Inositol phosphate production following α_1-adrenergic, muscarinic or electrical stimulation in isolated rat heart

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A possible participation of polyphosphoinositide metabolism in the excitation-contraction coupling in heart was investigated. Isolated rat ventricles prelabelled with *myo*-[2-^{3}H]inositol were stimulated by conditions that increase mechanical activity. Both noradrenaline and carbachol increased the basal level of IP_3, IP_2 and IP by the activation of α_1-adrenergic and muscarinic receptors, respectively. Electrical stimulation accelerated inositol lipid degradation by phospholipase C thus enhancing the IP_3 level as compared to quiescent ventricles. It is proposed that IP_3 may be involved in excitation-contraction coupling in cardiac tissue.

(Rat heart) *Noradrenaline* *Carbachol* *Electrical stimulation* *Inositol phosphate*

1. INTRODUCTION

Several tissues respond to external stimuli by an increase in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) located at the inner face of the plasma membrane. Binding of hormones or neurotransmitters to specific receptors leads to the activation of phospholipase C (PLC) which hydrolyzes PIP_2 into inositol 1,4,5-trisphosphate (IP_3) and diglyceride [1–3]. IP_3 acts, via a coupling mechanism which probably involves a GTP-binding protein, on specific receptors [4,5] on the endoplasmic reticulum and increases its permeability to Ca^{2+}. Increase in intracellular Ca^{2+} then triggers a variety of intracellular responses (glycogenolysis, secretion, aggregation, phototransduction; review [1]). Recently, it has been shown that IP_3 is increased by short tetanic stimulation in skeletal muscle [6] and is able to release Ca^{2+} from isolated vesicles of sarcoplasmic reticulum [7,8]. Thus in skeletal muscle, IP_3 fits some criteria required for a second messenger between depolarization of the plasma membrane and release of Ca^{2+} from internal stores. In heart tissues, a few reports have shown that phosphatidylinositol or polyphosphoinositide metabolism is increased by conditions that increase mechanical activity. These conditions include α_1-adrenergic and cholinergic stimulation and K^+ depolarisation ([9,10] but see also [11]). However, the role of IP_3 as an intracellular Ca^{2+}-mobilizing agent in cardiac muscle is a matter of controversy [12,13]. The data presented here demonstrate the presence of IP_3 in heart cells and show that IP_3 content is increased by α_1-adrenergic, muscarinic and electrical stimulation.

2. MATERIALS AND METHODS

2.1. *Materials*

The hormones, noradrenaline and carbachol, and inhibitors, propranolol and atropine, were from Sigma. Prazosin was a gift from Pfizer. *myo*-[2-^{3}H]Inositol (619 GBq/mmol) was obtained from New England Nuclear. D-*myo*-[2-^{3}H]Inositol 1,4,5-trisphosphate standard was obtained from Amersham (France). Dowex IX8 ion-exchange resin (formate form, 200–400 mesh) was from Bio-Rad.

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2.2. *Tissue incubation*

Thin ventricles (about 100 mg) were quickly isolated from the heart of young female Wistar rats (160–180 g) anaesthetized with pentobarbital. The ventricles were incubated for 3 h in a small volume (30 ml for 1.2 g tissue) of a vigorously shaken modified Krebs solution containing 11 μM *myo*-[2-^{3}H]inositol (270 MBq/ml) under an atmosphere of O_2/CO_2 (19:1), pH 7.4 at 35°C. The modified Krebs solution contained (mM): NaCl, 116; KCl, 5.4; $CaCl_2$, 0.9; $MgCl_2$, 0.81; NaH_2PO_4, 0.92; $NaHCO_3$, 25, supplemented with glucose (1 g/l) and mannitol (0.4 g/l). The muscles were then rinsed twice to remove the extracellular label and preincubated for 10 min in Krebs solution containing 10 mM LiCl.

The muscles were further incubated with or without hormone for a period varying from 15 s to 2 min. In some experiments, at the end of the preincubation period, thin threads were tied to the ends of the muscle and the muscle suspended under slight torsion while being superfused with Krebs solution. The muscles were stimulated by passing current between two wires touching the muscle surface. The experiment was stopped by freeze-clamping the muscles with Wollenberger clamps. The muscles were individually ground to a fine powder and homogenized in 1 ml of 5% ice-cold perchloric acid and centrifuged (15 min at 1000 × *g*).

