Norepinephrine-induced Inositol 1,4,5-Trisphosphate Formation in Atrial Myocytes is Regulated by Extracellular Calcium, Protein Kinase C, and Calmodulin

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SUMMARY

We investigated whether alteration of extracellular and intracellular Ca^{2+} concentrations, protein kinase C, and calmodulin modulate norepinephrine (NE)-induced inositol 1,4,5-trisphosphate (IP_3) formation in neonatal rat atrial myocytes. NE-induced IP_3 production in atrial myocytes was stimulated by elevation of extracellular Ca^{2+} in a dose-dependent manner. However, TMB-8 (an intracellular calcium antagonist) and A23187 (an intracellular calcium agonist) did not significantly affect NE-induced IP_3 production. PMA (a protein kinase C agonist) significantly decreased and staurosporine (a protein kinase C antagonist) significantly stimulated NE-induced IP_3 production. W7 (a calmodulin antagonist) significantly increased the NE-induced IP_3 . In conclusion, elevation of extracellular Ca^{2+} concentrations affects NE-induced IP_3 formation in atrial myocytes. Protein kinase C and calmodulin may control the IP_3 response to NE by a negative feedback mechanism. (Jpn Heart J 2003; 44: 547-556)

Key words: Atrial myocytes, IP_3 , Calcium, Protein kinase C, Calmodulin, Atrial natriuretic peptide

INOSITOL 1,4,5-trisphosphate (IP_3) plays an important role in excitation and contraction in atrial myocardium. In atrial cells, IP_3 is associated with secretion of atrial natriuretic peptide (ANP).^{1,2)} The stimulation of α -adrenergic receptors with norepinephrine (NE) increases ANP secretion.²⁾ The signal transduction of NE occurs through activation of phospholipase C (PLC), which leads to release of IP_3 . On the other hand, release of IP_3 by NE in atrial cells is also associated with the development of arrhythmias.³⁾ Thus, NE-stimulated IP_3 plays an important role in atrial cells, however, intracellular regulation of NE-stimulated IP_3 formation in atrial myocytes remains unclear.

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NE activates PLC and stimulates the rapid formation of IP₃ and diacylglycerol (DAG). IP₃ stimulates the release of Ca²⁺ from sarcoplasmic reticulum. The intracellular calcium binds to calmodulin. The calcium-calmodulin complex activates Ca²⁺/CaM-dependent protein kinase and contributes to phosphorylation of myosin light chain. DAG produces protein kinase C (PKC), which also plays an important role in phosphorylation of myosin light chain.⁴⁾ Activation of PKC, increases in extracellular and intracellular Ca²⁺, and calmodulin each enhances ANP secretion by α 1-adrenoceptor stimulation.¹⁾ Increases in intracellular and extracellular Ca²⁺, PKC, and calmodulin have a significant role in arrhythmias.⁵⁾ In this study, we investigated the effect of alteration of extracellular and intracellular Ca²⁺, PKC, and calmodulin on NE-induced IP₃ formation in neonatal rat atrial myocytes.

METHODS

Isolation of cardiomyocytes from neonatal rat heart: The research was conducted according to the Helsinki Declaration and approved by our animal use committee. Three-day-old neonatal rats were anesthetized with sevoflurane and killed by cervical dislocation. Myocytes were isolated using an aseptic technique. The hearts were rapidly removed and placed in ice-cold Ca²⁺- and Mg²⁺-free PBS containing 40 U mL⁻¹ sodium heparin, 4 mM glucose, and 25 mM HEPES. The hearts were washed with 4 liters of PBS. The atria and ventricles were divided and kept in ice-cold MEM and PBS, respectively. The atria were minced with scissors into 1 to 3 mm³ fragments, which were then washed with PBS by gently stirring in a 37 °C water-jacketed Erlenmeyer flask for 10 minutes. The tissue was then enzymatically digested five times for 10 minutes each with 10 mL PBS containing 0.1% trypsin, 0.1% collagenase (type 5), 15 g mL⁻¹ deoxyribonuclease 1, and 1% chicken serum. The liberated cells were collected by centrifugation at 200 × g and resuspended in PBS. The pooled and washed cells were plated in T-75 cell culture flasks in minimal essential medium (containing Hank's salts, L-glutamine, 5% chicken serum, 3 mM pyruvic acid, MEN vitamins, 1 g mL⁻¹ insulin, 1 g mL⁻¹ transferrin, 10 ng mL⁻¹ selenium, and 50 g mL⁻¹ gentamicin). The nonadherent cells were harvested after incubation at 37°C for 60 minutes in a humidified incubator with 5% CO₂ in air. The cells were counted and resuspended in minimal essential medium containing 0.1 mM 5-bromo-2'deoxyuridine to inhibit cell division and thereby control nonmyocyte cell growth. Fibroblasts were separated from cardiomyocytes. The suspension was then transferred to 0.1% gelatin-coated 25 cm² flasks for measurement of IP₃. The culture medium was changed after 48 hours to the above media and finally to serum-free medium 24 hours before the cells were studied. After four days, the cells were used at confluence

for this study to provide stable data. A highly enriched population of myocardiocytes was identified by light microscope examination and the cultures could be maintained for at least 14 days with little change in overall cell morphology.

IP₃ measurement: Twenty-four hours before the experiments, the culture medium was changed to calcium free minimal essential medium. After NE was added for 20 seconds, the reaction was stopped by addition of 5 mL ice-cold 1 M trichloroacetic acid (TCA) for each 1 mg of cells. The acid extract was homogenized and centrifuged at 0-4°C for 10 minutes at 1000 × g. TCA was removed from the extracts by adding 2 mL of a mixture of 3 vol of 1,1,2-trichloro-1,2,2-trifluoroethane plus 1 vol of trioctylamine for each mL of TCA extract. IP₃ content in the aqueous top layer was determined by means of a radioreceptor assay kit (NEN Research Products, Boston, USA).

