ATPase Activity and Calcium Uptake of Microsomes Isolated from Stomach Smooth Muscle after Exposure to Phospholipase C

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Summary

Sakai, Y., Ichikawa, S., Yoshida, M., Oouchi, M. and Miyagawa, M. *ATPase Activity and Calcium Uptake of Microsomes Isolated from Stomach Smooth Muscle after Exposure to Phospholipase C*. Japanese J. Smooth Muscle Res., 1982, 18, (5), 339-345. ATPase activity and Ca^{2+} uptake were examined in microsomal membrane fractions isolated from guinea pig stomach smooth muscle which had been exposed to phospholipase C (PLC). Basal Mg^{2+} -ATPase, Na^+ , K^+ -ATPase and Ca^{2+} , Mg^{2+} -ATP activities were inhibited in a time dependent manner by PLC treatment. There was positive correlation between each of these ATPase activities and total phospholipid content of the microsomal fraction. Phosphatidylcholine restored Ca^{2+} , Mg^{2+} -ATPase activity of the microsomal fraction isolated from the tissue which had been treated with PLC for 30 min but not after 60 min. Ca^{2+} uptake in the presence of ATP by microsomal fraction from tissue treated with PLC for 60 min was significantly decreased. The results provide a cellular basis for the inhibitory effect of PLC on contractility of stomach smooth muscle.

Introduction

The removal of Ca^{2+} from cytoplasm, a process that initiates relaxation of smooth muscle, is thought to be achieved by an energy dependent Ca^{2+} transport systems (Carsten & Miller, 1977; Daniel *et al.*, 1979). Such Ca^{2+} transport systems have been reported to be associated with isolated crude microsomal fractions (Carsten & Miller, 1977; Wuytack *et al.*, 1978) or highly purified plasma membrane fractions (Grover *et al.*, 1980; Kwan *et al.*, 1981; Sakai *et al.*, 1981). It is known that phospholipase C (PLC) inhibits action potential, simultaneously depolarizing resting membrane potential; and affects the ionic permeability of neuron and muscle cell membrane (Tobias, 1958; Albuquerque & Thesleff, 1967; Sakai, 1980). The treatment of skeletal microsomes with PLC has been shown to decrease ATPase activity, Ca^{2+}

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transport (Martonosi, *et al.*, 1968) and guanylate cyclase activity (Sulakha, *et al.*, 1976). The present work was undertaken to investigate ATPase activity and Ca^{2+} uptake in microsomal fractions isolated from guinea pig stomach smooth muscle treated with PLC. Results are discussed in relation to the effects of PLC on smooth muscle contractility.

Methods and Materials

Membrane Isolation : Guinea pigs weighing 250–350 g were stunned and bled. The pyloric region of the stomach was immediately removed and placed in ice-cold Krebs solution. Mucosa and connective tissues were carefully separated from the stomach muscle layer. Approximately 1 g of trimmed muscle from 4–5 guinea pigs was incubated in Krebs solution which was continuously aerated with 95% CO_2 and O_2 at $37 \pm 0.5^\circ\text{C}$, pH 7.4. Phospholipase C (50 $\mu\text{g}/\text{ml}$) was added to the incubation medium 60 min or 30 min before the end of the incubation period. Control samples were similarly incubated in Krebs solution in the absence of added PLC. The tissues were subsequently washed with ice-cold Krebs solution, blotted with filter paper and washed twice with buffer containing 250 mM sucrose, 0.15 mM EGTA and 40 mM histidine buffered at pH 7.0. The tissues were minced with a pair of scissors in 15 ml of the above buffer and homogenized for 15 sec at 15,000 rpm with a motor driven homogenizer (B. Braun Model ET-20). The tissue homogenate was centrifuged at 1,000 g for 10 min to remove cell debris and connective tissues. The supernatant was centrifuged at 10,000 g for 10 min and filtered through layers of gauze to remove mitochondria. The microsomal fraction was obtained as sediment after subsequent centrifugation at 105,000 g for 40 min. The pellet was washed and resuspended in 40 mM histidine buffer without EGTA.

ATPase Assays : Mg^{2+} -ATPase activity was measured as the release of inorganic phosphate in medium containing 100 mM KCl, 5 mM MgCl_2 , 0.15 mM EGTA, 5 mM Na_2ATP and 40 mM imidazole at pH 7.0, 37°C . Ca^{2+} , Mg^{2+} -ATPase activity was measured similarly in the same medium in the presence of various amounts of CaCl_2 . Na^+ , K^+ -ATPase activity was measured in 100 mM NaCl, 20 mM KCl, 5 mM Mg-ATP , 0.15 mM EGTA and 10 mM Tris buffer at pH 7.5. All ATPase reactions were started by the addition of 0.2 ml (80–100 μg protein) of the fraction to the reaction medium (2 ml final volume). Reaction was stopped after 10 min incubation at 37°C by addition of 2 ml cold 10% TCA.

