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Effect of Calcium Entry Blockers on the Calcium Transport in the Isolated Sarcolemmal membrane from the Porcine Small Intestine

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ABSTRACT

There are some evidence for the presence of more than one type of calcium channels. To investigate whether organic calcium antagonist sensitive calcium channels exist in the isolated sarcolemmal membrane, we prepared high KCl-loaded sarcolemmal vesicle from the procine small instine, and induced calcium transport by high K^+ concentration or by electrical stimulation after preincubation of KCl-loaded vesicle in the low potassium solution.

Calcium transport induced by high K^+ concentration (84.7mM) was significantly increased ($p<0.05$), compared with that by low K^+ concentration (2.08 mM), and not inhibited by diltiazem (10^{-6}M). Calcium transport was inactivated with time.

By continuous electrical stimulation (3V, 15Hz, 25m sec), calcium transport was markedly increased, and inhibited significantly by dilltiazem (10^{-6}M) and nifedipine (10^{-6}M) ($p<0.005$), compared with the value of control without electrical stimulation. Calcium transport by electrical stimulation was not inactivated with time for at least 2 min.

From these results, it was concluded that there was organic calcium antagonist sensitive channel in the isolated intestinal sarcolemma membrane, which was activated by electrical stimulation.

Key Words: Sarcolemma, calcium channel, calcium transport, calcium channel antagonist

INTRODUCTION

All cells maintain high gradient of free calcium ions across the cell membrane, and for the contraction of cardiac and smooth muscle, it may be necessary to increase the transmembrane influx of extracellular calcium ions (Bolton, 1979; Colis and Shepherd, 1979; Reuter and Beeler, 1969; Rosenberger *et al.*, 1979).

By this time, it has been known that there are two types of slow channels regulating the transmembrane influx of calcium ions; 1) a potential dependent calcium channel, activated by membrane depolarization, 2) a receptor-operated calcium channel, activated by specific agonist-receptor interaction. Calcium transports through these slow calcium channels are influenced by calcium antagonist or agonist (Bolton, 1979; Rosenberg *et al.*, 1979; Schramm *et al.*, 1983), neurotransmitters and cyclic AMP dependent-phosphorylation (Osterrieder *et al.*, 1982; Sperelakis, 1984; Sperelakis and Schneider 1976).

Binding of ^{3}H-nitrendipine to membranes from cardiac or smooth muscle was found to be specific, and nifedipine analogs inhibit ^{3}H-nitrendipine binding to membranes. The potencies of nifedipine analogs for inhibition of K^+-induced contraction are correlated those for inhibition of ^{3}H-nitrendipine binding (Bolger *et al.*, 1983; Depover *et al.*, 1982; Janis and Triggle, 1983; Schwartz and Triggle, 1984). Toll (1982) also reported that potassium induced calcium uptake in a clonal cell line was inhibited by

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calcium antagonist with approximately the same potencies as those needed to inhibit ^{3}H-nitrendipine binding to membranes. Thus, it is now widely believed that the transmembrane calcium influx is associated with slow calcium channels which are inhibited by organic calcium antagonists.

Futhermore, in the isolated cardiac sarcolemmal membrane, Rinaldi *et al.*, (1982) reported that K^+-induced calcium uptake was inhibited by verapamil and the function of this channel might be modulated by cycle AMP dependent-phosphorylation. However, several reports failed to show inhibition or enhancement of calcium transport by calcium entry blockers or by phosphorylation of membrane (Flockerzi, 1983; Seok and Lee, 1985).

In an attempt to find a calcium transport pathway which is inhibited by organic calcium entry blocker in the isolated sarcolemmal membrane, we prepared high KCl-loaded sarcolemmal vesicles from porcine small intestine, and tried to induce calcium transport by high K^+ concentration or electrical stimulation.

The present study reports that calcium transports induced by electrical stimulation were inhibited with the treatment of diltiazem or nifedipine, but those by high K^+ concentration were not inhibited.

