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α_1-Adrenergic Stimulation Mediates Ca^{2+}-Dependent Inositol Phosphate Formation Through the α_{1B}-Like Adrenoceptor Subtype in Adult Rat Cardiac Myocytes

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Abstract We studied the effects of increased Ca^{2+} influx on α_1-adrenoceptor-stimulated InsP formation in adult rat cardiac myocytes. We further examined if such effects could be mediated through a specific α_1-adrenoceptor subtype. [^{3}H]InsP responses to adrenaline were dependent on extracellular Ca^{2+} concentration, from 0.1 μM to 2 mM, and were completely blocked by Ca^{2+} removal. However, in cardiac myocytes preloaded with BAPTA, a highly selective calcium chelating agent, Ca^{2+} concentrations higher than 1 μM had no effect on adrenaline-stimulated [^{3}H]InsP formation. Taken together these results suggest that [^{3}H]InsP formation induced by α_1-adrenergic stimulation is in part mediated by increased Ca^{2+} influx. Consistent with this, ionomycin, a calcium ionophore, stimulated [^{3}H]InsP formation. This response was additive with the response to adrenaline stimulation implying that different signaling mechanisms may be involved. In cardiac myocytes treated with the α_{1B}-adrenoceptor alkylating agent, CEC, [^{3}H]InsP formation remained unaffected by increased Ca^{2+} concentrations, a pattern similar to that observed when intracellular Ca^{2+} was chelated with BAPTA. In contrast, addition of the α_{1A}-subtype antagonist, 5′-methyl urapidil, did not affect the Ca^{2+} dependence of [^{3}H]InsP formation. Neither nifedipine, a voltage-dependent Ca^{2+} channel blocker nor the inorganic Ca^{2+} channel blockers, Ni^{2+} and Co^{2+}, had any effect on adrenaline stimulated [^{3}H]InsP, at concentrations that inhibit Ca^{2+} channels. The results suggest that in adult rat cardiac myocytes, in addition to G protein-mediated response, α_1-adrenergic-stimulated [^{3}H]InsP formation is activated by increased Ca^{2+} influx mediated by the α_{1B}-subtype. J. Cell. Biochem. 84: 201–210, 2002.

Key words: α_1-adrenoceptors; inositol phosphates; cardiac myocytes; Ca^{2+}

In cardiac cells, responses to α_1-adrenergic stimuli include rapid changes in contractility, in electrophysiological properties or in metabolic responses [Terzic et al., 1993; Li et al., 1997; Varma and Deng, 2000]. α_1-Adrenergic stimulation may also have long time effects on cardiac structure and function since exposure to α_1-adrenergic agonists leads to the activation of growth related genes expression [Knowlton et al., 1993; Graham et al., 1996; Sugden and Clerk, 1998].

α_1-Adrenergic receptors belong to the larger family of $G_{q/11}$-protein coupled receptors which initiate signals by activating phospholipase C-dependent hydrolysis of membrane phosphoinositides thus leading to production of $Ins(1,4,5)P_3$ and diacylglycerol. The former regulates intracellular Ca^{2+} movements into a variety of tissues whereas the latter is the physiological activator of protein kinase C [Berridge, 1993; Zhong and Minneman, 1999]. Other signaling pathways have also been shown to be activated by α_1-adrenergic receptors among which is Ca^{2+} influx through voltage-dependent and independent Ca^{2+} channels,

Abbreviations used: BAPTA, 1,2-bis(2-aminophenoxy)-ethane-N′,N′,N′,N′-tetraacetic acid; BAPTA-AM, 1,2-bis(2-aminophenoxy) ethane-N′,N′,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester); CEC, cloroethylclonidine; DMSO, dimethyl sulfoxide; PLC, phospholipase C.

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arachidonic acid release and phospholipase D activation [Suzuki et al., 1990; Xing and Insel, 1996; Ruan et al., 1998; Zhang et al., 1998].

Pharmacologically distinct α_1-adrenergic receptor subtypes have been described and molecular cloning and expression of the cDNA for three α_1 subtypes, namely α_{1A}, α_{1B}, and α_{1D}, have been reported in rat heart [Stewart et al., 1994; Graham et al., 1996]. At the RNA level, all three subtypes appear to be present in the heart [Stewart et al., 1994; Scofield et al., 1995; Rokosh et al., 1996; Wenham et al., 1997]. At the protein level, both the α_{1A}- and α_{1B}-adrenoceptor subtypes have been reported to be present in cardiac tissue, using selective receptor antagonists [Hanft and Gross, 1989; Lazou et al., 1994; Michel et al., 1994]. However, for α_{1D}-adrenoceptors, it seems that there is no obvious relationship between mRNA and protein expression in heart and several other rat tissues [Deng et al., 1996a; Yang et al., 1997]. Furthermore, α_{1D}-adrenoceptors are not functionally expressed in rat heart and cardiac myocytes [Deng and Varma, 1997; Wenham et al., 1997; Zhang et al., 1999; Seraskeris et al., 2001]. In contrast, the α_{1D} subtype seems to play an important role in several rat arteries contributing to contraction and growth of vascular smooth muscle [Kenny et al., 1995; Piascik et al., 1995; Deng et al., 1996a; Xin et al., 1997; Hrometz et al., 1999].