2.3. *Separation of [^{3}H]inositol phosphates and [^{3}H]inositol lipids*

The perchloric supernatants were neutralized, diluted and the [^{3}H]inositol phosphates separated by anion-exchange chromatography on a Dowex column [14]. Free inositol was not retained on the column. [^{3}H]Inositol phosphates were eluted sequentially with 20 ml of the eluting solution for glycerophosphoinositol, 10 ml each of the eluting solutions for IP and IP_2 and 6 ml of the eluting solution for IP_3 [15]. A 2 ml portion of each fraction was mixed with 12 ml Lumagel (Lumac) and counted for radioactivity by liquid scintillation. The [^{3}H]inositol lipids were extracted from the perchloric acid pellet [16]. After deacylation the resulting glycerophosphoryl esters were then separated as in [17].

2.4. *Identification and measurement of [^{3}H]-inositol phosphates (IP_2 and IP_3) by high-performance liquid chromatography (HPLC)*

In these experiments, after freeze-clamping 2 muscles were ground with 0.5 ml ice-cold 7% perchloric acid. After centrifugation the supernatants were neutralized with 2 N KOH, diluted with an equal volume of HPLC eluant, and centrifuged again. Samples (100 μl) were injected into a 30 cm μBondapak C18 HPLC column (Waters) and eluted at 1 ml/min, with 0.025 M tetrabutylammonium hydrogenosulfate (TBA, Merck), 0.1 M KH_2PO_4 (pH 5.5). Fractions were collected every 30 s and mixed with scintillant for ^{3}H or ^{32}P radioactivity measurement. IP_3 was identified by comparison with authentic (1,4,5)-[^{3}H]IP_3 and (1,4,5)-[^{32}P]IP_3 standards, and IP_2 by comparison with (1,4)-[^{32}P]IP_2 (fig.1). (1,4,5)-[^{32}P]IP_3 and (1,4)-[^{3}H]IP_2 were obtained from erythrocyte ghosts as described [14,18].

Results were expressed as cpm/mg wet wt or as percentage of stimulation compared to controls. Statistical analysis of the data was done by Student's *t*-test.

3. RESULTS AND DISCUSSION

We first attempted to detect the presence of [^{3}H]polyphosphoinositides and [^{3}H]inositol phosphates in heart cells. After a 3 h labelling period the incorporation of [^{3}H]inositol into inositol lipids was constant (not shown). Their relative contents expressed as % of total [^{3}H]phosphoinositides were 89.9, 4.3 and 5.8 for [^{3}H]phosphatidylinositol (PI), [^{3}H]phosphatidylinositol 4-phosphate (PIP) and PIP_2, respectively. This repartition is in agreement with literature values [19]. [^{3}H]IP_3, [^{3}H]inositol diphosphate (IP_2) and [^{3}H]inositol monophosphate (IP) were also detected. No attempt was made to separate the two isomers of IP_3 which coelute using this anion-exchange chromatography method. Their relative contents expressed as % of total [^{3}H]inositol phosphates were respectively 75.9, 16.9 and 7.1. In some experiments perchloric acid extracts obtained from labelled hearts were analyzed by HPLC. Good separation was obtained for [^{3}H]IP_2 and [^{3}H]IP_3 (fig.1). The identity of the peaks was based on comparison with standards, [^{3}H]IP_3 and