MATERIALS AND METHODS

Isolation of the sarcolemma preparation

A vesicular membrane preparation was isolated from porcine small intestine by a slight modification of the method described by Van Alstyne *et al.*, (1980). Porcine small intestine was obtained in ice box from slaughterhouse as soon as killed.

Tissue (50g) was cut and minced with scissors in medium A (containing 10 mM $NaCHO_3$, 5 mM NaN_3 and 0.25 M sucrose at a pH of 7.4). The minced tissue was suspended in 4 volumes of medium A and homogenized with Omni-mixer (2 times for 5 sec with 60% maximum speed). The homogenate was passed with motor driven glass teflon pestle and centrifuged for 20 min at 8700g. The pellet obtained was suspended in 4 volumes of medium B containing 5 mM Ethylene Glycol bis-N,N,N,N-Tetra acetic acid (EGTA), 150 mM KCl, 10 mM (N-Morpholino) Propane Sulfonic acid (MOPS)/Tris at a pH of 7.2.

Microsomal and sarcolemmal membrane fraction were obtained as the same of Van Alstyne *et al.*, and suspended in medium B without EGTA.

All of the above procedures were carried out at 4°C. Protein concentration was determined according to the method described by Lowry *et al.*, (1951) with bovine serum albumin as standard. The average yield of sarcolemmal membrane from 50g of tissue was about 4 mg.

Enzyme activity (Na^+, K^+-ATPase) of membranes of homogenate and sarcolemma was determined by the method described by Schwartz *et al.*, (1969) to see membrane properties.

Effect of KCl on the ^{45}Ca-transport

The polarized membrane or the depolarized membrane of sarcolemma was produced by the method of Bartschat *et al.*, (1980).

The sarcolemma loaded with high potassium solution (5 mM EGTA, 150 mM KCl and 10 mM MOPS/Tris, pH 7.2) was preincubated in low potassium solution (153 mM NaCl, 2 mM KCl, 5 mM Tris-base or Tris-HCl, pH 7.4) to produce the polarized membrane for 10 min and reacted with ^{45}Ca in low potassium solution for a given time at 37°C (^{45}Ca-transport in polarized state of membrane) (*Na-Na*).

In order to examine the ^{45}Ca-transport by high KCl concentration, the polarized membrane with preincubation (10 min) in low potassium solution was reacted with ^{45}Ca in high potassium solution (155 mM KCl, 5 mM Tris-base or Tris-HCl, pH 7.4) for given time at 37°C (^{45}Ca-transport in depolarized state of membrane) (Na-K).

Effect of electrical stimulation

The sarcolemma was incubated and induced ^{45}Ca-transport in low potassium solution containing 138 mM NaCl, 10 mM $NaCHO_3$, 3 mM KH_2PO_4, 1 mM $MgSO_4$, 5.5 mM glucose and 0.1 mM $^{45}CaI_2$, pH 7.4, for 5.5, 6, 7 min at 37°C (^{45}Ca-transport of control).

To examine the electrical stimulation, sarcolemml suspension in low potassium solution was stimulated with electrical stimulation (3V, 15Hz, 25m sec) for a given period after preincubation of sarcolemma in same solution without electrical stimulation for 5 min.

To test the effect of calcium channel blockers on the ^{45}Ca-transport, nifedipine or diltiazem (10^{-6}M) was treated in the preincubation medium.

After inducing ^{45}Ca-transport for a given time, the reaction was stopped by adding the terminating solution containing 200 mM KCl, 0, 1 mM EGTA and 5 mM MOPS/Tris, pH 7.4 at 4°C, and by filtration immediately on Gelman filter paper (0.45 μm). This paper was washed with terminating solution (4 times), and dropped in counter bottle containing the scintillation cockeail (6 ml). The transported ^{45}Ca was counted by liquid scientillation counter (Packard

Tri-CARB 300C).

Statistical significance was tested with Student's t-test. The chemicals used were $^{45}CaCl_2$ (NEN), nifedipine (Sigma), d-dis diltiazem (Sigma), EGTA (Sigma), Tris-base or Tri-HCl (Sigma), MOPS (Sigma), and sodium dedecyl sulfate (SDS) (Sigma).

