Vol. 99, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 31, 1981 Pages 737-744

CALMODULIN STIMULATION OF CALCIUM UPTAKE AND (Ca^{2+}-Mg^{2+})-ATPASE ACTIVITIES IN MICROSOMES FROM CANINE TRACHEAL SMOOTH MUSCLE

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Received January 9,1981

SUMMARY: The role of calmodulin in the regulation of microsomal $^{45}Ca^{2+}$ transport in canine tracheal smooth muscle was studied. Calmodulin stimulated ATP-dependent $^{45}Ca^{2+}$ uptake and (Ca^{2+}-Mg^{2+})-ATPase activities in microsomes treated with 0.5 mM EDTA and 0.5 mM EGTA. Oxalate also stimulated ATP-dependent $^{45}Ca^{2+}$ uptake and (Ca^{2+}-Mg^{2+})-ATPase activities and the stimulation was additive to the effects of calmodulin. The (Ca^{2+}-Mg^{2+})-ATPase and ATP-dependent $^{45}Ca^{2+}$ uptake activities are probably related as they exhibited similar $[Ca^{2+}]_{free}$- and [calmodulin]-dependencies. These results indicate that calmodulin may play a role in the control of the cytosolic $[Ca^{2+}]_{free}$ in canine tracheal smooth muscle.

The regulation of the cytosolic $[Ca^{2+}]_{free}$ is important in the control of contractile and metabolic processes in many tissues (1). In skeletal (2), cardiac (3), and smooth (4) muscles, and in erythrocyte membranes (5), ATP-dependent Ca^{2+} transport by various organelles maintains the $[Ca^{2+}]_{free}$ below 10 nM.

Recent evidence indicates that a heat stable, Ca^{2+}-binding protein, calmodulin, mediates the effects of Ca^{2+} on many intracellular enzyme systems and cellular functions (6). Among these are the stimulation by calmodulin of ATP-dependent Ca^{2+} transport in human erythrocytes (7) and canine sarcoplasmic reticulum (8), as well as human erythrocyte (Ca^{2+}-Mg^{2+})-ATPase (9).

In smooth muscle, calmodulin is involved in the Ca^{2+}-dependent regulation of the contractile process through its interactions with myosin light chain kinase (10). Essential to this process is the control of the cytosolic

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2. Abbreviations used: ECTA, ethylene glycol bis (β-aminoethyl ether) N,N-tetraacetic acid; SI, 250 mM sucrose + 10 mM imidazole, pH 7.0 (4°C); gww, gram wet weight of tissue.

0006-291X/81/060737-08$01.00/0

$[Ca^{2+}]_{free}$ by energy-dependent Ca^{2+} transport. Smooth muscle Ca^{2+} transport has been characterized (4). However, only recently has a (Ca^{2+}-Mg^{2+})-ATPase been demonstrated to be related to Ca^{2+} transport in smooth muscle (11). As the stimulation of smooth muscle microsomal Ca^{2+} transport by a cytosolic factor (12) has been reported, it was important to investigate the interactions of calmodulin with microsomal Ca^{2+} transport and its related (Ca^{2+}-Mg^{2+})-ATPase. In the present study we demonstrate the existence of a Ca^{2+}-transport ATPase in tracheal smooth muscle which is stimulated directly by calmodulin.

METHODS: $^{45}CaCl_2$ was purchased from New England Nuclear. MgATP and ouabain were purchased from Sigma Chemical Co. Ro 2-2985/1 (the sodium salt of X537A) was a generous gift of Hoffman-La Roche, Inc.

Tracheal smooth muscle was obtained from male mongrel dogs anesthesized with sodium pentobarbital (40 mg/kg i.v.). The trachea was excised and immersed in a cold modified Krebs-Hensleit solution of the following composition (mM): NaCl, 113; KCl, 4.8; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 5.5; gassed 95% O_2-5% CO_2; pH 7.4.

The homogenization and centrifugation scheme used was a modification of previously described methods (12) and was carried out at 4°C. The trachealis muscle was minced with a McIlwain tissue chopper. The mince was blotted on filter paper, weighed, and placed in 10 ml SI/gww^2. The mince was Polytron-treated at low speed for 15 s and hand-homogenized in a Duall teflon-in-glass homogenizer. The homogenate was filtered through 4 layers of cotton gauze. The filtrate was saved and the filter residue placed in 5 ml SI/gww, homogenized as above, filtered, and the filtrates combined. The combined filtrate was centrifuged at 1,300g x 10 min. The supernatant was saved and the pellet resuspended in 5 ml SI/gww and centrifuged at 1,300g x 10 min. The 1,300g supernatants were combined and the pellet discarded. The 1,300g supernatant was centrifuged at 10,000g x 30 min and the resulting supernatant centrifuged at 27,000g x 10 min. The supernatant was centrifuged at 105,000g x 60 min. The pellet was resuspended in 2.5 ml SI + 0.5 mM EDTA + 0.5 mM EGTA/gww and centrifuged at 105,000g x 60 min. The pellet was resuspended in 2.5 ml SI/gww, frozen, and stored for use.