The physiological rationale for multiple α_1-adrenoceptor subtypes is largely unclear. Although it was originally proposed [Han et al., 1987; Minneman, 1988] that α_1-adrenergic receptor subtypes may utilize distinct transmembrane signaling pathways, α_{1B}-adrenoceptor being coupled to direct G protein activation of phospholipase C while α_{1A}-subtype is coupled to an extracellular Ca^{2+} entry activating a Ca^{2+} sensitive isoform of phospholipase C, it is now clear that there is not always such a subtype distinction. Studies in heart and other tissues have shown that both α_{1A}- and α_{1B}-adrenoceptors are coupled to G-protein mediated phosphoinositide hydrolysis [Lazou et al., 1994; Deng et al., 1996b; Zhong and Minneman, 1999]. In addition, studies with expressed cloned α_1-adrenoceptors have shown that all three subtypes can both increase Ins(1,4,5)P_3 generation mobilizing intracellular Ca^{2+} and increase Ca^{2+} influx via voltage-dependent Ca^{2+} channels [Schwinn et al., 1991; Perez et al., 1993; Wise et al., 1995]. Thus, it seems that the relationship between specific subtypes and signaling mechanisms is more complex.

In an earlier study, it was shown that in adult rat cardiac myocytes, both α_{1A}- and α_{1B}-adrenoceptor subtypes are coupled to phosphoinositide hydrolysis in proportion to their relative abundance [Lazou et al., 1994]. However, the involvement of Ca^{2+} in this process was not examined. In the present study, we investigated the possible role of extracellular Ca^{2+} in the InsP responses to α_1-adrenergic stimulation. We also addressed the possibility that one of the α_1-adrenoceptor subtypes may have a greater sensitivity to Ca^{2+} concentration. Thus, we examined, using selective antagonists for α_1-adrenoceptor subtypes, if any Ca^{2+} dependence of α_1-adrenergic-stimulated InsP formation could be attributed to a specific α_1-adrenoceptor subtype.

METHODS

Materials

Collagenase (Worthington Type 1) was from Lorne Diagnostics (Twyford, UK). 5′-methyl urapidil and CEC were from Research Biochemicals (Natick, MA). BAPTA-AM was obtained from Calbiochem (La Jolla, CA). *myo*-[^{3}H]inositol was from Amersham Pharmacia Biotech (Merck Hellas, Glyfada, Greece). All other biochemical reagents were from Sigma-Aldrich (Deisenhofen, Germany). General laboratory chemicals were from Merck (Darmstadt, Germany). BSA (fraction V) was extensively dialyzed against water before use. Aqueous solutions were prepared with double-distilled deionized water. Adrenaline was dissolved in 5 mM L-ascorbic acid. CEC and 5′-methyl urapidil were dissolved in water. Stock solutions of nifedipine and ionomycin were prepared in dimethylsulfoxide.

Animals

Male Wistar rats weighing between 250–300 g were used. All animals received humane care in accordance to the Guidelines for the Care and Use of Laboratory Animals published by the Greek Government (160/1991) based on EU regulations (86/609).

Preparation of Cardiac Myocytes

Ventricular myocytes were isolated by collagenase digestion of hearts as previously described [Lazou et al., 1994]. Freshly isolated

cells from a single heart were washed twice with collagenase-free Krebs-Henseleit medium (hereafter referred to as incubation medium) containing 25 mM $NaHCO_3$, 4.7 mM KCl, 118.5 mM NaCl, 1.2 mM $MgSO_4$ $7H_2O$, 1.2 mM KH_2PO_4, 2% bovine serum albumin (BSA), 10 mM glucose, and 0.1 mM added Ca^{2+} (as $CaCl_2$) equilibrated with 95% O_2–5% CO_2. They were finally resuspended in 10 ml of incubation medium, in which added Ca^{2+} was increased to 1 mM. Approximately 3×10^6 cells were obtained from each heart and were used for experiments immediately. After isolation and resuspension, 70–90% of the myocytes were rod shaped and quiescent.

Measurement of [^{3}H]-Inositol Phosphate Formation

Cardiac myocytes were preincubated with *myo*-[^{3}H]inositol for 60 min at 37°C. During this incubation, the myocytes were shaken and gassed continuously with 95% O_2–5% CO_2. At the end of the incubation period, they were allowed to settle, the supernatant medium was discarded, and the myocytes washed three times with fresh incubation medium. Finally the cells were resuspended in 10-ml incubation medium containing 1 mM added Ca^{2+}. A total of 10 mM LiCl were added 15 min before the experiments were initiated.