RESULTS

Na^+, K^+-ATPase activities of isolated membrane

Na^+, K^+-ATPase is one of the putative sarcolemmal markers. Na^+, K^+-ATPase activity of sarcolemma is increased about 2.3 times and 4.4 times, in the absence and in the presence of SDS, respectively, compared with that of homogenate. The Na^+, K^+-ATPase activity of sarcolemma in the absence of SDS was 4.5 μm pi/mg protein/hr and it was increased to 10.5 μm pi/mg protein/hr in the presence of SDS as detergent. It means that the vesicles of right-side-out type are about 57% (Table 1).

KCl-induced ^{45}Ca-transport

^{45}Ca-transport in the depolarized state by high K^+ concentration was increased significantly as compared with that in the polarized state (Fig. 1, Table 2) ($p<0.05$). But the amount of increased ^{45}Ca-transport was inactivated slowly with time. This ^{45}Ca-transport was not blocked significantly by pretreatment of diltiazem ($10^{-6}M$) ($p>0.05$). It suggests that a channel which is not blocked by calcium channel blockers may exist in this sarcolemmal preparation.

^{45}Ca-transport by electrical stimulation

^{45}Ca-transport by electrical stimulation with 3V (15 Hz, 25 msec) for a given period of 0.5, 1, and 2 min were markedly increased (10.72 ± 3.36, 11.45 ± 3.38 and 24.38 ± 2.89 nmol/mg protein), compared with the value of control calcium transport without electrical stimulation (6.08 ± 0.48, 6.48 ± 0.67 and 6.98 ± 0.53 nmol/mg protein) (Fig. 2).

Table 1. ATPase activity of isolated intestinal homogenate and sarcolemma

Treatment	ATPase activity in the absence of SDS (μmol pi/mg protein/hr)	ATPase activity in the presence of SDS (μmol pi/mg protein/hr)
Homogenate		
Azide + EGTA	12.33	11.57
Azide + EGTA + Ouabain	10.40	9.18
Ouabain-sensitive activity	1.93	2.39
Sarcolemma		
Azide + EGTA	16.09	21.61
Azide + EGTA + Ouabain	11.59	11.11
Ouabain-sensitive activity	4.50	10.50

Right-side out type of sarcolemma is about 57% in this preparation.

Table 2. Effect of diltiazem on KCL-induced ^{45}Ca-uptake of sarcolemmal membrane

Membrane state	^{45}Ca-uptake in the absence of diltiazem (nmol/mg protein)	^{45}Ca-uptake in the presence of diltiazem (nmol/mg protein)
Polarized membrane (Na-Na)	1.17 ± 0.5	1.34 ± 0.20
Depolarized membrane (Na-K)	2.29 ± 2.37*	2.03 ± 0.37*

Diltiazem ($10^{-6}M$) was pretreated in preincubation medium for 10 min. The reaction was 30 sec. Mean value and standard error were obtained from 4 experiments. * Significantly different from corresponding value of polarized membrane ($p<0.05$).

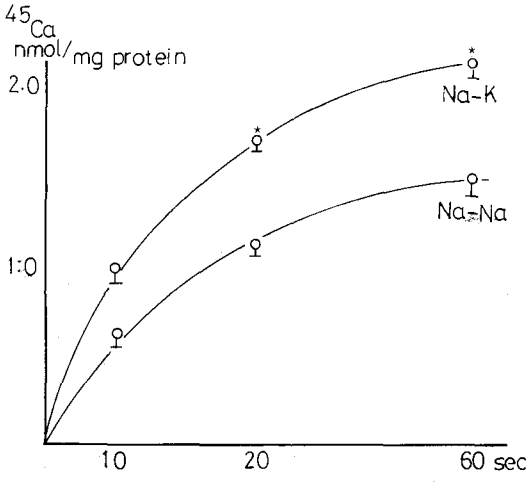

Fig. 1. Time course of ^{45}Ca-uptake in the sarcolemma vesicles of polarized or depolarized state. *; significantly different from the corresponding values of the polarized membrane ($p<0.05$). Legends are described in the method.