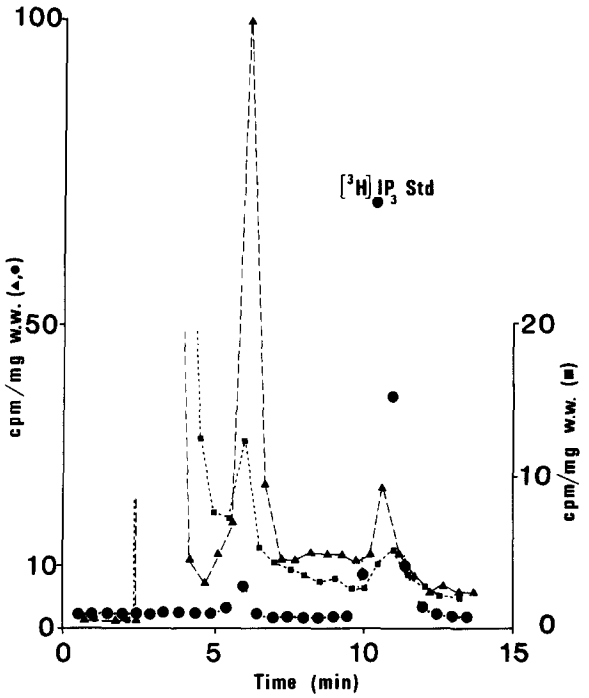

Fig.1. Analysis of inositol phosphates by HPLC. For experimental details see section 2. Inositol phosphates from ventricles pre-labelled with *myo*-[2-^{3}H]inositol stimulated (▲—▲) or not (■---■) with noradrenaline (100 μM) for 3 min were loaded onto the column. The identification of inositol phosphates is based on comparison with standards of (1,4,5)-[^{32}P]IP_3 and (1,4)-[^{32}P]IP_2, prepared as described in the text, and of (1,4,5)-[^{3}H]IP_3 purchased from Amersham. [^{3}H]IP_3 and [^{32}P]IP_3 standards (Std) were eluted from the column at 10.5 min. [^{3}H]IP_3 from controls and noradrenaline-stimulated cells was eluted at 10.7 and 10.5 min respectively. [^{32}P]IP_2 standard was eluted at 7 min (not shown). [^{3}H]IP_2 from controls and stimulated cells was eluted at 6 and 6.1 min respectively. The approximate elution of [^{3}H]IP is marked.

[^{32}P]IP_2 prepared from ^{32}P-labelled red cell ghosts incubated with Mg^{2+} and [^{3}H]inositol 1,4,5-trisphosphate from Amersham. This HPLC method has never been described as separating inositol phosphates. It employes an ion pair chromatography system with isocratic elution and permits identification of IP_3 and IP_2 and measurement of their radioactivity directly on perchloric acid extracts in less than 20 min per sample.

This constitutes the first report on the presence of IP_3 in heart cells, indicating that even under these particular conditions (quiescent ventricles) there is an active metabolism of the polyphosphoinositides since [^{3}H]inositol is incorporated into PI which is then phosphorylated into PIP and PIP_2 and [^{3}H]PIP_2 is then hydrolysed to form [^{3}H]IP_3. It seems likely that [^{3}H]IP_2 and [^{3}H]IP result from the hydrolysis of IP_3 by a phosphomonoesterase activated by the internal Mg^{2+} [20,21].

Fig.2 illustrates the time course of the effects of noradrenaline at 5 μM a half-maximal dose [9], in the presence of 10 μM propranolol and 10 μM atropine, on [^{3}H]IP_3, [^{3}H]IP_2 and [^{3}H]IP levels (A) and [^{3}H]PIP_2, [^{3}H]PIP and [^{3}H]PI levels (B). The addition of noradrenaline increased [^{3}H]IP_3 content to 144 ± 12% (n = 5) and 189 ± 12% (n = 4) of controls after 15 s and 2 min, respectively. [^{3}H]IP_2 accumulated more slowly than [^{3}H]IP_3; no statistically significant increase was observed before 30 s of application of the α_1-agonist. However, after 2 min [^{3}H]IP_2 content rose to 218 ± 32% (n = 4) of controls. The [^{3}H]IP increase was even more delayed, reaching 228 ± 49% (n = 4) of controls after 2 min. A 10 min preincubation of the ventricles with prazosin (0.1 μM) inhibited the noradrenaline effect. Since the effect was observed in the presence of the β-blocker propranolol and was blocked by prazosin, this indicates that these effects are specifically mediated through α_1-receptor activation. In two experiments performed in duplicate, the perchloric acid extract of labelled ventricles incubated for 3 min with noradrenaline 100 μM, a maximal dose [9], was analyzed by HPLC (fig.1). Comparison of the elution profiles of controls and hormone-stimulated cells confirms the large accumulation of both [^{3}H]IP_2 and [^{3}H]IP_3 which increased by 1.8- and 3.1-fold, respectively. Simultaneously, noradrenaline (5 μM) elicited a decrease of [^{3}H]PIP_2 whose content diminished to 57 ± 14% (n = 4) of controls after 15 s and then tended to increase. [^{3}H]PIP and [^{3}H]PI were both lowered with a slight delay as compared to [^{3}H]PIP_2. The observation that [^{3}H]IP_3 appeared first and [^{3}H]PIP_2 disappeared first after hormonal stimulation suggests that phospholipase C hydrolyses [^{3}H]PIP_2 preferentially. Whether [^{3}H]PIP and [^{3}H]PI were hydrolysed directly by phospholipase C or decreased by phosphorylation by PIP and PI kinases respectively to