Calmodulin was purified from rat testis (13). The protein was assayed by its ability to stimulate rat brain calmodulin-deficient phosphodiesterase.

The calcium uptake assay was modified from the method described by Janis, et al. (4). The uptake media contained 40 mM imidazole·HCl, pH 7.0 (37°C), 100 mM KCl, 0.1 mM ouabain, 5 mM KN_3, 0.125 mM $CaCl_2$, 5 µCi $^{45}CaCl_2$/ml, 25 µg protein/ml, and where appropriate, 4 mM MgATP, 2 mM $MgCl_2$, and 5 mM oxalate in a total volume of 300 µl. Calcium uptake was begun with the addition of protein and terminated by filtering 200 µl of the assay media through a Millipore filter. The filtered microsomes were immediately washed with 10 ml of 250 mM sucrose + 40 mM imidazole·HCl, pH 7.0 (37°C). The filters were dissolved in 1 ml of methyl cellosolve. 10 ml of scintillation fluid was added and the solutions were counted for radioactivity.

ATP-dependent $^{45}Ca^{2+}$ uptake (Ca^{2+} uptake) is defined as the amount of $^{45}Ca^{2+}$ that is accumulated by microsomal vesicles in an energy-dependent manner. ATP-independent $^{45}Ca^{2+}$ binding (Ca^{2+} binding) is defined as an ATP-independent association between $^{45}Ca^{2+}$ and microsomal membrane sites.

ATPase activity was measured in the presence of 40 mM imidazole·HCl, pH 7.0 (37°C), 100 mM KCl, 0.1 mM ouabain, 5 mM KN_3, 0.125 mM $CaCl_2$, 2 mM $MgCl_2$, 4 mM MgATP, 25 µg protein/ml, and where appropriate, 1 mM EGTA, in a total volume of 200 µl. The reaction was started with MgATP and terminated by heating at 100°C for 1 min in a heat block. The reaction tubes were cooled in ice and 1 ml of ice-cold distilled water was added. Approximately 50 mg of activated charcoal was added, the tubes were vortexed and then centrifuged to sediment the charcoal. The supernatant was removed and the inorganic phosphate determined by the method of Chen et al. (14). Basal Mg^{2+}-dependent ATPase was determined in the presence of 1 mM EGTA.

The $[Ca^{2+}]_{free}$ was buffered with the use of EGTA. The [EGTA] required to produce the desired $[Ca^{2+}]_{free}$ were calculated using the equations described by Katz et al. (15) and the stability constants of Godt (16).

Protein was assayed, with slight modification, by the method of Bradford (17) using bovine serum albumin as standard.

The data are presented as means ± S.E. and N is the number of separate experiments. Statistical evaluation was made by the Students t-test for paired samples. The 0.05 level of probability was accepted as significant.

RESULTS: The time-course of ATP-dependent Ca^{2+} uptake and ATP-independent Ca^{2+} binding was studied. Ca^{2+} uptake was found to be energy-dependent. The ionophore X537A (10 µM) decreased Ca^{2+} uptake to 20% of control levels (results not shown, N = 2) indicating the accumulation of Ca^{2+} against an ion gradient (18). X537A (10 µM) also stimulated the (Ca^{2+}-Mg^{2+})-ATPase activity (results not shown, N = 2) as previously observed in skeltal muscle (18). Similar effects of X537A on Ca^{2+} uptake and (Ca^{2+}-Mg^{2+})-ATPase activities have been observed in other smooth muscle preparations (4, 11).

As seen in Figure 1, oxalate and calmodulin significantly increased total Ca^{2+} uptake at all time points. The effect of oxalate and calmodulin together was additive at 30 min to that of calmodulin alone or oxalate alone. Calmodulin alone, oxalate alone, or calmodulin and oxalate together had no effect on Ca^{2+} binding.

