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Inositol Tetrakisphosphate Stimulates a Novel ATP-independent Ca^{2+} Uptake Mechanism in Cardiac Junctional Sarcoplasmic Reticulum

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SUMMARY. The effects of inositol phosphates on Ca^{2+} uptake in cardiac junctional sarcoplasmic reticulum (JSR) vesicles was investigated. Inositol 1,3,4,5-tetrakisphosphate (IP_4) selectively increased Ca^{2+} uptake 2.8 fold over basal levels whereas 1,3,4-IP_3, 1,4,5-IP_3 or 1,4-IP_2 were without effect. Stimulation of Ca^{2+} uptake by IP_4 was maximal within 15-60 sec at 30°C and ATP-independent. Following incubation of JSR with ruthenium red, Ca^{2+} uptake in the presence or absence of IP_4 was further enhanced 2.5 fold. Both basal and IP_4-stimulated Ca^{2+} uptake were half-maximal and maximal in the presence of 60 nM and 180 nM Ca^{2+}, respectively. These studies indicate that IP_4 stimulates an ATP-independent Ca^{2+} uptake mechanism in cardiac JSR which may function to promote rapid Ca^{2+} loading.

Introduction. In cardiac muscle, phosphoinositide-specific phospholipase C is stimulated by muscarinic and α_1-adrenergic receptor agonists to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) (1-3). 1,4,5-IP_3 stimulates Ca^{2+} release from sarcoplasmic reticulum (SR) (4-7) which may contribute to a modest increase in contractility (8,9). Furthermore, α_1-adrenergic (10,11) and muscarinic receptor (12) agonists stimulate increases in inositol 1,3,4,5-tetrakisphosphate (IP_4) the phosphorylated product of 1,4,5-IP_3. Although increases in IP_4 were suggested to be involved in sustaining the positive inotropic response associated with receptor stimulation (12), the mechanism of action was not described. Since IP_4 has been proposed to modulate Ca^{2+} fluxes in non-cardiac cells by a wide variety of mechanisms (13-16), it was considered here that IP_4 could potentially affect cardiac contractility by modulating Ca^{2+} fluxes in JSR.

MATERIALS AND METHODS

Materials. Alamethicin and ruthenium red were from Sigma Chem. Co, St. Louis MO. Myo-inositol 1,3,4,5-tetraphosphate, myo-inositol 1,4,5 triphosphate, myo-inositol 1,3,4-triphosphate

Abbreviations used: EGTA, [Ethylenebis(oxyethylenenitrilo)]tetraacetic acid ; RR, ruthenium red; 1,3,4,5-IP_4, Inositol 1,3,4,5-tetrakisphosphate.

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and myo-inositol 1,4-diphosphate were from Calbiochem, La Jolla, CA. Filters (Type HA, 0.45 μm) were from the Millipore Corp. Bedford, MA.

Preparation of Cardiac Membrane Vesicles. Mongrel dogs were anesthetized with pentobarbital and ventricular tissue was immediately immersed in cold isotonic saline and 0.1 mM EGTA. Ventricular tissue was homogenized and layered on an 8% and 22.5% step sucrose gradient and centrifuged (17). Membranes collected on the sucrose interface were washed and stored at -80°C. Protein content was determined by the Bradford method (18).

Calcium Uptake Measurements. Membrane vesicles were resealed by suspension in isotonic medium containing 1.0 mM $MgCl_2$, 20 mM HEPES, pH 7.4, 100 mM KCl, 1 mM DTT, 1 mM NaN_3, 30 mM choline Cl, 0.1 mM EGTA, 10 mM potassium oxalate, 4 μg aprotinin and 120-200 μg of membrane protein in a final volume of 1.0 ml. Ruthenium red was included in some experiments. In most studies 5 mM creatine phosphate and 3 units of creatine phosphokinase were included, although they were not required for Ca^{2+} uptake in the absence of ATP. Vesicles were incubated 10-30 min on ice to facilitate resealing. One volume (25 μl) of resealed membranes (3-5 μg of protein) was suspended in two volumes of medium yielding in final concentration 1.0 mM $MgCl_2$, 20 mM HEPES, pH 7.4, 100 mM KCl, 1 mM DTT, 1.0 mM NaN_3, 30 mM choline Cl, 0.1 mM EGTA, 3.3 mM potassium oxalate, 5 mM creatine phosphate and 3 units of creatine phosphokinase. The concentrations of ATP, inositol phosphates and $^{45}CaCl_2$ were varied as indicated. The concentration of free Ca^{2+} in the presence of an EGTA buffering system was calculated as previously described (19). Suspensions were incubated at 30°C. Reactions were stopped with 25 μl of medium containing 1 mM $LaCl_3$ and 150 mM choline Cl and tubes were kept on ice. Aliquots (60 μl) were then pipeted onto Millipore filters and rinsed 3 times with 3 ml of 150 mM choline Cl, 10 mM HEPES, pH 7.4 and 0.1 mM $LaCl_3$ under gentle vacuum. La^{3+} was included in the above media to stop Ca^{2+} fluxes after incubation and to remove externally bound $^{45}Ca^{2+}$ during rinsing (20). Filters were counted in 8 ml of Ecolume (ICN, Irvine CA). All experiments were repeated at least three times and data points were determined in duplicate or triplicate. Data points are represented as the means $\pm$ standard deviations.

