Inositol trisphosphate does not release Ca^{2+} from permeabilized cardiac myocytes and sarcoplasmic reticulum

Matthew A Movsesian*, Andrew P Thomas, Mary Selak and John R Williamson+

*Laboratory of Molecular Cardiology, National Institutes of Health, Bethesda, MD 20205, and Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

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The possibility that inositol 1,4,5-trisphosphate (IP_3) may act as a Ca^{2+}-mobilizing second messenger in cardiac muscle in a manner analogous to its actions in other cell types has been examined using saponin-permeabilized myocytes and isolated cardiac sarcoplasmic reticulum Myocytes permeabilized in the presence of $MgATP^{2-}$ sequestered Ca^{2+} to a level of about 200 nM, similar to the cytosolic free Ca^{2+} concentration of intact cells, but addition of IP_3 was ineffective in causing Ca^{2+} release from intracellular stores Similarly, IP_3 (up to 50 μM) was unable to inhibit Ca^{2+} uptake or cause Ca^{2+} release from isolated canine cardiac sarcoplasmic reticulum vesicles in the presence of either EGTA or sodium vanadate These results indicate that IP_3 is unlikely to mediate mobilization of intracellular Ca^{2+} stores in myocardial cells

Ca^{2+} uptake *Ca^{2+} release* *Cardiac myocyte* *Sarcoplasmic reticulum* *Inositol 1,4,5-trisphosphate*

1. INTRODUCTION

An increased rate of hydrolysis of phosphatidylinositol 4,5-bisphosphate has been shown to be induced by a variety of hormones or stimuli that increase the cytosolic free Ca^{2+} concentration in a large number of different cell types (reviews [1,2]). Recent experiments have shown that one of the products of phosphatidylinositol 4,5-bisphosphate hydrolysis, inositol 1,4,5-trisphosphate (IP_3), is able to release Ca^{2+} from a non-mitochondrial, ATP-dependent, intracellular calcium store in permeabilized hepatocytes and pancreatic acinar cells and to release Ca^{2+} from membrane vesicles derived from rat insulinoma endoplasmic reticulum, suggesting that IP_3 may act as a second messenger in the hormonal mobilization of intracellular Ca^{2+} [3–7].

Cardiac myocyte contraction is regulated by changes in the cytosolic free Ca^{2+} concentration, which, in turn, is controlled by sequestration and release of Ca^{2+} by the sarcoplasmic reticulum. It has been reported that administration of IP_3 to skinned frog skeletal muscle fibers elicited contractions, suggesting that it may be involved in excitation-contraction coupling by release of Ca^{2+} from the sarcoplasmic reticulum [8]. α_1-Adrenergic stimulation has been shown to have positive inotropic effects in heart [9,10] and to stimulate phosphoinositide turnover in isolated myocytes [11,12] Consequently, it was of interest to investigate whether IP_3 could act as a Ca^{2+}-mobilizing agent in permeabilized myocytes. We report here that Ca^{2+} was ineffective in eliciting Ca^{2+} release with this system, and in contrast to the findings of Hirata et al. [13] we have been unable to demonstrate any effect of IP_3 on Ca^{2+} uptake or release by isolated cardiac sarcoplasmic reticulum vesicles.

2. MATERIALS AND METHODS

Calcium-tolerant myocytes were prepared from male, Sprague-Dawley rats (250–300 g) as de-