Myocytes were incubated at 37°C in siliconized stoppered glass tubes. Adrenaline and antagonists were added and the cells were incubated for 30 min more. In each set a control was included (without adrenaline added). At the end of the incubation period the cells were centrifuged in an Eppendorf centrifuge for 1–2 sec. The medium was discarded and 1 ml of 0.8 M $HClO_4$ was added. The samples were vortexed and placed on ice for 30 min. Precipitated protein was removed by bench-centrifugation and the supernatant fractions were neutralized with 0.8 M KOH/0.2 M Tris. Precipitated $KClO_4$ was removed by bench centrifugation and the supernatant fractions were retained.

[^{3}H] inositol phosphates were separated by a method based on that of Berridge et al. [1983]. The entire neutralized cell extract was chromatographed on a 0.5×2.5 cm column of Bio-Rad AG1 × 8 formate form (100–200 mesh) equilibrated with water. Each column was washed with water (2 ml), 5 mM disodium tetraborate/60 mM sodium formate (3 ml), and finally with 3 ml 0.1 M formic acid/1.0 M ammonium formate, which were retained. Fifteen milliliters of scintillation fluid was added to the final wash. Radioactivity was counted on a liquid scintillation counter.

Extracellular and Intracellular Ca^{2+} Buffering

Calcium buffers were prepared in Krebs-Henseleit saline containing 1 mM 1,2-bis(2-aminophenoxy)ethane-N′,N′,N′,N′-tetraacetic acid (BAPTA) and 0.5% BSA. The amount of $CaCl_2$ required to give the appropriate free Ca^{2+} concentration was calculated from published binding constants using a computer program (EqCal Biosoft, Cambridge, UK).

In experiments where the intracellular Ca^{2+} concentration was buffered, myocytes were preincubated in the presence of 10 μM 1,2-bis(2-aminophenoxy)ethane-N′,N′,N′,N′-tetraacetic acid tetra (acetoxy methyl) ester (BAPTA-AM) for 15 min at 37°C. Cells were washed three times with fresh incubation medium and then they were resuspended in the same medium containing the appropriate Ca^{2+} concentration.

Treatment of Ventricular Myocytes With CEC

Cardiac myocytes were isolated and prelabeled with *myo*-[^{3}H]inositol as described above. They were washed with incubation medium containing 1 mM Ca^{2+} from which BSA had been omitted. The cells were incubated with BSA-free incubation medium containing 0.1 mM CEC for 15 min at 37°C and were resuspended in 10 ml of the same medium (1 mM Ca^{2+}, containing BSA) and subsequently centrifuged. The cells were washed with fresh incubation medium three more times. Cells for control experiments were treated similarly except that CEC was omitted. When intracellular concentration of Ca^{2+} was buffered, myocytes were first incubated with incubation medium containing 10 μM BAPTA-AM for 15 min.

Data Analysis

Curve fitting was performed using the nonlinear, least-squares regression analysis program Prism (Graph Pad Software, San Diego, CA). Statistical analyses (ANOVA with Dunnet post test or two-tailed Student's *t*-test where appropriate) were performed using Instat (Graph Pad Software, San Diego, CA) with

significance taken as being established at $P < 0.05$. All data are expressed as mean±SEM.

RESULTS

Effects of Extracellular Ca^{2+} Concentration on the Stimulation of [^{3}H]InsP Formation by Adrenaline

Ca^{2+} dependence of [^{3}H]InsP formation in cardiac myocytes was tested by incubating the cells at various extracellular Ca^{2+} concentrations and stimulating with 100 μM adrenaline. Adrenaline was used as an α_1-adrenergic agonist since it was known from previous studies that it gives the largest relative stimulation of [^{3}H]InsP generation compared with phenylephrine or noradrenaline [Lazou et al., 1994].

Generation of [^{3}H]InsPs by adrenaline was dependent on Ca^{2+} concentration in the extracellular medium (Fig. 1). Removal of Ca^{2+} from extracellular medium by chelation with BAPTA, a highly selective calcium chelating agent, abolished the stimulation by adrenaline. A slight increase in adrenaline-induced [^{3}H]InsP formation was observed when Ca^{2+} concentration was raised to 0.1 μM (2.206±0.34-fold compared with the non stimulated control). [^{3}H]InsP formation became prominent when Ca^{2+} concentration was raised to 1 μM (5.08±0.27-fold) and was further increased as external Ca^{2+} concentration was raised in a graded manner from 1 μM to 2 mM (Fig. 1). Ca^{2+} concentration had no effect on basal [^{3}H]InsP formation in the absence of adrenaline. The change in the maximal activation of [^{3}H]InsP formation by adrenaline at the various Ca^{2+} concentrations did not result from loss of myocyte viability because there was no differences in ATP contents and myocytes maintained their morphology under any incubation condition (results not shown).