Ca^{2+} uptake : Ca^{2+} uptake was measured by Millipore filtration technique (Janis *et al.*, 1977). The reaction medium was similar to that used for Ca^{2+} , Mg^{2+} -ATPase assay except for the addition of 0.5 μCi $^{45}\text{CaCl}_2$. Reactions were started by the addition of microsomal fraction to the medium and stopped by filtering through Millipore filters (0.45 μm pore size). The filters were then washed with 10 ml of a solution containing 250 mM sucrose and 40 mM imidazole. ^{45}Ca accumulated on the filters without the membrane fraction has been accounted for in calculation. Filters were dissolved in 10 ml of Bray's solution (Bray, 1960).

Other Assays : The phospholipid content of each microsomal fraction was determined by the method of Chen *et al.* (1965). Inorganic phosphate was measured by the method of Fiske and Subbarow (1925). Protein was determined by the method of Lowry *et al.* (1951).

Reagents : Phospholipase C, Phosphatidylcholine, Phosphatidylethanolamine, Na_2ATP and Mg-ATP were purchased from Sigma. $^{45}\text{CaCl}_2$ was obtained as solution in 0.5 M HCl from

International Chemical Nuclear Co.

Results

Effect of PLC on ATPase activity in microsomal fraction.

Table 1 shows that both Mg^{2+} -ATPase and Na^+ , K^+ -ATPase activities of the microsomal membrane fraction were significantly inhibited by PLC treatment. Mg^{2+} -ATPase activity was about four times that of the Na^+ , K^+ -ATPase. Mg^{2+} -ATPase was inhibited to about 45% of the control value after 60 min PLC treatment ($P > 0.001$). Na^+ , K^+ -ATPase was inhibited to 60% of the control value ($P < 0.01$).

Relationship between ATPase activities and phospholipid content in microsome fractions.

Phospholipid content changed after PLC treatment. ATPase activity and total phospholipid content were linearly related up to 60 min treatment for both Mg^{2+} -ATPase activity and Na^+ , K^+ -ATPase activity as shown in Fig. 1. This could indicate that ATPase activity, in both cases, is dependent on available phospholipid, either directly or as a secondary effect of another process or factor.

Ca^{2+} modification of Mg^{2+} -ATPase activity in microsomal fractions.

Figure 2 shows changes in Mg^{2+} -ATPase activity in the presence of Ca^{2+} . Ca^{2+} enhanced Mg^{2+} -ATPase activity in control samples and samples treated with PLC for 30 min, but not in samples treated with PLC for 60 min. The differences observed here could be due to limitation of the available membrane reaction sites, or to limited availability of energy, or both.

Effects of PLC on Ca^{2+} uptake.

As shown in Table 2, Ca^{2+} uptake in the presence and absence of ATP by microsomal

Table 1. ATPase activity in microsomal fractions isolated from guinea pig stomach smooth muscle treated with phospholipase C (PLC). Data are expressed as Mean $\pm$ S.E. The number of separate experiments are shown in the parentheses. Assay conditions are described in detail in the text.

	ATPase Activity (μ mol/mg/min)	
	Mg^{2+} -ATPase	Na^+ , K^+ -ATPase
Control	2.10 ± 0.06 (6)	0.50 ± 0.06 (5)
PLC, 30 min	$1.63 \pm 0.15^*$ (3)	0.42 ± 0.08 (3)
PLC, 60 min	$0.92 \pm 0.08^{**}$ (6)	$0.29 \pm 0.03^*$ (5)

*: $P < 0.01$, **: $P < 0.001$

Fig 1. Relationship between ATPase activity and total phospholipid content in microsomal fractions isolated from guinea pig stomach smooth muscle treated with phospholipase C. A, Mg^{2+} -ATPase; B, Na^+ , K^+ -ATPase. Data are expressed as mean value from 3 to 6 separate experiments.

Table 2. Calcium uptake in the absence and the presence of ATP by microsomal fractions isolated from PLC treated and untreated stomach smooth muscle. The number of experiments are shown in the parentheses. Data are expressed as Mean $\pm$ S.E.

	Calcium uptake ($\mu\text{mol/g/10 min}$)	
	No ATP added	5 mM ATP added
Control	1.7 $\pm$ 0.2 (6)	13.5 $\pm$ 3.0 (6)
PLC, 30 min	1.5 $\pm$ 0.1 (2)	10.1 $\pm$ 2.7 (3)
PLC, 60 min	1.3 $\pm$ 0.3 (6)	7.1 $\pm$ 1.4* (6)

*: $P < 0.05$

Fig. 2. Calcium concentration dependence of Mg^{2+} -ATPase activity in microsomal fractions isolated from PLC treated stomach smooth muscle. Data are expressed as mean values from 4 to 5 separate experiments.

fractions was decreased by PLC treatment. However, this depressant effect was statistically significant only in the presence of ATP after 60 min treatment with PLC.

Effect of phospholipids on Ca^{2+} , Mg^{2+} -ATPase.

