EFFECT OF REMOVAL OF EXTERNAL CALCIUM ON PHOSPHOINOSITIDE HYDROLYSIS IN CULTURED MYOTUBES OF EMBRYONIC CHICKEN\(^3\)

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ABSTRACT The effect of removal of external Ca\(^{2+}\) on phosphoinositide hydrolysis was investigated in cultured myotubes from 9-day-old Leghorn embryonic chicken. In the myotubes exposed to Ca\(^{2+}\)-free Ringer's solution, the turnover of phosphoinositide was exponentially decreased with a time constant of about 26 min. In the presence of external Ca\(^{2+}\), the hydrolysis of phosphoinositide was significantly increased by exposure to 80 mmol/L K\(^+\) solution. After removal of external Ca\(^{2+}\), 80 mmol/L K\(^+\) exposure caused a slight decrease of phosphoinositides hydrolysis in comparison with the control (normal Ringer). It is indicated that hydrolysis of phosphoinositide in cultured myotubes can be enhanced by high K\(^+\) exposure. External Ca\(^{2+}\) is essential for this effect, which is different from mature muscle fibres.

Key words: high K\(^+\); Ca\(^{2+}\)-free; phosphoinositide; myotube

Inositol phospholipids play an important role in signal transduction of various cells\(^1\). Under the action of phosphatidylinositol specific phospholipase C (P2PLC), the phospholipids are hydrolyzed into two important second messengers\(^2\): diacylglycerol (DAG) and 1,4,5-trisphosphate (IP\(_3\)), which can activate PKC\(^3\) and mobilize Ca\(^{2+}\) from intracellular Ca\(^{2+}\) store\(^1\), respectively. Several subtypes of P2PLC have been identified, some of which are Ca\(^{2+}\)-dependent\(^4\). Thus, increasing cytoplasmic free calcium ([Ca\(^{2+}\)]\(_i\)) may elicit hydrolysis of phosphoinositide. Recently, we have shown that, besides the increase of [Ca\(^{2+}\)]\(_i\), depolarization may also enhance phosphoinositide hydrolysis in frog skeletal muscle\(^5\,#6\). However, phosphoinositide hydrolysis in immature muscle cells may be regulated differently, since cultured myotubes exposed to Ca\(^{2+}\)-free high K\(^+\) medium showed a decrease of phosphoinositide hydrolysis\(^7\). Owing to the fact that about 75\% of the embryonic myotubes cultured for 3 days could respond to Ca\(^{2+}\)-free high K\(^+\) exposure with raised [Ca\(^{2+}\)]\(_i\) (unpublished data), an in2crease of phosphoinositide hydrolysis should be expected, considering Ca\(^{2+}\) dependence of P2PLC. In the present study, the effect of removal of external Ca\(^{2+}\) on phosphoinositide hydrolysis was investigated in cultured myotubes of embryonic chicken.

The methods of cell culture and extraction of inositol phosphates were as described previously\(^7\). In brief, the cells were prepared from the thigh muscles of 9-day-old embryos of Leghorn chickens. After cultured in DMEM ( Gibco) with 10\% fetal cattle serum and 5\% embryo extract at
37 °C in 5% CO₂ and 95% air for 3 days, the cells were incubated in 2 mL DMEM containing 10μCi [³H]myo2inositol (Shanghai Institute of Nuclear Research) for another 14 hr. Then, the cells were washed with DMEM 3 × 15 min at 37 °C and loaded with Li⁺ by incubating the cells in 20 mM Li⁺ Ringer (see below) for 20 min. After provided with different challenges, the cells were extracted with trichloroacetic acid (TCA). The TCA extract was loaded on a column of Dowex 1X8 (Bio2Rad), and the total labeled inositol phosphates (IPs) were eluted with 16 mL of 110 mM ammonium formate/0.11 M formic acid. The radioactivity was determined with a liquid scintillator (Beckman LS6000IC). Student t test was used for statistical comparison between groups.