+ To whom reprint requests should be sent

Abbreviation. IP_3, *myo*-inositol 1,4,5-trisphosphate

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scribed [14]. The cells were washed with Ca^{2+}-free Krebs bicarbonate medium and stored with oxygenation at a concentration of about 40 mg dry wt/ml. The myocytes were diluted into medium at pH 7.2 and 37°C containing 110 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $KHCO_3$, 0.3 mM $MgCl_2$, 5 mM succinate, 3 μg/ml each of rotenone and oligomycin, 20 mM Hepes, 0.2% (w/v) bovine serum albumin, 3 mM $MgATp^{2-}$, 20 mM creatine phosphate, 20 μg/ml of creatine phosphokinase and 75 μM quin2. The cells (3 mg dry wt/ml) were permeabilized by addition of 45 μg/ml of saponin and changes in quin2-Ca^{2+} fluorescence were measured using a filter fluorometer constructed by the Biomedical Instrumentation Group (University of Pennsylvania) with excitation at 339 nm and emission at 490–570 nm as described in [5]. The free Ca^{2+} concentration in the medium was calculated using a quin2-Ca^{2+} dissociation constant of 115 nM [15].

Cardiac sarcoplasmic reticulum was prepared as in [16] Oxalate supported Ca^{2+} uptake and Ca^{2+} release from cardiac sarcoplasmic reticulum were measured by filtration assay ($^{45}Ca^{2+}$) at a free Ca^{2+} concentration of 1 5 μM or by changes in arsenazo III spectral absorbance as described by Movsesian et al. [16,17]. Ca^{2+}-ATPase activity was assayed under similar conditions following the method of Pollard and Korn [18] Ca^{2+}-independent ATPase activity was measured in the presence of 2.5 mM excess EGTA relative to Ca^{2+}, and Ca^{2+}-ATPase activity was determined by subtracting the Ca^{2+}-independent from the total ATPase activity. Free Ca^{2+} concentrations were calculated according to a computer program based upon that published by Perrin and Sayce [19] using dissociation constants reported by Fabiato and Fabiato [20] and Scarpa [21]. IP_3 was prepared from human erythrocytes by the method of Downes et al. [22] and was also obtained as a gift from Dr B Agranoff (University of Michigan) using the method of Agranoff and Seguin [23].

3 RESULTS

The effect of IP_3 on free Ca^{2+} concentrations in saponin-permeabilized myocytes was examined using quin2 as a fluorescent Ca^{2+} indicator (fig.1) Permeabilization of the cells in the presence of $MgATP^{2-}$ resulted in removal of Ca^{2+} from the

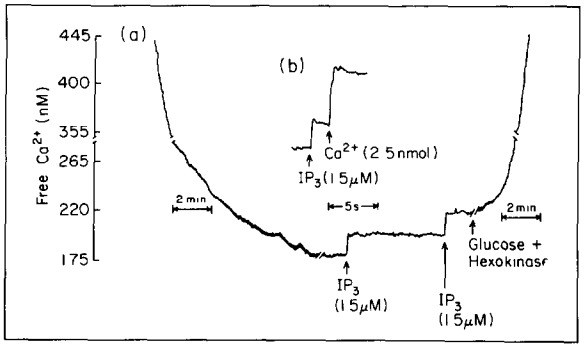


Fig.1 Lack of IP_3 effect on Ca^{2+} release from saponin-permeabilized myocytes. Myocytes were suspended in a concentration of 3 mg dry wt/ml in the medium described in section 2 and Ca^{2+} changes were assayed with quin2 Myocytes were permeabilized (as verified by trypan blue staining) by addition of 45 μg/ml of saponin, following which sequestration of Ca^{2+} occurred (trace a) Trabe b shows the effect of IP_3 addition in the same medium from which myocytes had been omitted A similar lack of effect of IP_3 addition was obtained when Ca^{2+} was monitored with a Ca^{2+} electrode

medium down to approx. 180 nM. The subsequent addition of IP_3 (1.5 μM) resulted in a small (20 nM) increase in the free Ca^{2+} concentration, which could be reproduced by a second similar addition to the permeabilized cells (fig 1a) or to incubation medium in the absence of cells (fig.1b). These results indicate that the increase in the ambient free Ca^{2+} concentration does not represent a release of Ca^{2+} from an intracellular storage site but is attributable to an artifact of IP_3 addition. Additions of glucose and hexokinase to remove ATP (fig 1a) or the Ca^{2+} ionophore ionomycin (not shown) were effective in causing Ca^{2+} release, showing that the permeabilized myocytes contained intracellular sequestered calcium. Calcium from this pool could be partially released by 20 mM caffeine but was not affected by addition of 1 mM sodium vanadate to inhibit Ca^{2+}-ATPase activity (not shown)