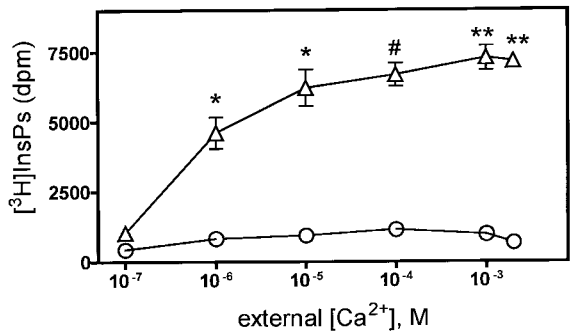

Fig. 1. Ca^{2+} dependence of the adrenaline-induced [^{3}H]InsP generation in cardiac myocytes. *myo*-[^{3}H] inositol prelabeled cardiac myocytes were incubated for 30 min with adrenaline in the presence of 10 mM LiCl. Ca^{2+} was buffered to the indicated concentrations with BAPTA. Results are the means±SEM of eight determinations from different experiments. Where no error bar is shown, the error bar is within the size of the symbol. Open circles represent incubations in the absence of adrenaline (basal rate). Open triangles represent incubations in the presence of 100 μM adrenaline. $^*P < 0.01$ compared with the previous value, $\#P < 0.01$ compared with the value at 10^{-6} M Ca^{2+}. $^{**}P < 0.01$ compared with the value at 10^{-5} M Ca^{2+}.

In order to examine if Ca^{2+} dependence of adrenaline-induced [^{3}H]InsP formation was related to changes in adrenaline efficacy, concentration-response curves for adrenaline were constructed under various extracellular Ca^{2+} concentrations and EC_{50} values were computed (Table I). There was very little variation in the computed EC_{50} values for adrenaline at the various concentrations of extracellular Ca^{2+} (0.81–1.17 μM).

Chelation of Intracellular Ca^{2+} Concentration

In order to investigate further the role of Ca^{2+} in [^{3}H]InsP formation in cardiac myocytes,

TABLE I. Stimulation of [^{3}H]InsP Generation by Adrenaline at Various External Ca^{2+} Concentrations

External [Ca^{2+}], M	pEC_{50}	n_H	Maximal response (% of basal rate)
10^{-7}	5.93±0.14	1.15±0.10	212±27
10^{-6}	6.26±0.13	0.86±0.11	549±32
10^{-5}	6.39±0.11	1.12±0.11	590±75
10^{-4}	6.39±0.16	0.94±0.13	640±45
10^{-3}	6.09±0.12	0.91±0.08	772±53

Cardiac myocytes were stimulated with increasing concentrations of adrenaline (15 concentrations from 10^{-9} to 10^{-3} M) at the indicated concentrations of Ca^{2+}. Values of pEC_{50}, n_H, and maximal response were derived from fitted curves obtained as described in Methods and are given as means±SEM for observations with six to nine separate preparations of myocytes. pEC_{50}, -log of half-maximum effective concentration, n_H, Hill coefficient. Maximal response is given as the percentage increase in rate of adrenaline stimulated [^{3}H]InsP formation compared with the basal rate.

intracellular Ca^{2+} concentration was chelated using BAPTA-AM. BAPTA-AM is an esterified membrane permeant form of BAPTA which is de-esterified in the cytosol whereupon binds Ca^{2+}. Cells were stimulated with adrenaline at various external Ca^{2+} concentrations. In cells preloaded with 10 μM BAPTA-AM, the pattern of extracellular Ca^{2+} dependence of the adrenaline-stimulated [^{3}H]InsP generation was different to that observed in non-treated cells (Fig. 2). Even though [^{3}H]InsPs increased as the extracellular Ca^{2+} concentration was raised to 1 μM (5.15±0.25-fold compared with basal), no further increases were observed as Ca^{2+} was raised even more. At 1 mM and 2 mM external Ca^{2+} concentration, adrenaline-stimulated [^{3}H]InsP formation in BAPTA-AM preloaded cardiac myocytes was about 65–70% of that observed in myocytes stimulated with adrenaline under no intracellular Ca^{2+} buffering conditions (545±47 vs. 781±53% and 568±59 vs. 887±94%, respectively) (Fig. 2). It is evident that, at these concentrations of external Ca^{2+}, approximately 30–35% of the adrenaline induced [^{3}H]InsP formation is dependent on Ca^{2+} influx.