The composition of Ringer’s solution was (in mM/L): 155 NaCl, 5 KCl, 5 CaCl₂, 2 MgCl₂, and 5 HEPES. It was titrated to pH 7.12 with NaOH. 20 mM/L Li⁺ Ringer was made by equivalent replacement of Na⁺ in Ringer with Li⁺. To examine the effect of high K⁺ exposure on phosphoinositol2 tide turnover, the K⁺ concentration was elevated to 80 mM/L and the ionic strength of the solution was maintained by reducing Na⁺. Cl⁻ was partially replaced by CH₃SO₃⁻ to keep the product of [K⁺]₀ and [Cl⁻]₀ constant. For preparing Ca²⁺ free solution, CaCl₂ was omitted, but 2 mM/L EGTA was added. Moreover, MgCl₂ was increased to 5 mM/L. The free Ca²⁺ concentration in the Ca²⁺ free solution was about 10⁻⁸ M/L.

In consistence with the previous result[7], the labeled IPs of the myotubes exposed to 80 mM/L K⁺ for 15 min increased to 34613 ± 132 % (mean ± SD, n = 12) of the control (Ringer’s solution for 15 min), indicating that in the presence of external Ca²⁺ high K⁺ exposure can increase phosphoinositol2 tide hydrolysis in cultured myotubes. Moreover, as seen previously[7], the hydrolysis of phosphoinositol2 tide was decreased in the myotubes exposed to Ca²⁺ free Ringer’s solution for 15 min. As a new finding of the present study, this decrease of phosphoinositide hydrolysis induced by removal of external Ca²⁺ was observed to be time dependent (Fig11). The time course of this reduction could be fit with a single exponential, as shown in Fig121. The time constant was about 26 min, which may represent a translocation of Ca²⁺ ions from cytoplasm into extracellular compartment. Since it is difficult to measure slow change of [Ca²⁺]ᵢ, whether the decrease of phosphoinositide turnover is due to reduction of [Ca²⁺]ᵢ remains to be clarified.

Different from matured muscle fibres[5,6], the present study clearly indicated that the presence of external Ca²⁺ was essential for high K⁺ induced increase of phosphoinositide hydrolysis in the cultured myotubes. In comparison with the control (normal Ringer), the exposure to Ca²⁺ free 80 mM/L K⁺ medium caused a slight decrease of phosphoinositides hydrolysis (Fig11). But, if the myotubes treated with Ca²⁺ free Ringer’s solution for the same period of time were used as a control, different results can be seen. For instance, the labeled IPs of the myotubes exposed to Ca²⁺ free Ringer’s solution and Ca²⁺ free 80 mM/L K⁺ for 15 min was 5711 ± 1116 % and 8215 ± 2111 % of the control (normal Ringer), respectively. Their difference became significant (P < 0.01), representing the effect of high K⁺ exposure in the absence of external Ca²⁺. The difference was still significant (P < 0.01) when the exposure time was decreased to 5 min. However, with an exposure of 2 min, their difference was
no longer significant ($P > 0.0105$). Thus, it is evident that, being different from mature skeletal muscle fibers, depolarization alone cannot increase the hydrolysis of phosphoinositide in the myotubes. The coupling between depolarization and activation of P2HLC which probably exists in mature fibers may be absent in cultured myotubes. As shown in Fig11, the time dependence of the effect of high K+ exposure mainly resulted from that phosphoinositide hydrolysis in the myotubes exposed to Ca$^{2+}$-free Ringer’s solution was decreased with a rate faster than that in the myotubes exposed to Ca$^{2+}$-free 80 mmol/L K$^+$. In conclusion, it is indicated that the hydrolysis of phosphoinositide in cultured myotubes can be enhanced by exposure to high K+ medium, but external Ca$^{2+}$ is essential for this effect.

**REFERENCES**


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