Further confirmation that IP_3 was unable to release Ca^{2+} from intracellular stores in cardiac myocytes was obtained by studying the effect of IP_3 on Ca^{2+} uptake and release in purified cardiac sarcoplasmic reticulum vesicles. At concentrations as high as 50 μM, IP_3 had no effect upon Ca^{2+} uptake in cardiac sarcoplasmic reticulum as deter-

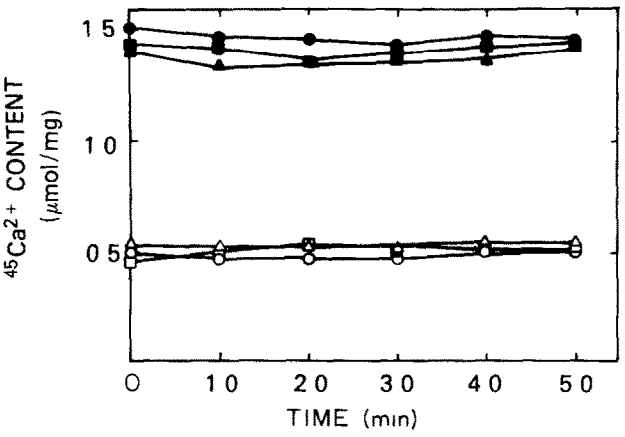
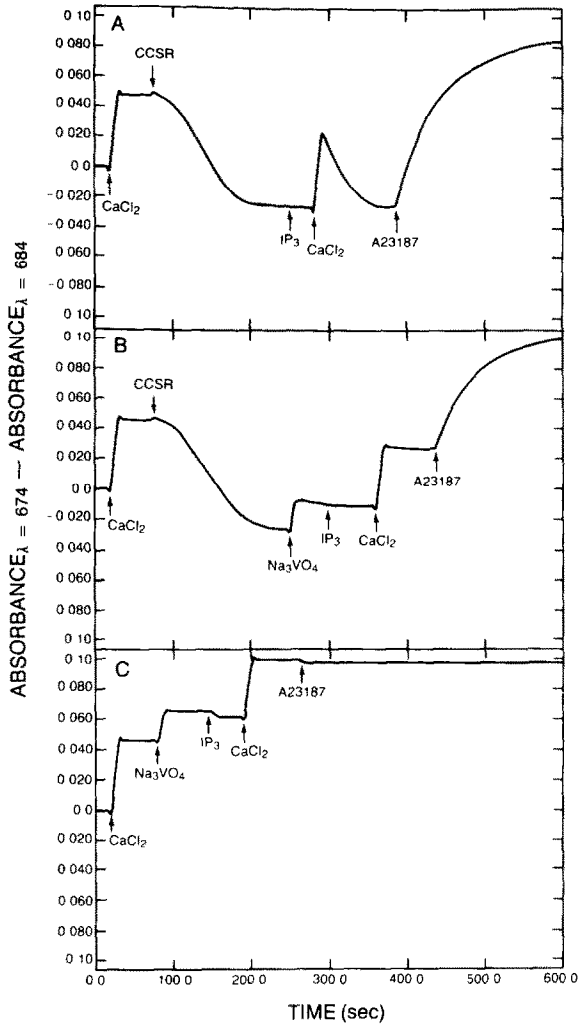