RESULTS. In membrane vesicles, enriched in junctional sarcoplasmic reticulum (JSR), Ca^{2+} was significantly taken up in the absence of inositol phosphates or ATP (Fig. 1). Ca^{2+} uptake was further increased by 2.8 fold in the presence of 10 μM 1,3,4,5-IP_4. These changes were representative of Ca^{2+} accumulation rather than binding since; i) Ca^{2+} uptake was increased approximately 16 fold in the presence of 3.3 mM oxalate, ii) Ca^{2+} accumulation was reduced to less than 1 nmoles/mg in the presence of the membrane permeabilizing agent alamethicin (17) and iii) accumulated Ca^{2+} was not removed by washing with La^{3+} containing solution. The stimulation by 1,3,4,5-IP_4 was half maximal at a concentration of 2.5 μM and plateaued at approximately 7.5 μM. Other inositol phosphates, 1,4,5-IP_3, 1,3,4-IP_3 and 1,4-IP_2 had no effect on Ca^{2+} uptake over this same concentration range indicating that the stimulatory effects of IP_4 are relatively specific and that 1,3,4-IP_3, a metabolite of IP_4 was not responsible for stimulating Ca^{2+} uptake.

In JSR, ATP-independent Ca^{2+} uptake was rapid as evidenced by plateauing within 15-60 sec in the presence and absence of IP_4 (Fig. 2). ATP-independent Ca^{2+} uptake displayed high affinity for Ca^{2+} in that both basal and IP_4-stimulated Ca^{2+} uptake were half maximal at

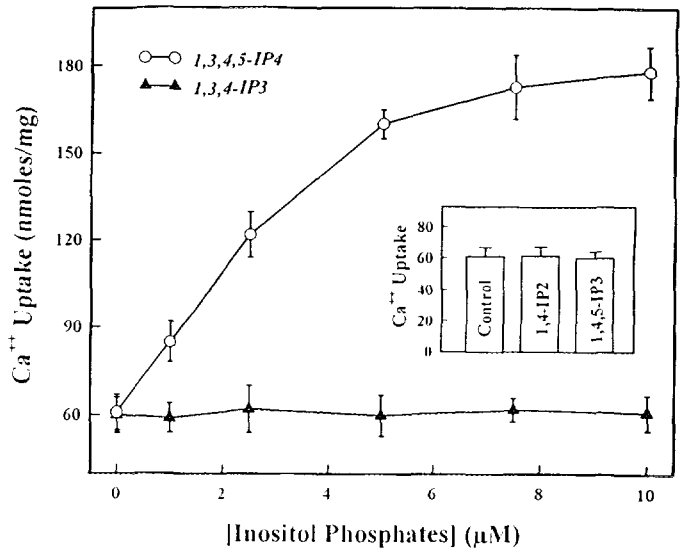

Figure 1. The effects of 1,3,4,5-IP_4 (○) and 1,3,4-IP_3 (▲) on ATP-independent Ca^{2+} uptake in junctional sarcoplasmic reticular vesicles. Inset, the effects of 10 μM concentrations of 1,4,5-IP_3 and 1,4-IP_2 in comparison to uptake in the control or absence of inositol phosphates. Uptake time was 2.5 min at 30°C and [Ca^{2+}] was 180 nM.

approximately 60 nM Ca^{2+} and maximal at 150-200 nM Ca^{2+} (Fig. 3A). The similar Ca^{2+} concentration dependencies of Ca^{2+} uptake in the presence and absence of IP_4 suggests that IP_4 acts by stimulating the basal Ca^{2+} uptake mechanism. In the presence of ruthenium red, a ryanodine channel antagonist, Ca^{2+} uptake was enhanced both in the presence and absence of

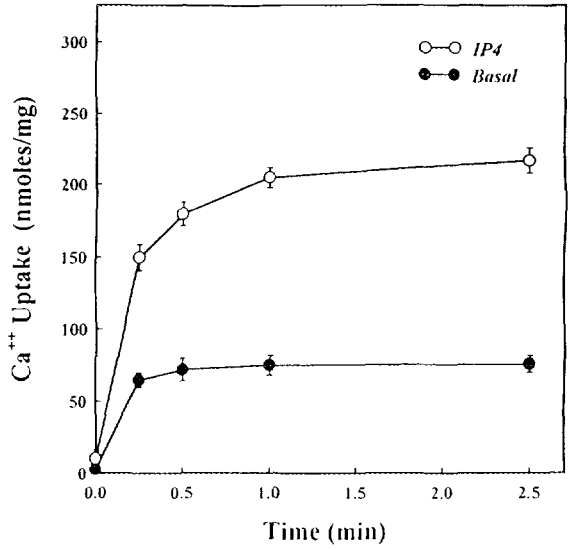

Figure 2. Time courses of Ca^{2+} uptake in the absence (●) and presence of 10 μM 1,3,4,5-IP_4 (○) at 30°C. The concentration of Ca^{2+} was 180 nM.