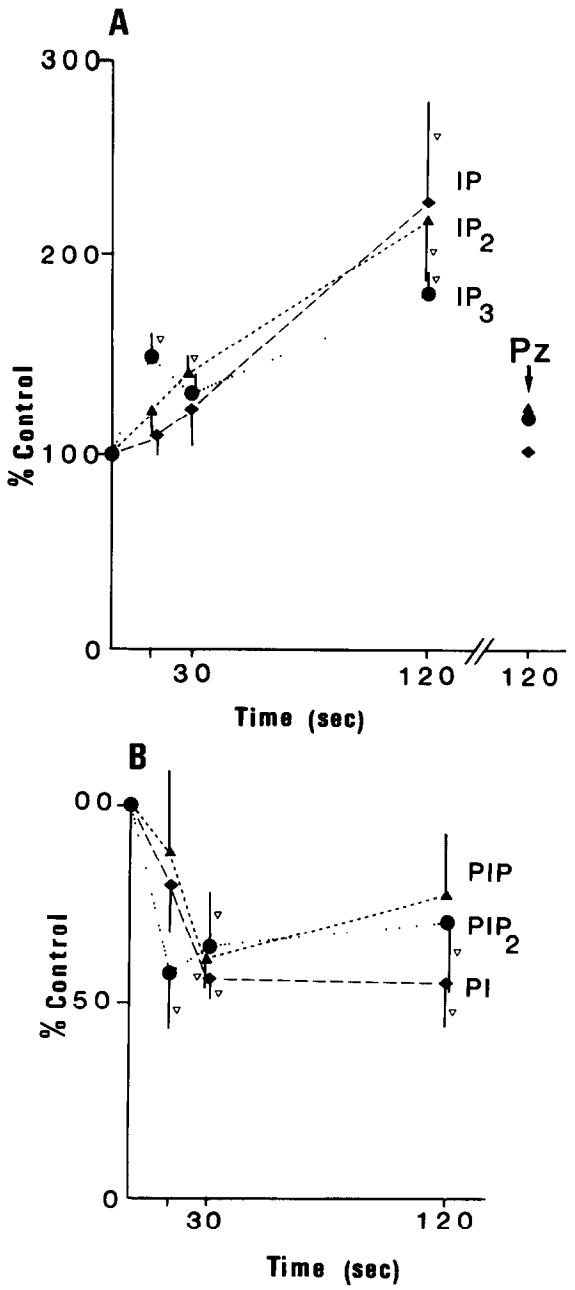

Fig.2. Time course of noradrenaline-induced decrease of phosphoinositides and accumulation of inositol phosphates. [^{3}H]Inositol-labelled ventricles were incubated with noradrenaline (5 μM) in the presence of 10 μM propranolol and 10 μM atropine. Incubation was stopped at the indicated times. (A) Increases in IP_3 (●), IP_2 (▲) and IP (◆) are expressed as a percentage of the controls (IP_3 = 2.71 ± 0.34 (6); IP_2 = 5.4 ± 0.5 (6) and IP = 25.4 ± 2.1 (5) cpm/mg wet wt, respectively). The points below Pz refer to inositol phosphate contents of ventricles preincubated 10 min with 0.1 μM prazosin before a challenge of 2 min in the presence of 5 μM noradrenaline. (B) Changes in content of PIP_2 (●), PIP (▲) and PI (◆) are expressed as a percentage of the controls incubated without agonist (PIP_2 = 10.3 ± 2.1 (7); PIP = 7.9 ± 1.5 (6) and PI = 176 ± 54 (6) cpm/mg wet wt, respectively). (▽) Statistically different from controls ($p < 0.05$).

replenish the PIP_2 pool remains an open question.