Fig 2. Effect of IP_3 on $^{45}Ca^{2+}$ release by cardiac sarcoplasmic reticulum Sarcoplasmic reticulum vesicles were loaded with $^{45}Ca^{2+}$ in the presence of 5 mM $MgCl_2$, 6 mM NaN_3, 5 mM NaF, 0.6 mM $^{45}Ca^{2+}$, 0 8 mM EGTA, 5 mM oxalic acid, 0 12 M KCl, and 40 mM Mops (pH 6.8, 27°C) for 5 min (open symbols) or 30 min (closed symbols). Ca^{2+} uptake was stopped by addition of EGTA (2 5 mM in excess of $^{45}CaCl_2$), following which IP_3 was immediately added Intravesicular $^{45}Ca^{2+}$ was determined at 1-min intervals in the absence (○, ●) or presence of 2.5 μM (△, ▲), or 50 μM (□, ■) IP_3 No difference was noted when uptake was stopped by the addition of 5 mM Na_3VO_4 rather than EGTA (not shown)

Fig 3 Effect of IP_3 on Ca^{2+} uptake and release in cardiac sarcoplasmic reticulum $CaCl_2$ was added to a final total concentration of 40 μM to a solution containing 5 mM $MgCl_2$, 5 mM oxalic acid, 5 mM NaF, 6 mM NaN_3, 0.12 M KCl, 60 μM arsenazo III, and 40 mM Mops (pH 6.8, 28°C) The free Ca^{2+} concentration was calculated to be 5 2 μM Ca^{2+} uptake was initiated by the addition of canine cardiac sarcoplasmic reticulum (CSSR) to a final concentration of 0 11 mg/ml When Ca^{2+} uptake was complete, IP_3 was added to a final concentration of 50 μM (A) Following addition of IP_3, $CaCl_2$ was again added to augment the total concentration by 40 μM, following which A23187 was added to a final concentration of 10 μM This experiment was repeated in the same manner save for the addition of 5 mM Na_3VO_4 prior to addition of IP_3 (B), Na_3VO_4 blocked by Ca^{2+} uptake but had no effect upon Ca^{2+} release. As a control, the effects of the varying additions on arsenazo III absorbance in a reaction mixture from which canine sarcoplasmic reticulum had been omitted are shown (C)

mined by filtration assay. Similarly, no release of $^{45}Ca^{2+}$ was observed when IP_3 was added to cardiac sarcoplasmic reticulum that had been loaded with $^{45}Ca^{2+}$ in the presence of oxalate with Ca^{2+} uptake subsequently terminated by the addition of EGTA (fig.2) Loading the vesicles to a higher $^{45}Ca^{2+}$ content did not overcome this unresponsiveness to IP_3. Because Ca^{2+} release from cardiac sarcoplasmic reticulum may be Ca^{2+} mediated, we repeated this experiment using sodium vanadate in place of EGTA as an inhibitor of Ca^{2+} uptake. Ca^{2+} uptake and Ca^{2+}-ATPase activity were inhibited half-maximally at 0.1 and 0 026 mM sodium vanadate, respectively. In the presence of 5 mM vanadate, however, IP_3 remained unable to



release Ca^{2+}. It may be noted that use of similar techniques with endoplasmic reticulum microsomes derived from Syrian hamster insulinoma cells showed a large and maximal release of Ca^{2+} with 0.5 μM IP_3 ([7] and unpublished).

As the filtration assay measures intravesicular Ca^{2+} content, the possibility cannot be discounted that IP_3 releases Ca^{2+} in amounts insufficient to cause a measurable change in the intravesicular Ca^{2+} content but sufficient to cause a significant increase in the extravesicular Ca^{2+} concentration. This possibility seemed particularly important in view of the high calcium content of the cardiac sarcoplasmic reticulum vesicles. For this reason, the effect of IP_3 upon Ca^{2+} uptake and release was examined using arsenazo III as a spectrophotometric indicator of the extravesicular Ca^{2+} concentration (fig.3). Addition of 50 μM IP_3 was again shown to have no effect on the release of Ca^{2+} from sarcoplasmic reticulum in either the absence (fig.3A) or presence (fig.3B) of sodium vanadate. Addition of the Ca^{2+} ionophore A23187, however, resulted in a release of Ca^{2+} from the calcium oxalate-loaded sarcoplasmic reticulum vesicles. In this system, addition of 50 μM IP_3 to medium in the absence of sarcoplasmic reticulum caused a negligible addition artifact (fig.3C).