It has been reported that PLC hydrolyzes sphingomyeline, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Rosenberg & Condrea, 1968). The latter two phospholipids were the major components observed in membrane phospholipids of guinea pig stomach smooth muscle in the pyloric region (Sakai, 1980). To test the hypothesis that chemical modification of these phospholipids is related to microsome ATPase activity, the effects of exogenously added PC and PE on Ca^{2+} , Mg^{2+} -ATPase was investigated. Figure 3A shows that PC was able to effectively restore the ATPase activity of microsomal fractions which has been treated with PLC for 30 min, but had only small effect on fractions treated for 60 min. PE, on the other hand, did not significantly affect the ATPase activity of microsomal fractions isolated from treated muscle, but slightly inhibited the ATPase activity of the control tissue at concentrations higher than $37 \mu\text{g/ml}$ (Figure 3B). This effect of PE has also been reported by others (Martonosi *et al.*, 1968; Goldman & Albers, 1973).

Discussion

Both ATPase activity and phospholipid content decreased with increased time of PLC treatment. There was positive, linear correlation between ATPase activity and phospholipid content (Fig. 1). This could indicate that ATPase activity depends on phospholipid which is available, either directly or as a secondary result of PLC treatment. PC was able to restore Ca^{2+} , Mg^{2+} -ATPase activity of microsome fractions to near the control level after 30 min of tissue exposure to PLC (Fig. 3A). PE was not able to restore the ATPase activity of microsome fractions but there appeared to be a tendency to prevent activity reduction at higher

Fig. 3. Effects of phospholipids on Ca^{2+} , Mg^{2+} -ATPase activity in microsomal fractions isolated from PLC treated stomach smooth muscle. A, phosphatidylcholine added; B, phosphatidylethanolamine added. The reaction mixture contained 0.1 mM CaCl_2 , 5 mM MgCl_2 , 100 mM KCl and 40 mM imidazole at pH 7.0. Phospholipid suspension was facilitated by supersonic treatment.

PE concentrations (Fig. 3B). This suggests that at least one PLC effect on membrane phospholipid is destructive. It also suggests that these phospholipids play a major role in ATPase activity. In skeletal and cardiac muscle microsomes, the Ca^{2+} , Mg^{2+} -ATPase has been demonstrated to be associated with Ca^{2+} transport system (Hasselbach, 1964). Wuytack and Casteels (1980) have recently shown that Ca^{2+} , Mg^{2+} -ATPase activity is also present in microsomal fraction isolated from vascular smooth muscle and may be related to the active Ca^{2+} transport system. The present work also demonstrates Ca^{2+} , Mg^{2+} -ATPase activity in stomach smooth muscle microsomes (Fig. 2). Prior treatment of tissue with PLC for 30 min significantly reduced microsomal Ca^{2+} , Mg^{2+} -ATPase activity and 60 min PLC treatment almost completely inhibited all ATPase activity. This suggests that phospholipids which serve as substrates for PLC are also essential for catalytic activity of Ca^{2+} , Mg^{2+} -ATPase. Although PLC treatment inhibited both Ca^{2+} , Mg^{2+} -ATPase and ATP supported Ca^{2+} uptake of isolated microsomal fractions, a significant amount of Ca^{2+} uptake was resistant to PLC treatment even after 60 min preincubation. It is possible, however, that the residual ATP supported Ca^{2+} uptake seen after 60 min PLC treatment might have been due to ATP dependent binding which was substantially higher than Ca^{2+} binding in the absence of ATP (Kwan *et al.*, 1981), since binding of Ca^{2+} by microsomal fractions was slightly but not significantly affected by PLC treatment (Table 2).

The decreased Na^+ , K^+ -ATPase activity may be related to our previous observation that PLC treatment of stomach smooth muscle caused membrane depolarization and inhibition of slow potential generation (Sakai, 1980). The decrease in Ca^{2+} , Mg^{2+} -ATPase activity and ATP supported Ca^{2+} uptake after PLC treatment also suggests that disturbance of Ca^{2+} movement in the smooth muscle cell may also be involved.

Although a heterogenous microsomal fraction was used in this study, the biochemical

changes induced by PLC treatment is likely to be associated with plasma membrane because (a) PLC was applied exogenously to intact smooth muscle and subsequently removed prior to homogenization, (b) Na^+ , K^+ -ATPase and basal Mg^{2+} -ATPase have been shown to be associated with smooth muscle plasma membrane (Hurwitz *et al.*, 1973; Kwan & Ramlal, 1982) and (c) most smooth muscles have sparse content of endoplasmic reticulum (Devine *et al.*, 1972) and the distribution of ATP supported Ca^{2+} uptake parallels that of plasma membrane markers in isolated subcellular membrane fractions from various types of smooth muscles (Janis *et al.*, 1977; Grover *et al.*, 1980; Kwan *et al.*, 1981) including stomach smooth muscles (Sakai *et al.*, 1981; Kawn *et al.*, 1982). Our present study shows that PLC is a useful tool for study of the functional role of membrane phospholipids in membrane associated enzymes and ion transport systems and the results are consistent with our previous report that PLC treatment causes smooth muscle cell membrane damage leading to inhibition of smooth muscle activity.

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