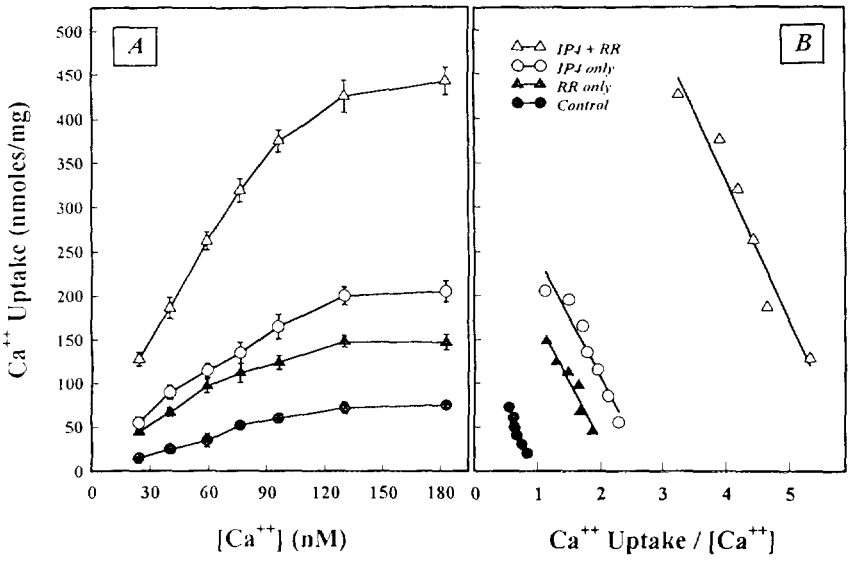

Figure 3. A. Ca^{2+} concentration dependence of basal and 1,3,4,5-IP_4-stimulated Ca^{2+} uptake in presence and absence of ruthenium red. Ca^{2+} uptake in the absence (●) and presence of 10 μM 1,3,4,5-IP_4 (○). In vesicles preincubated with 5 μM ruthenium red, Ca^{2+} uptake in the absence (▲) and presence of 1,3,4,5-IP_4 (△). Ca^{2+} uptake time was 2.5 min at 30°C.
B. A replot of data in Fig. 3A according to Eadie-Hofstee.

IP_4. The slopes of the curves were parallel under all conditions in Fig. 3B indicating that ruthenium red does not affect the Ca^{2+} affinity of the uptake mechanism. In accordance with previous suggestions (21,22), ruthenium red probably enhances Ca^{2+} uptake by blocking ryanodine channels which are in an "open" configuration in isolated JSR vesicles. Thereby, Ca^{2+} efflux through these channels would be reduced and net Ca^{2+} accumulation increased. By this mechanism, ruthenium red enhanced ATP-dependent Ca^{2+} uptake (22) and reduced Ca^{2+} efflux (23) in JSR vesicles. The ability of ruthenium red to induce an increase in IP_4-stimulated Ca^{2+} uptake also shows that IP_4 does not indirectly enhance basal Ca^{2+} uptake by blocking efflux through ryanodine channels.

To further understand the functional relationship between ATP-independent and ATP dependent Ca^{2+} uptake in JSR vesicles, the Ca^{2+} concentration dependencies of these mechanisms were compared. In the absence of ATP, Ca^{2+} uptake plateaued at 180 nM Ca^{2+} whereas Ca^{2+} uptake in the presence of ATP was greater and plateaued at higher Ca^{2+} concentrations (Fig. 4). The ATP-dependent component of Ca^{2+} uptake was half maximal at 400 nM Ca^{2+} and maximal uptake occurred at approximately 2 μM Ca^{2+}. These results for ATP-dependent Ca^{2+} uptake were in excellent agreement with studies using canine SR vesicles (24,25).