Figure 2 demonstrates the effects of calmodulin and oxalate on the time-course of Mg^{2+}-ATPase and (Ca^{2+}-Mg^{2+})-ATPase activities. Ca^{2+} (10 µM)

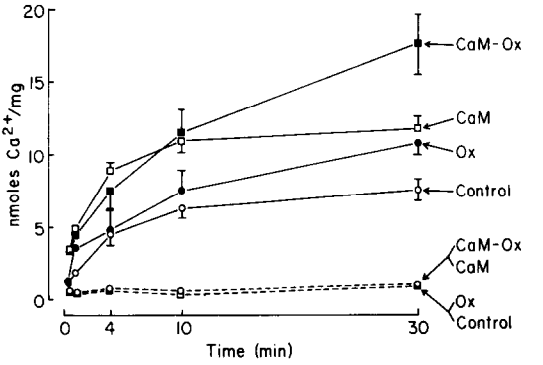


Figure 1. The effect of calmodulin (CaM; 150 nM) and oxalate (Ox; 5 mM) on the time-course of calcium uptake. Dashed lines: the effect of oxalate (□), calmodulin (○), and oxalate and calmodulin (○) on control (□) calcium binding. Solid lines: control (○) total calcium uptake and the effects of oxalate (●), calmodulin (□), and oxalate and calmodulin (■). $[Ca^{2+}]_{free}$ = 10 μM. N = 4 for all figures.

slightly, but significantly stimulated Mg^{2+}-ATPase activities at 10 and 30 min. Oxalate alone and calmodulin alone significantly increased the (Ca^{2+}-Mg^{2+})-ATPase activities at all time points but had no effect on the Mg^{2+}-ATPase activity. The effects of oxalate and calmodulin together were pronounced and additive at all time points.

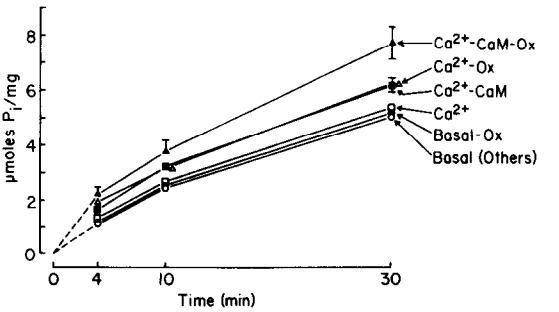
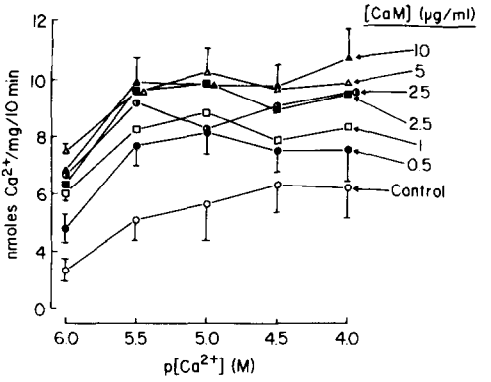
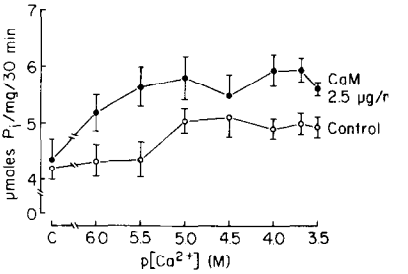


Figure 2. The effect of oxalate (Ox; 5 mM) and calmodulin (CaM; 150 nM) on the time-course of Mg^{2+}-ATPase and (Ca^{2+}-Mg^{2+})-ATPase activities. Calcium (□) significantly stimulated Mg^{2+}-ATPase activities (○) at 10 and 30 min. Calmodulin alone (■) and oxalate alone (△) significantly stimulated (Ca^{2+}-Mg^{2+})-ATPase activities at all time points but had no effect on Mg^{2+}-ATPase activities (●,○). Calmodulin and oxalate together (▲) significantly stimulated (Ca^{2+}-Mg^{2+})-ATPase activities above calmodulin alone and oxalate alone at all time points but had no effect on Mg^{2+}-ATPase activities (○).



(3)



(4)

Figure 3. The concentration-dependent stimulation by calmodulin of total calcium uptake at different $[Ca^{2+}]_{free}$. As compared to control (○), 30 nM (●), 60 nM (□), 150 nM (■), 300 nM (△), 600 nM (▲), and 1500 nM (◐) calmodulin significantly stimulated total Ca^{2+} uptake at all $[Ca^{2+}]_{free}$.

Figure 4. The effect of calmodulin (CaM; 150 nM) on $(Ca^{2+}$-$Mg^{2+})$-ATPase and Mg^{2+}-ATPase activities. C on abscissa: Mg^{2+}-ATPase activities in the absence (○) and presence (●) of calmodulin. At all free Ca^{2+} concentrations (○, control), calmodulin (●) significantly stimulated the ATPase activity.

The total Ca^{2+} uptake increased as the $[Ca^{2+}]_{free}$ was increased from 1 to 100 µM (Fig 3). At each $[Ca^{2+}]_{free}$, calmodulin significantly increased the total Ca^{2+} uptake activity with the maximal effect occurring at a calmodulin concentration of 300 - 600 nM.