4. DISCUSSION

The ability of externally added IP_3 to cause Ca^{2+} release from a non-mitochondrial pool of sequestered calcium after permeabilization of the plasma membrane is a property exhibited by many cell types and has been demonstrated in hepatocytes [4,5], pancreatic acinar cells [3], GH_3 pituitary cells [24], insulinoma cells [7,25], neutrophils [26], macrophages [13], Swiss 3T3 cells [27], platelets [28], and smooth muscle cells [29,30]. In each of these tissues specific agonists have been shown to cause an increased rate of hydrolysis of phosphatidylinositol 4,5-bisphosphate and formation of inositol trisphosphate [1]. Furthermore, in most of these cells the agonist has been shown to produce a characteristic increase of cytosolic free Ca^{2+} in association with the divergent functional response of the tissues. These data, therefore, have been interpreted as demonstrating a second messenger role for IP_3 in releasing intracellular Ca^{2+} by activation of a specific Ca^{2+} conductance in membranes of the endoplasmic reticular system [1,2]. It would perhaps be surprising for cardiac muscle, which has well developed voltage-sensitive Ca^{2+} channels in the plasma membrane [31], to use a similar mechanism for the release of activator calcium. Studies with quin2-loaded myocytes have in fact shown no response of the cytosolic free Ca^{2+} to phenylephrine or methoxamine when these agents were added to resting cells, although an electrical depolarization-dependent Ca^{2+} transient was readily observed [32]. Furthermore, the effect of α_1-adrenergic agents on the height of the stimulus-induced Ca^{2+} transient was negligible compared with the effect of β-adrenergic agents and BAY k 8644 ([32] and M. Selak and J.R. Williamson, unpublished).

The present studies demonstrating a lack of effect of IP_3 in causing Ca^{2+} release from permeabilized myocytes and isolated sarcoplasmic reticulum suggests that cardiac muscle cells may lack the putative IP_3 receptor in the sarcoplasmic reticulum that in other cells regulates Ca^{2+} efflux. It should also be pointed out that although α_1-adrenergic stimulation increases the hydrolysis and turnover of phosphatidylinositol in cardiac muscle [11,12] there is no evidence that IP_3 is formed, suggesting that phospholipase C has a preferential specificity for phosphatidylinositol compared with the phosphatidylinositol polyphosphates in this tissue. Hormone-stimulated phosphatidylinositol hydrolysis may be involved in the regulation of cardiac contractility through other mechanisms. The diacylglycerol product of phosphatidylinositol hydrolysis may exert signalling mechanisms that indirectly affect Ca^{2+}-transport processes through activation of protein kinase C [33]. For instance, a protein kinase C-mediated phosphorylation of phospholamban accompanied by a stimulation of Ca^{2+} sequestration by cardiac sarcoplasmic reticulum has been demonstrated [17]. The physiological significance of protein kinase C-mediated phosphorylation of phospholamban is unclear, however, since pretreatment of isolated myocytes with up to 100 ng/ml of phorbol myristate acetate failed to increase the rate of decline of the Ca^{2+} transient induced by electrical depolarization in quin2-loaded cells (M. Selak and J.R. Williamson, unpublished). These results suggest a lack of effect

of protein kinase C-mediated protein phosphorylations on Ca^{2+} sequestration by the sarcoplasmic reticulum during the Ca^{2+}-activated contraction-relaxation cycle, but further studies will be necessary with intact heart preparations to assess whether diacylglycerol production through enhanced inositol phospholipid metabolism has physiologically relevant effects on myocardial contractility. Our results indicate that IP_3 has little, if any, role in this process.

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