Fig.3 illustrates the time course of the effects of a maximal dose of carbachol (100 μM) on [^{3}H]-inositol phosphate accumulation (A) and [^{3}H]phosphoinositide content (B). Carbachol was less potent than noradrenaline in stimulating [^{3}H]IP_3 accumulation as observed for total inositol phosphates [9]. A 40% increase in [^{3}H]IP_3 could be observed as soon as 15 s after carbachol application. The increase was statistically significant after 2 min (139 ± 7% of controls, n = 4). [^{3}H]IP_2 content increased less than [^{3}H]IP_3 after 15 s, but then accumulated faster and to a greater extent than [^{3}H]IP_3. [^{3}H]Phosphoinositide analysis shows a marked reduction of [^{3}H]PIP_2, [^{3}H]PIP and even [^{3}H]PI. After 2 min [^{3}H]PIP_2 was more reduced (36 ± 2% of controls, n = 4) than [^{3}H]PIP and [^{3}H]PI (55 ± 9 and 59 ± 4% of controls respectively, n = 4). Atropine (10 μM for 5 min) abolished the accumulation of [^{3}H]inositol phosphates and the hydrolysis of [^{3}H]inositol lipids indicating that this is a response mediated by muscarinic receptors. These results are in agreement with the observations reported in [22] on heart showing that muscarinic receptors can be coupled either to adenylate cyclase via N_i or to phospholipid metabolism depending on the dose of agonist, the second effect requiring a higher concentration of carbachol (K_d 2 × 10^{-5} M) than the first (K_d 2 × 10^{-7} M). The high carbachol concentration used here corresponds to the coupling of muscarinic receptors to phosphoinositide degradation.

The above results support the hypothesis that neurotransmitters may regulate cardiac contraction by an IP_3-induced Ca^{2+} release from sarcoplasmic reticulum. During cardiac activity the membrane potential varies from −90 to +30 mV.

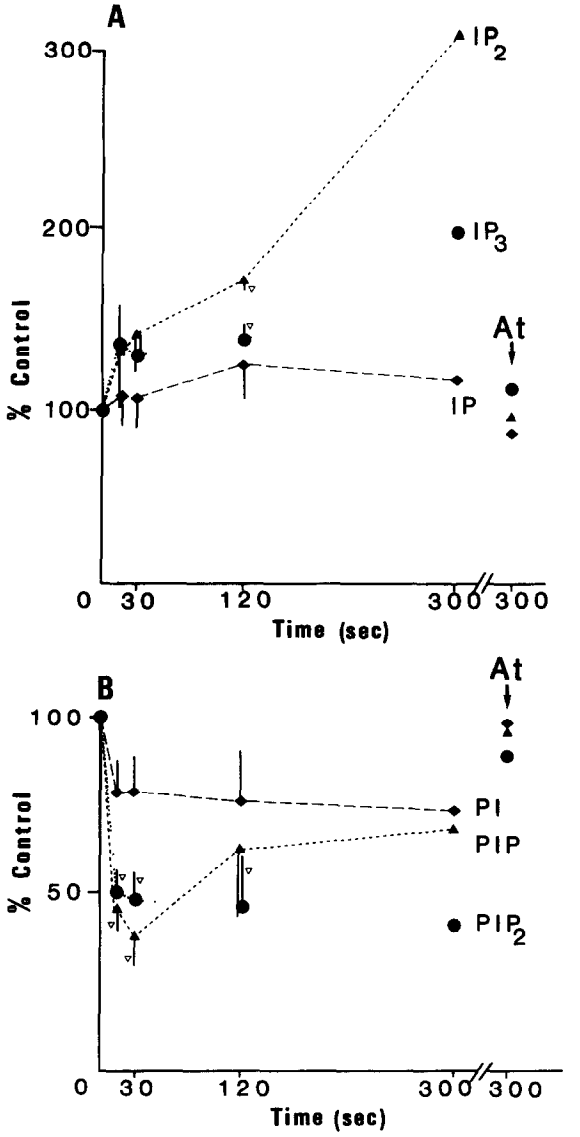

Fig.3. Time course of carbachol-induced decrease of phosphoinositides and accumulation of inositol phosphates. [^{3}H]Inositol-labelled ventricles were incubated with a maximal dose of carbachol (100 μM). Incubation was stopped at the indicated times. (A) Increases in IP_3 (●), IP_2 (▲) and IP (◆) are expressed as a percentage of the controls (IP_3 = 1.97 ± 0.13 (5), IP_2 = 4.8 ± 1.0 (5) and IP = 27.2 ± 3.6 (5) cpm/mg wet wt, respectively). (B) Changes in content of PIP_2 (●), PIP (▲) and PI (◆) are expressed as a percentage of the controls incubated without hormone (PIP_2 = 6.1 ± 1.8 (5); PIP = 4.4 ± 0.9 (5) and PI = 84 ± 17 cpm/mg wet wt, respectively). The points below At correspond to cells preincubated for 5 min with 10 μM atropine before the addition of carbachol for 5 min. (▽) Statistically different from controls ($p < 0.05$).