Effects of Ionomycin on the Basal and Adrenaline-Stimulated [^{3}H]InsP Formation

The effect of Ca^{2+} influx on the adrenaline-stimulated [^{3}H]InsP generation in cardiac myocytes was further examined using the Ca^{2+} ionophore ionomycin. Ionomycin alone stimulated [^{3}H]InsP formation in a concentration-dependent manner, with an apparent EC_{50} about 3.3 μM (Fig. 3). The maximal stimulation was approximately 1.7-fold relative to the non-treated control and was about 25% of that caused by activation of α_1-adrenoceptors with a maximal concentration of adrenaline. In control experiments, DMSO (which was used as a carrier for ionomycin) alone had no effect on [^{3}H]InsP formation. In cells stimulated with 1 μM adrenaline, ionomycin also induced increases in [^{3}H]InsP formation in a concentration dependent manner with an apparent EC_{50} around 1.5 μM. Maximal response was 8.5-fold higher than the basal rate of [^{3}H]InsP formation and 1.7-fold compared with adrenaline stimulated cells in the absence of ionomycin. These results imply that [^{3}H]InsP responses to α_1-adrenergic stimulation and Ca^{2+} influx caused by ionomycin were exactly additive.

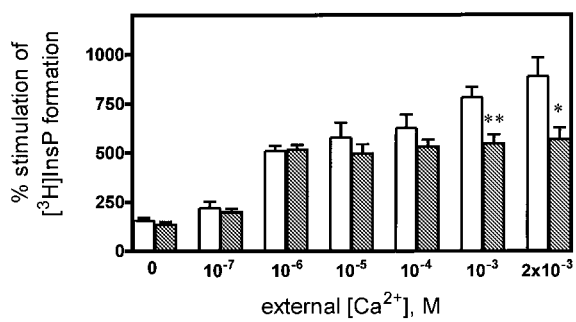

Fig. 2. Effect of BAPTA preloading on the external Ca^{2+} dependence of [^{3}H]InsP formation. Cardiac myocytes were incubated for 30 min with 100 μM adrenaline at extracellular Ca^{2+} buffered to the indicated concentrations. Open bars represent control cells stimulated with adrenaline, hatched bars represent cells incubated with 10 μM BAPTA-AM for 15 min at 37°C before adrenaline stimulation. Data in each group are expressed as percentage of the respective basal rate, in the absence of adrenaline stimulation, at the particular Ca^{2+} concentration. Values are the means±SEM from 8–10 different experiments. $^*P<0.05$ and $^{**}P<0.01$ compared with the respective value in control cells.

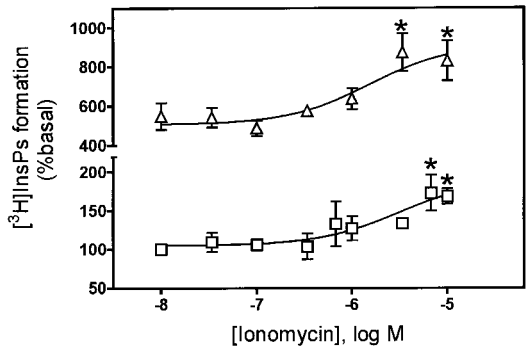

Fig. 3. Stimulation of [^{3}H]InsP formation by ionomycin. Cardiac myocytes were incubated for 30 min with the indicated concentrations of ionomycin in the absence (squares) or presence (triangles) of 1 μM adrenaline at 1 mM extracellular Ca^{2+}. Data are expressed as percentage of the basal rate in the absence of any drug. Where no error bar is shown, the error bar is within the size of the symbol. Each value is the mean±SEM from three experiments. $^*P<0.05$ compared with control incubations in the absence of ionomycin.

Effects of α_1-Adrenoceptor Subtype Antagonists on the Adrenaline-Stimulated [^{3}H]InsP Generation

To examine if the Ca^{2+} dependence of adrenaline-stimulated [^{3}H]InsP formation in cardiac myocytes is related to a specific α_1-adrenergic receptor subtype, CEC, an irreversible inhibitor of α_{1B}-subtype and 5′-methyl urapidil, a selective antagonist of α_{1A}-subtype were used. Since, both CEC and 5′-methyl urapidil reduced

[^{3}H]InsP production induced by adrenaline compared with the non-treated cells, in order to facilitate comparison among the various treatments, the rate of [^{3}H]InsP formation in each group was expressed as percentage of the rate at zero concentration of external Ca^{2+}.

In cells pretreated with 100 μM CEC, [^{3}H]InsP formation in response to 100 μM adrenaline required a minimum of extracellular Ca^{2+} concentration between 0.1 and 1 μM as is the case with non-treated control cells (Fig. 4A). However, raising extracellular Ca^{2+} concentration more than 10 μM did not significantly affect [^{3}H]InsP formation. A similar pattern was observed when cells, preloaded with BAPTA and pre-treated with CEC, were stimulated with adrenaline at increasing extracellular Ca^{2+} concentrations (Fig. 4A).