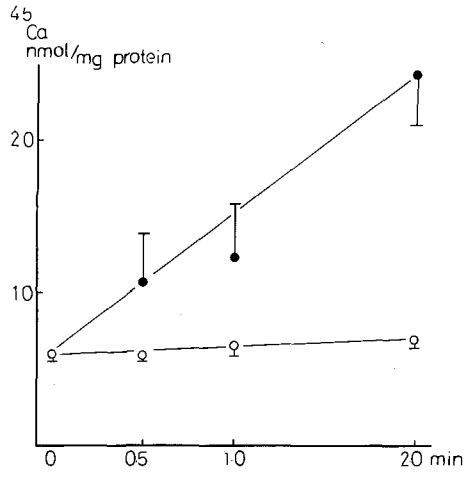

Fig. 2. Time course of ^{45}Ca-uptake of intestinal sarcolemma by the electrical stimulation. Calcium transport was induced in the absence (○) or in the presence (●) of electrical stimulation (3V, 15Hz, 25m sec) after 5 min preincubation in the low K^+ solution containing $^{45}CaCl_2$.

Table 3. The effect of calcium blockers on ^{45}Ca-uptake by electrical stimulation

	^{45}Ca-uptake in the absence of Ca-blocker (nmol/mg protein)	^{45}Ca-uptake in the presence of diltiazem (nmol/mg protein)	^{45}Ca-uptake in the presence of nifedipine (nmol/mg protein)
1) control	6.98 ± 0.53	6.87 ± 0.85	6.86 ± 0.74
2) electrical stimulation	24.38 ± 2.89	16.90 ± 0.95*	13.88 ± 2.88*

Diltiazem or nifedipine (10^{-6}M) were pretreated in preincubation medium for 5 min. The electrical stimulation was done with 3V (15Hz, 25m sec) for 2 min. Mean value and standard error were obtained from 5 experiments.
* Significantly different from the values of ^{45}Ca-uptake by electrical stimulation in the absence of Ca-blocker ($p<0.005$)

In the presence of calcium channel antagonist, ^{45}Ca-transport by electrical stimulation for 2 min were inhibited significantly; inhibited about 30.7% by diltiazem (10^{-6}M), and 43.1% by nifedipine (10^{-6}M), compared with those in the absence of calcium channel antagonist (Table 3) ($p<0.005$).

DISCUSSION

In intact cell, it is well known that the changes of calcium channel current or calcium uptake through calcium channels are modulated by calcium antagonist or agonist and phosphorylation of membrane (Osterrieder *et al.*, 1982; Reuter *et al.*, 1969; Sperelakis and Schneider, 1976; Toll, 1982).

However, in an isolated sarcolemmal membrane, it is not clear whether there are functional calcium channels which are inhibited by organic calcium antagonist. Bartschat *et al.*, (1980) reported first that potassium induced Ca*-uptake in a cardiac sarcolemma preparation is related to the depolarization of membrane and inhibited by verapamil, and Rinaldi *et al.*, (1981, 1982) reported that this depolarization-induced calcium transport might be modulated by phosphorylation of calcium channel. But Schilling and Lindenmayer (1984) showed that high potassium-induced voltage-sensitive calcium influx was not inhibited by the organic calcium antagontsts. Flockerzi *et al.*, (1983) and Seok and Lee (1985) also failed to show the inhibition or enhancement of KCl induced calcium uptake by organic calcium an-

tagonist or by cyclic AMP dependent phosphorylation in the cardiac sarcolemma preparations.

There are some evidence that some cells have sensitive and insensitive calcium channels to dihydropyridine calcium antagonists or agonist. That is, Nilius *et al.*, (1985) and Nowycky *et al.*, (1985) reported that channel of long-lasting current activated by strong depolarization is sensitive to dihydropyridine derivatives, but channel of transient current activated by weak depolarization is not sensitive in cardiac or neuronal cells.

