplasmic reticulum.\(^{9,10}\) Calmodulin is present in human milk and in the mammary glands of rats and cows.\(^{8,9}\) Speculating from the origin of MFGM, it is considered that the Ca$^{2+}$-calmodulin-Ca$^{2+}$-ATPase system may be involved in regulating the cytosolic calcium concentration required for milk secretion in the mammary gland.

This work was undertaken to investigate the stimulatory effects of calmodulin and the inhibitory effects of calmodulin antagonists (trifluoperazine, calmidazolium, and N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7)) on Ca$^{2+}$-ATPase at neutral pH in the membrane fraction of human milk.

Mature human milk obtained from healthy mothers was fractionated by centrifugation. The floating cream layer was churned after dilution, and the separated butter milk rich in MFGM was used.\(^{10}\) The neutral Ca$^{2+}$-ATPase activity was measured by incubation for 30 min at 37°C in 0.9 volume of 30 mM imidazole-histidine buffer (pH 7.0), 1 mM NaCl, 3 mM KCl, and 0.5 mM CaCl$_2$, 2 μg of protein, and 0.1 vol. of 10 mM ATP (Tris salts, equine muscle, Sigma), as described previously.\(^{11}\) The reaction was stopped by adding 1 ml of a mixture of chloroform and methanol (2:1, v/v) and inorganic phosphate was measured by the method of Luthra.\(^{12}\)

The membrane fraction (5 mg of protein/2 ml) of human milk was washed twice with 15 volumes of 20 mM Tris-HCl (pH 7.0) containing 1 mM ethyleneglycol-bis-(6-aminoethyl) ether)-tetraacetic acid (EGTA) by centrifugation (100,000 × g, 30 min, 4°C) for removal of endogenous calmodulin.\(^{12}\) The final pellet was suspended in 8 ml of 20 mM Tris-HCl (pH 7.0) in the presence or absence of 1% Tween 20 (0.142 mg of protein/ml). Calmidazolium (Sigma) or W-7 (Sigma) dissolved in dimethylformamide were added to the suspension to make a final concentration of 0.01 to 100 μM. Trifluoperazine (Sigma) was freshly made up before the assay, and care was taken in protecting the stock solution from light to prevent the formation of free radicals.\(^{12}\) The calmodulin-deficient membrane fraction (2 μg of protein/ml) in the assay mixture (0.9 ml) for neutral Ca$^{2+}$-ATPase was first incubated for 5 min at 37°C with and without calmodulin (1 μg, bovine brain, Sigma) and then, if necessary, for another 5 min with 10 μl of various inhibitors, before starting the reaction by adding 0.1 ml of an ATP solution.

The effects of calmodulin on neutral Ca$^{2+}$-ATPase activity by its presence (1 μg/ml) or absence at various concentrations of the calcium ion are shown in Fig. 1. The depletion of extracellular calcium ions inhibited the activity of neutral Ca$^{2+}$-ATPase, but calmodulin failed to increase the neutral Ca$^{2+}$-ATPase activity significantly. However, the calmodulin dependency of this enzyme was confirmed with calmodulin antagonists (Fig. 2). The half-maximal inhibition of neutral Ca$^{2+}$-ATPase occurred with 0.3 μM of trifluoperazine and with 25 μM of calmidazolium, and activity being completely inhibited with 100 μM of these two drugs. This result is similar to that reported for Ca$^{2+}$-ATPase in other systems.\(^{14,15}\) The half-maximal inhibition of W-7 was found at 0.75 μM, but the maximal inhibition rate was 70% at 10 μM. These results for calmodulin antagonists strongly suggest that neutral Ca$^{2+}$-ATPase may be regulated by calmodulin.

Calmodulin did not enhance neutral Ca$^{2+}$-ATPase activity. However, calmodulin antagonists strongly inhibited neutral Ca$^{2+}$-ATPase. Such behavior was also found in the membrane fraction solubilized with Tween 20 (data not shown), and is similar to the results for other Ca$^{2+}$-ATPases.\(^{12,14-18}\) The discrepancy between the ineffectiveness of added calmodulin and the inhibition by calmodulin antagonists may have been caused by the presence in the membrane fraction of human milk of a calmodulin-binding protein or by nonspecific inhibitory effect of calmodulin antagonists.\(^{19}\) Calmodulin-binding protein has inhibited the calmodulin action on neutral Ca$^{2+}$-ATPase activity and active calcium transport, as was found in the red cell membrane.\(^{20}\) Moreover, calmodulin antagonists may bind to Ca$^{2+}$-binding proteins other than calmodulin, in which these proteins probably have a common hydrophobic binding site for the drugs.\(^{21}\) Trifluoperazine had a specific effect on basal ATPase activity unrelated to the interaction with calmodulin.\(^{20}\) However, calmidazolium is the most effective anti-calmodulin compound, removing the stimulation of Ca$^{2+}$-ATPase by calmodulin with a K$_i$ of <0.5 μM.\(^{22}\) The inhibition of calmidazolium implies that neutral Ca$^{2+}$-ATPase may be regulated by calmodulin.

In conclusion, neutral Ca$^{2+}$-ATPase activity was not enhanced by calmodulin, but was strongly inhibited by calmodulin antagonists. It is, therefore, still difficult to conclude that there is an interaction between calmodulin and Ca$^{2+}$-ATPase, which may mediate the calcium transport.

Fig. 1. Effects of Calmodulin on Neutral Ca$^{2+}$-ATPase at Different Concentrations of the Calcium Ion.

The calmodulin-deficient membrane fraction (2 μg of protein) in an assay mixture containing the indicated concentration of calcium ions in the presence (●●●●) or absence (○○○○) of calmodulin (1 μg/ml) was incubated for 30 min at 37°C. Bars show the mean ± SD (n = 3).

Fig. 2. Inhibition of Neutral Ca$^{2+}$-ATPase by Trifluoperazine (A), Calmidazolium (B) and W-7 (C).

The incubation time was 30 min at 37°C, and all assays were run under dim light. Black squares indicate the basal activity without calmodulin, and bars represent the mean ± SD (n = 3).
between cytoplasm and extracellular space in the human mammary gland.\textsuperscript{23)}

References