Ca^{2+} dependence of the adrenaline-induced [^{3}H]InsP generation was also tested in the presence of the α_{1A}-adrenergic receptor antagonist 5′-methyl urapidil. A concentration of 10 nM of 5′-methyl urapidil was used, which lies close to the IC_{50} value for the inhibition of the adrenaline stimulated [^{3}H]InsP formation through α_{1A}-adrenoceptors in adult rat cardiac myocytes [Lazou et al., 1994]. When cardiac myocytes were stimulated with 1 μM adrenaline in the presence of 5′-methyl urapidil, [^{3}H]InsP formation was found to be dependent on the external Ca^{2+} concentration (Fig. 4B). Stepwise increases of extracellular Ca^{2+} from 0.1 μM to 1 mM, resulted in proportional increases in [^{3}H]InsP generation (116±30 to 280±40%) following a pattern similar to that observed in cells stimulated with adrenaline in the absence of 5′-methyl urapidil. In contrast, in cells preloaded with BAPTA-AM and stimulated with adrenaline in the presence of 5′-methyl urapidil, [^{3}H]InsP generation did not show any dependence on extracellular Ca^{2+} concentrations (Fig. 4B). Taken together these results imply that [^{3}H]InsP generation through α_{1A}-adrenergic stimulation is not dependent on Ca^{2+} influx while the opposite situation applies to stimulation mediated by the α_{1B}-adrenoceptor subtype.

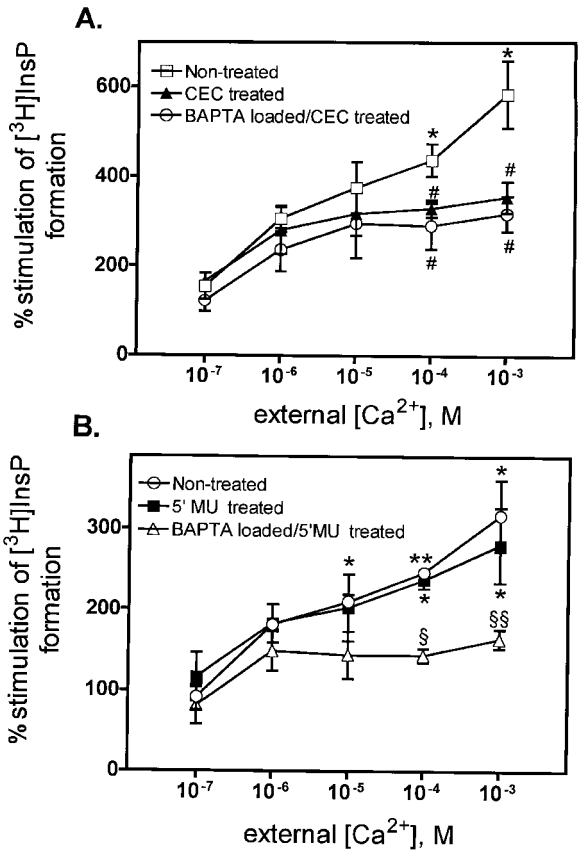

Fig. 4. Effects of α_1-adrenergic subtype inhibitors on the Ca^{2+} dependence of the adrenaline stimulated [^{3}H]InsP formation in cardiac myocytes. **A**: Control myocytes, in the absence of any treatment (open squares), myocytes treated with 100 μM CEC for 15 min at 37°C (closed triangles) or treated with CEC and preloaded with BAPTA-AM (open circles) were stimulated with 100 μM adrenaline at the indicated external Ca^{2+} concentrations. **B**: Control, non-treated myocytes (open circles), myocytes treated with 5′-methyl urapidil (5′ MU) (closed squares) or myocytes preloaded with BAPTA-AM and treated with 5′-methyl urapidil (open triangles) were stimulated with 1 μM adrenaline. For each group, the rate of [^{3}H]InsP formation is presented as percentage of the rate at zero concentration of external Ca^{2+}. Values are the mean±SEM from 3–6 experiments on separate batches of cells. * $P<0.05$ and ** $P<0.01$ compared with the value at 10^{-6} Ca^{2+}, $^{\#}P<0.05$ compared with the value at the same Ca^{2+} concentration in the control group, $^{\S}P<0.01$ compared with the value at the same Ca^{2+} concentration in the control and 5′-methyl urapidil-treated groups, $^{\S\S}P<0.05$ compared with the value at the same Ca^{2+} concentration in the control and 5′-methyl urapidil-treated groups.

Effects of Ca^{2+} Channel Blockers (Nifedipine, Ni^{2+}, Co^{2+}) on the Adrenaline-Stimulated [^{3}H]InsP Formation

To examine further the role of Ca^{2+} entry on the adrenaline induced [^{3}H]InsP formation, cardiac myocytes were incubated with 100 μM adrenaline in the presence of either the L-type voltage-dependent Ca^{2+} channel blocker nifedipine or divalent cations, Ni^{2+} and Co^{2+}. [^{3}H]InsPs responses to adrenaline, at 1 mM external Ca^{2+} concentration, were not sensitive to nifedipine with significant inhibition being observed at 50 μM nifedipine (Table II). In addition, pretreatment of cardiac myocytes with

TABLE II. Effects of Nifedipine and Inorganic Ca^{2+} Channel Blockers on the Adrenaline-Stimulated [^{3}H]InsP Formation