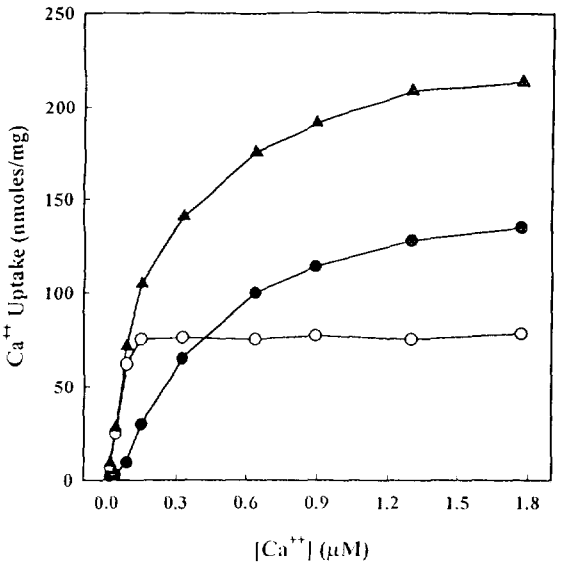

Figure 4. The Ca^{2+} concentration dependence of ATP-independent and ATP-dependent Ca^{2+} uptake in junctional sarcoplasmic reticular vesicles. Ca^{2+} uptake in the absence (○) and presence of 100 μM ATP (▲). ATP dependent Ca^{2+} uptake (●) determined by subtraction of ATP-independent Ca^{2+} uptake. Incubation time was 10 min at 30°C in the presence of an ATP regenerating system.

DISCUSSION. This investigation describes a novel ATP-independent Ca^{2+} uptake mechanism in cardiac JSR which is selectively stimulated by 1,3,4,5-IP_4. Although IP_4 had been reported to stimulate Ca^{2+} sequestering in liver endoplasmic reticulum, an ATP-dependent mechanism was proposed in that tissue (14). In cardiac JSR, the high rate of ATP-independent Ca^{2+} uptake and the high affinity for Ca^{2+} suggest that Ca^{2+} uptake occurs via a facilitated transporter.

In order for Ca^{2+} to be taken up into JSR by an ATP-independent mechanism under physiological conditions, the free cytosolic Ca^{2+} concentration would be expected to be greater than inside the JSR for at least some interval during the contraction-relaxation cycle. Under the in vitro conditions used here, the energy for ATP-independent Ca^{2+} uptake was due to the Ca^{2+} gradient established from outside to inside the vesicle. The observation that Ca^{2+} uptake was enhanced 16 fold by oxalate indicates that the Ca^{2+} gradient can be created by intravesicular Ca^{2+} chelating anions. Physiological fluctuations in the intravesicular concentrations of chelating anions such as oxalate or phosphate could therefore modulate the direction of Ca^{2+} gradients across JSR membranes. Moreover, intravesicular Ca^{2+} binding proteins could play a contributory role. An intriguing possibility is that changes in membrane potential during excitation-relaxation could cyclically modulate the charge and the Ca^{2+} chelating properties of Ca^{2+} binding proteins. It was determined that Na^+ co-transport as in the case of the Na^+/Ca^{2+} antiporter in sarcolemma (26) was not required for Ca^{2+} uptake into JSR since these studies were performed in very low

Na^+ medium. However further studies are required to determine the potential role of other ions in ATP-independent Ca^{2+} uptake. The possibility that Ca^{2+} may be compartmentalized in cardiac cytosol to enhance the Ca^{2+} gradient during some phase of the cardiac cycle could also be considered.

Because of the high Ca^{2+} affinity and rapid Ca^{2+} uptake characteristics of the ATP independent Ca^{2+} transporter in JSR, this mechanism could function to facilitate JSR loading with Ca^{2+} and simultaneously reduce cytosolic Ca^{2+}. Ca^{2+} loading by this mechanism would be anticipated to be most efficient during muscle relaxation or diastole when ryanodine channels are in a closed configuration. Thereafter the cytosolic Ca^{2+} loaded into JSR would be available for release through ryanodine channels during excitation. The presence of an ATP-independent Ca^{2+} uptake mechanism would also imply that the ATP-dependent Ca^{2+} pump is not solely responsible for loading JSR with Ca^{2+} between beats. In fact, the ATP-independent Ca^{2+} uptake mechanism may be more suited for rapid Ca^{2+} refilling of JSR on a beat to beat basis ($\leq$ 1 sec) than the ATP-dependent Ca^{2+} pump.

Stimulation of Ca^{2+} uptake into JSR by an IP_4 stimulated Ca^{2+} mechanism could also account for the sustained contractile response correlated with IP_4 production in myocardium (10,11). Accordingly, enhanced Ca^{2+} loading of JSR during diastole could increase the amount of Ca^{2+} released during excitation to elicit a stronger contraction. It is clear that the ATP independent Ca^{2+} transporter in cardiac JSR could have important physiological significance. The surprising finding that an ATP-independent mechanism may support Ca^{2+} influx into JSR would imply that Ca^{2+} movements in functioning cardiac muscle may be under dynamic control, influenced by equilibrium changes in several ions and metabolites as well as to variations in Ca^{2+} binding sites.

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