Ca^{2+} produced a concentration-dependent stimulation of the Mg^{2+}-ATPase activity that was increased further by calmodulin at all $[Ca^{2+}]_{free}$ used (Fig 4). The calmodulin concentration used here produced near-maximal stimulation of Ca^{2+} uptake activity (see Fig 3).

Total Ca^{2+} uptake and $(Ca^{2+}$-$Mg^{2+})$-ATPase activities were significantly stimulated in a [calmodulin]-dependent manner at all $[Ca^{2+}]_{free}$ used (Figs 5 and 6). At each $[Ca^{2+}]_{free}$, maximal stimulation of total Ca^{2+} uptake and $(Ca^{2+}$-$Mg^{2+})$-ATPase activities occurred at a [calmodulin] of 300 - 600 nM.

DISCUSSION: The present findings indicate that calmodulin, at physiological concentrations (19), significantly stimulates Ca^{2+} uptake and $(Ca^{2+}$-$Mg^{2+})$-ATPase activities in canine tracheal smooth muscle microsomes. The Ca^{2+} uptake and

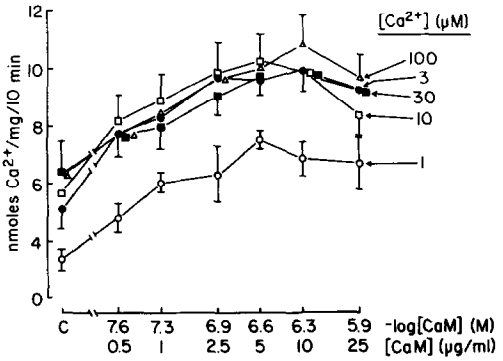


Figure 5. The [calmodulin]-dependent stimulation of total Ca^{2+} uptake at various free calcium concentrations. C on abscissa: control total Ca^{2+} uptake at 1 (O), 3 (●), 10 (□), 30 (■), and 100 μM (△) $[Ca^{2+}]_{free}$.

(Ca^{2+}-Mg^{2+})-ATPase activities appear to be related as exemplified by 1) similar $[Ca^{2+}]_{free}$- and [calmodulin]-dependencies (Figs 3-6), 2) similar effects of oxalate (Figs 1, 2), 3) the effect of the ionophore X537A, and 4) the calmodulin-stimulated increase in the affinity for Ca^{2+} and Vmax of both activities. The results provide direct biochemical evidence that tracheal smooth muscle microsomes contain an ATP-driven Ca^{2+} transport system that is directly stimulated by calmodulin and which may be involved in the maintenance of the myoplasmic $[Ca^{2+}]_{free}$ below 10 nM.

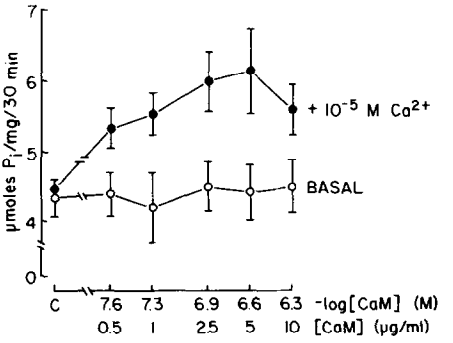


Figure 6. The [calmodulin]-dependent stimulation of (Ca^{2+}-Mg^{2+}) ATPase activities. C on abscissa: Control Mg^{2+}-ATPase (O) and (Ca^{2+}-Mg^{2+})-ATPase (●) activities measured in the absence of calmodulin (CaM). In the presence of 10 μM $[Ca^{2+}]_{free}$ (●), calmodulin significantly stimulated the (Ca^{2+}-Mg^{2+})-ATPase activity at all concentrations.

The regulation of the intracellular free [Ca^{2+}] is important in the control of the contractile state of tracheal smooth muscle. Upon excitation of the muscle, the myoplasmic free [Ca^{2+}] rises to 1-10 μM and contraction of the smooth muscle ensues. Calmodulin has recently been determined to directly mediate Ca^{2+}-dependent contractile processes in smooth muscle (10). In the present study, the results indicate that calmodulin directly stimulates the activity of a microsomal Ca^{2+}-transport ATPase. The findings suggest that, in addition to the initiation of contraction, Ca^{2+}·calmodulin interactions may regulate the rate and degree of smooth muscle relaxation by stimulating the removal of Ca^{2+} from the myoplasm and facilitating the dissociation of Ca^{2+} from the contractile proteins.

ACKNOWLEDGEMENTS: We are grateful to Dr. Fred Butcher for providing equipment and facilities for the isolation and purification of calmodulin and to Dr. K. Ku for providing calmodulin-deficient phosphodiesterase. This investigation was supported in part by NIGMS 5 T32 GM 07039.

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