		[^{3}H]InsP formation (% of control)		
		Non-treated cells	CEC-treated cells	5′-methyl urapidil treated cells
Nifedipine	0.1 μM	90.46±3.14	98.15±4.29	95.56±2.67
	1 μM	87.81±6.59	93.35±2.81	90.42±4.62
	10 μM	90.31±4.29	95.30±1.24	92.01±7.69
	30 μM	83.86±6.24	85.78±4.39	81.93±1.53
	50 μM	76.11±4.37[a]	70.67±5.44[b]	77.03±3.18[a]
	100 μM	70.66±5.37[a]	73.68±5.83[b]	77.46±5.08[a]
Ni^{2+}	1 mM	90.90±6.55	95.86±3.85	—
	2 mM	67.23±3.04[b]	74.39±7.80	—
Co^{2+}	0.5 mM	83.33±12.71	—	—
	1 mM	71.63±13.39	—	—
	2 mM	50.53±7.08[b]	—	—

Cardiac myocytes were stimulated for 30 min with 100 μM adrenaline (non-treated and CEC-treated cells) or 1 μM adrenaline (5′ methyl urapidil-treated cells) in the presence of the indicated concentrations of nifedipine, $NiCl_2$ or $CoCl_2$. Results are expressed as percentage of control incubations in the absence of Ca^{2+} channel blockers. In each group, values represent the mean±SEM from 3–9 separate experiments. [a]$P < 0.05$ and [b]$P < 0.01$ vs. control incubations.

CEC or addition of 5′-methyl urapidil did not change the sensitivity of [^{3}H]InsP formation to nifedipine.

Ni^{2+} at 1 mM and Co^{2+} at 0.5 and 1 mM did not significantly reduce [^{3}H]InsP formation. A significant effect for both Ni^{2+} and Co^{2+} was observed only at a higher concentration (Table II). Furthermore, [^{3}H]InsP formation in the presence of Ni^{2+}, was not significantly different between CEC treated and non-treated cardiac myocytes.

DISCUSSION

The aim of the present study was to determine whether α_1-adrenergic stimulated InsP generation in rat cardiac myocytes is dependent on increased Ca^{2+} influx and whether this effect involves preferentially a specific α_1-adrenoceptor subtype. Previous work in other cell systems has shown that the α_{1A}-adrenoceptor subtype is linked with responses requiring Ca^{2+} influx [Tsujimoto et al., 1989; Han et al., 1990]. Early studies proposed that G-protein coupled receptor activation of phospholipase C and Ca^{2+} entry (and subsequent activation of Ca^{2+}-sensitive phospholipase C) could be distinctly mediated by α_{1B}- and α_{1A}-adrenoceptor subtypes, respectively [Wilson and Minneman, 1990; Wigginton and Minneman, 1991]. However, more recent data indicate that there is no clear distinction between subtypes with respect to their transmembrane signaling mechanisms. In particular, in adult rat cardiac myocytes, it has been shown that stimulated InsP generation is coupled to both α_{1A}- and α_{1B}-adrenergic receptor subtypes [Lazou et al., 1994; Deng et al., 1996b]. The results of the present study demonstrate that, in addition to G-protein mediated response, α_1-adrenergic induced inositol phosphate generation is in part mediated by increased Ca^{2+} entry and this effect is attributed to α_{1B}-adrenoceptor subtype.

As in prior studies, the effects of α_1-adrenoceptor subtypes were dissected in the present study using nonselective α_1-adrenergic stimulation with adrenaline in conjunction with α_1-adrenoceptor subtype blockers, 5′-methyl urapidil and CEC, at concentrations that provided maximal physiological effects. Adrenaline was chosen as an α_1-adrenergic agonist because it gives the highest stimulation of inositol phosphate formation in adult rat cardiac myocytes [Lazou et al., 1994]. The conclusions drawn in this study are based on the assumption that the effects of CEC were the result of irreversible inhibition of α_{1B}-adrenoceptors with little or no effect on the α_{1A} subtype. CEC is known to irreversibly inactivate α_{1B}-adrenoceptors [Minneman, 1988] and to a lesser extent α_{1D}-subtype [Perez et al., 1991] but was found to exert little effect on α_{1A}. Because α_{1D}-adrenoceptors are minimally expressed in hearts [Deng et al., 1996a, Yang et al., 1997] and recent data suggest that they do not contribute to InsP formation in adult rat cardiac myocytes [Seraskeris et al., 2001], it was assumed that the effects of CEC treatment were not due to inactivation of the α_{1D}-subtype.