Table 1

Effect of KCl depolarization on inositol phosphate contents in rat ventricles

	Incubation time (min) in 100 mM KCl	
	1	3
IP (% of control)	109 ± 6	187 ± 53
IP_2 (% of control)	142 ± 17	175 ± 17[a]
IP_3 (% of control)	119 ± 15	120 ± 20

[a] $p < 0.05$

The [^{3}H]inositol-labelled ventricles were preincubated for 10 min in Krebs medium containing 10 mM LiCl, propranolol (10 μM), phentolamine (10 μM) and atropine (10 μM) and then in KCl-rich medium containing 10 mM LiCl and the same concentrations of inhibitors. Values are means ± SE of 3 experiments. Control values: IP, 30.4 ± 3.7; IP_2, 6.4 ± 0.8; IP_3, 3.6 ± 0.5 cpm/mg wet wt, respectively

At the beginning of the depolarisation phase a small Ca^{2+} inward current increases locally the Ca^{2+} which is expected to increase the Ca^{2+} permeability of the sarcoplasmic reticulum: the so-called 'Ca^{2+}-induced Ca^{2+} release' [23]. Experiments were performed to test whether membrane depolarisation could increase [^{3}H]IP_3 formation.

In preliminary experiments ^{3}H-labelled ventricles were incubated for 1 or 3 min in isotonic K^+-rich (100 mM) Krebs solution (calculated membrane potential 0 mV according to the Nernst equation). As shown in table 1, KCl caused an appreciable accumulation of [^{3}H]inositol phosphates in the tissue. In these experiments antagonists of the α,β-adrenergic and muscarinic receptors were added to prevent any effect of neurotransmitter release by K^+ depolarisation. The possibility that membrane depolarisation could modify polyphosphoinositide metabolism was further investigated in experiments where the ventricles were rapidly stimulated (5/s) for 15 s. The results are shown in fig.4. All [^{3}H]inositol phosphates increased, despite the short-term stimulation; [^{3}H]IP_3 accumulated to a lesser extent than [^{3}H]IP_2 and [^{3}H]IP. No net change in the content of [^{3}H]-inositol lipids was observed (not shown). The ac-

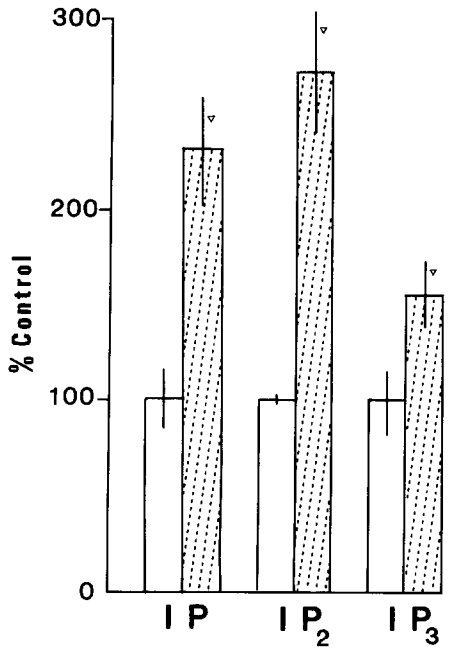

Fig.4. Effect of an electrical stimulation on inositol phosphates accumulation in isolated rat ventricles. The muscles were electrically stimulated (hatched bars) or not,(white bars) (5/s) for 15 s. The results are expressed as a percentage of the non-stimulated controls (IP_3 = 2.6 ± 0.4 (4); IP_2 = 5.4 ± 0.2 (4) and IP = 25.4 ± 2.7 (4) cpm/mg wet wt, respectively). (▽) Statistically different from controls ($p < 0.05$).

cumulation of [^{3}H]inositol phosphates is proof of phospholipase C activation under electrical stimulation. The absence of effect on the corresponding [^{3}H]phospholipids may reflect an immediate resynthesis balancing their hydrolysis.

The above results indicate that IP_3 is produced in cardiac cells under conditions that produce positive inotropic effects [24,25], i.e. α_1-adrenergic, muscarinic and electrical stimulation. These observations and recent reports showing that addition of IP_3 to sarcoplasmic reticulum induced a release of Ca^{2+} [12,26,27] suggest that IP_3 might be involved in the excitation-contraction coupling in heart cells.

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