Calcium channels undergo transitions between resting (closed, available for activation), open (active) and inactive (closed, unavailable for activation) states. Depolarization of membrane activates calcium channel to open state and open channel is changed to inactive state (Sperelakis, 1984). Sanguinetti and Kass (1984) suggested that the binding of calcium antagonist to membrane may be stronger to inactive channel than resting channel, and open channel block can occur by calcium antagonist in the presence of repetitive depolarization, due to increase rate of channel inactivation, by repetitive depolarization.

In the smooth muscle, it was reported that contraction induced by high KCl concentration or electrical stimulation was inhibited by calcium channel antagonist, and there were correlations between nitrendipine binding to membrane and inhibition of contraction by calcium antagonists (Bogler *et al.*, 1983; Karaki and Weiss, 1984).

In the result of the present study, Ca-transport in the high K^+ concentration (depolarized membrane) was increased, compared with that in low K^+ concentration (polarized membrane), but not inhibited by diltiazem and inactivated with time. These results are consistent with findings of Flockerzi *et al.*, 1983), Schilling and Lindenmayer (1984), and Seok and Lee (1985).

In contrast with these results, calcium transport in the presence of continous electrical stimulation did not inactivate with time, and was inhibited by diltiazem or nifedipine. It is difficult to interpret our results why electrical stimulation-induced Ca-transport was inhibited by calcium antagonist, but high K^+-induced calcium transport was not inhibited in the same sarcolemmal membrane. But in the dihydropyridine sensitive channel of long-lasting current, strong depolarization (10 or 20 mV) is needed to activate the calcium channel, from the holding potential of −40 mV (Nilius *et al.*, 1985; Nowycky *et al.*, 1985; Sanguinetti and Kass, 1984). It appears therefore that high K^+-induced depolarization may not be enough to activate the channels of long-lasting current, but electrical stimulation can activate these channels, and activated open channels or changed inactive channels are sensitive to organic calcium channel antagonists.

However, it is a subject of controversy whether calcium antagonist sensitive channel activated by electrical stimulation would be modulated by channel phosphorylation or other endogenous factors, or changed during membrane preparation.

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=국문초록=

돼지 소장 평활근 세포막에서의 Calcium 이동에 미치는 Calcium entry blockers의 영향

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최근 심근세포 또는 신경세포에서 발표된 여러 종류의 calcium channel중 calcium antagonist로 차단되는 channel 또는 차단되지 않는 channel 등이 있는지 알아보기 위해 실험을 시행하였다.

돼지 소장 평활근으로부터 고농도의 KCl (150mM)로 부하된 세포 포막낭을 만들어 고농도의 K^+ 또는 전기자극으로 ^{45}Ca의 이동을 유발시켜 다음과 같은 성적을 얻었다.

저농도의 K^+용액에서의 ^{45}Ca이동보다 고농도의 K^+-용액에서의 ^{45}Ca이동이 유의하게 증가되었으며($p<0.05$) 이때 유입되는 ^{45}Ca의 양은 시간에 따라 서서히 감소되었다.

전기자극(3V, 15Hz, 25msec)을 하였을때 유입되는 ^{45}Ca의 양은 전기자극을 하지 않은 대조군에 비하여 현저하게 증가되었고, 자극시간에 따른 ^{45}Ca의 유입량은 2분 동안 계속 증가되었다.

Diltiazem 또는 nifedipine을 처치하였을때, 고농도의 K^+-용액에 의한 ^{45}Ca의 유입은 억제되지 않았으나 전기자극에 의해 유도되는 ^{45}Ca의 유입은 유의하게 억제되었다($p<0.005$).

상기의 실험성적으로 돼지 소장 평활근으로부터 분리한 세포막에서의 calcium이동 중 전기자극에 의해 이루어지는 것은 calcium antagonist로 차단되는 calcium channel을 통하여 이루어지는 것으로 사료된다.