In cardiac myocytes, [^{3}H]InsP generation stimulated by adrenaline appeared to be dependent on the extracellular Ca^{2+} concentration and was completely blocked by removal of extracellular Ca^{2+}. A minimal amount of external Ca^{2+} (0.1 μM) was necessary to mediate the response while further increases in Ca^{2+} concentration (up to 2 mM) resulted in increasing [^{3}H]InsP formation (Fig. 1). However in BAPTA preloaded myocytes, [^{3}H]InsP responses to adrenergic stimulation remained unaffected by increases in extracellular Ca^{2+} concentration greater than 1 μM (Fig. 2). Taken together these results indicate that adrenaline stimulated [^{3}H]InsP formation is in part mediated by Ca^{2+} influx. In line with such conclusion, ionomycin was found to stimulate [^{3}H]InsP generation (Fig. 3). Furthermore, [^{3}H]InsP responses to ionomycin and adrenaline were additive suggesting that α_1-adrenergic receptor activation and ionophore treatment stimulate InsP formation through different mechanisms. Consistent with these results, it has been recently shown that, in neonatal cardiac myocytes, Ca^{2+}-stimulated PLC and G protein-activated PLC act on different phosphoinositide pools resulting in the generation of different inositol phosphate products [Matkovich and Woodcock, 2000]. In the present study, no attempt was made to discriminate between the various inositol phosphates formed but the additivity of responses to the ionophore and α_1-adrenergic stimulation support the possibility that different isoforms of PLC are activated.

If α_{1A}-adrenoceptors were associated with Ca^{2+} entry and Ca^{2+}-mediated PLC activation, one might expect that Ca^{2+} dependence of [^{3}H]InsP formation would be evident in CEC treated myocytes where only the α_{1A}-adrenoceptors are active. Furthermore, chelation of intracellular Ca^{2+} concentration in CEC-treated cells, would abolish the [^{3}H]InsP dependence on Ca^{2+} influx. However, adrenaline-induced [^{3}H]InsP formation appeared to be independent of Ca^{2+} influx after inactivation of α_{1B}-adrenoceptors in adult rat cardiac myocytes (Fig. 4A). In addition, preloading the CEC-treated cells with BAPTA did not alter [^{3}H]InsP responses to adrenaline. On the other hand, cardiomyocytes treated with 5′-methyl urapidil responded to α_1-adrenergic stimulation in a Ca^{2+}-dependent manner (Fig. 4B). These observations suggest that in adult rat cardiac myocytes, Ca^{2+}-mediated [^{3}H]InsP formation is not secondary to α_{1A}-adrenoceptor stimulation as it has been previously suggested for other tissues [Wilson and Minneman, 1990; Wigginton and Minneman, 1991]. It seems more likely that the Ca^{2+} effects on α_1-adrenoreceptor stimulated [^{3}H]InsP formation are mediated through the α_{1B}-like subtype.

The mechanisms by which Ca^{2+} influx is increased following α_1-adrenergic stimulation are not always clear. An increase in Ca^{2+} levels linked to inositol lipid hydrolysis could be achieved by different mechanisms and could involve the opening of receptor-operated Ca^{2+} channels or voltage-dependent Ca^{2+} channels [Barritt, 1999; Zhong and Minneman, 1999]. In heart, the whole area of regulation of Ca^{2+} entry into cells by α_1-adrenoceptors is controversial [Fedida et al., 1993; Terzic et al., 1993]. However, the results of the present study do not support the possibility that voltage-dependent Ca^{2+} channels are involved in the Ca^{2+} effects on α_1-adrenoreceptor-stimulated [^{3}H]InsP formation in adult rat cardiac myocytes. [^{3}H]InsP formation was insensitive to both nifedipine and inorganic Ca^{2+} channel blockers at concentrations that are known to inhibit the voltage-dependent Ca^{2+} channels (Table II). Recently, in some cell types, agonist-induced Ca^{2+} influx through store-operated channels (SOCs) which open on depletion of calcium stores have received much attention [Berridge, 1997; Barritt, 1999; Putney and McKay, 1999]. However, the mechanisms and regulation of this Ca^{2+} influx remains elusive. The molecular structures of these channels have not been identified but potential candidates are encoded by members of the TRP family, a class of ion channels initially discovered in *Drosophila* [Hardie and Minke, 1992; Freichel et al., 1999]. Although capacitative Ca^{2+} entry has not been shown in cardiac myocytes, mammalian TRP homologues are expressed in the heart [Wes et al., 1995]. In addition, evidence was provided that bFGF induces an increase in cytoplasmic Ca^{2+} concentration, which was generated in part by direct IP_3 activation of voltage-independent Ca^{2+} channels in rat cardiac myocytes [Merle et al., 1997]. The whole area is largely unexplored in the heart. Whether or not SOCs are present in cardiomyocytes and whether they are involved in these signaling pathways needs to be shown in future experiments.

In conclusion, the results presented here support the possibility that some of the effects

of α_1- adrenergic stimulation on InsP formation in adult rat cardiac myocytes could be secondary to Ca^{2+} influx from the extracellular medium. The response to adrenaline is blocked by removal of extracellular Ca^{2+}, however it is not significantly affected by the presence of inorganic and organic Ca^{2+} entry blockers. Ca^{2+}-dependent InsP responses are mediated through the α_{1B}-adrenoceptors.

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