The cultures were incubated at extracellular Ca²⁺ concentrations of 0, 0.25, 0.5, 1, and 5 mM for 60 minutes before adding NE. Cells were treated with phorbol 12 myristate 13 acetate (PMA) (a PKC agonist) for 10 minutes, and staurosporine (a PKC antagonist), 8-(N,N diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) (an intracellular calcium antagonist), A23187 (an intracellular calcium agonist), and N-(6-aminoethyl)-5-chloro-naphthalenesulphonamide (W7) (a calmodulin antagonist) for 30 minutes before exposure to NE. The PMA, staurosporine, and W7 experiments were conducted in the presence of 0.5 μM of extracellular calcium.

Protein measurements: Protein was determined by the method of Bradford using bovine serum albumin as standard.⁶⁾

Measurement of intracellular calcium mobilization: To evaluate whether TMB-8 and A23187 cause mobilization of intracellular calcium, we used the fluorescent calcium indicator chlortetracycline. Cell suspensions were incubated with 10 μM chlortetracycline for two hours at 25°C. Four samples were withdrawn one minute after the addition of NE. TMB-8 or A23187 was added 15 minutes before the addition of NE. The samples were placed into plastic conical centrifuge tubes and centrifuged at 7000 × g for one minute. Immediately after centrifugation, the supernatant was aspirated, leaving the undisturbed pellets in the tube tips. The tube tips were removed with a hot surgical blade and placed in acrylic holders for fluorescence determination with a photon-counting microspectrofluorometer.

Protein kinase C assay: Cardiomyocytes were exposed to NE, PMA, or staurosporine for the indicated times. Cells were then homogenized in buffer containing 20 mM Hepes, 5 mM EGTA, 0.25 mM sucrose, 5 mM DTT, 1 g L⁻¹ BSA, and 50 μg mL⁻¹ leupeptin by sonication (five times for 10 seconds) followed by 9 × 4 strokes in a glass potter homogenizer. The whole homogenate was considered as the cytosolic fraction. The pellet was solubilized in homogenizing buffer containing 0.4 % Triton X-100 and centrifuged at 10,000 × g for 10 minutes. Both frac-

tions were then applied to DEAE-cellulose columns, which were washed with 2.5 mL of a buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 5 mM EGTA, and 2 mM PMSF. PKC was eluted with the same buffer containing 100 mM NaCl. PKC activity was then determined by measuring the Ca^{2+} and phospholipid-dependent transfer of ^{32}P from [^{32}P]ATP to histone III-S. One hundred μL of the eluate was added to 150 μL of a solution containing 20 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 25 μg histone III-S either in the absence or presence of 0.75 mM CaCl_2 , 12 μg of phosphatidylserine, and 1.6 μg of 1,2-diolein. The reaction was started by addition of 50 μM ATP containing 5 μCi of [^{32}P]ATP per assay and conducted for 30 minutes at room temperature. Histone was then precipitated by the addition of ice-cold 25% trichloroacetic acid and separated by filtration on 0.45 μm nitrocellulose filters. Filters were then counted by liquid scintillation. Translocation of PKC was determined by measuring the ratio of membrane associated activity/total activity (total activity is cytosolic activity plus particulate activity).

Measurement of ANP secretion: Twenty-four hours before the experiments, the culture medium was changed to serum-free minimal essential medium. Cardiomyocytes were exposed to 1 μM NE, 0.25, 0.5, 1, and 5 μM extracellular calcium, 10 μM A23187, and 10 nM PMA or 1 μM W7 for the indicated times at 37°C in 5% CO_2 , after which the entire supernatant was collected. ANP was measured by radioimmunoassay (RIA)(Eiken, Tokyo). The mean intra-assay and interassay CV were 10.5 % and 12.5 %, respectively.

Solutions and drugs: Norepinephrine, ANP, the stable analog of TMB-8, A23187, PMA, staurosporine, and W7 were purchased from Sigma Chemical Co. (St Louis, USA). A radioreceptor assay kit for measurement of IP_3 was purchased from NEN Research Products (Boston, USA). PMA and staurosporine were dissolved in 0.1% dimethyl sulfoxide.

Statistical analysis: The data are expressed as the mean $\pm$ standard error of the mean. Each experiment was carried out in duplicate. Every experiment was repeated six times with different cell preparations unless otherwise specified. Statistical comparisons were made using ANOVA for repeated measures and the Student-Newman-Keuls multiple comparison test. Differences in mean values were considered significant at $P < 0.05$.

RESULTS

Exposure to 100 nM, 1, 10, and 100 μM of NE increased IP_3 production from a basal level of 106.5 ± 9.1 to 272.2 ± 23.1 , 344.7 ± 19.4 , 416.0 ± 24.8 , and 474.0 ± 34.8 pmol mg protein $^{-1}$, respectively ($P < 0.001$).

Figure 1 shows the effect on NE-induced IP₃ production under various concentrations of extracellular calcium. After exposure to 0.25, 0.5, 1, and 5 mM Ca²⁺, IP₃ production increased significantly from 275.2 ± 15.3 pmol mg protein⁻¹ in the absence of Ca²⁺ to 305.3 ± 22.0, 344.7 ± 19.4, 431.7 ± 21.4, and 506.3 ± 22.6 pmol mg protein⁻¹, respectively.

We investigated the effects of TMB-8 (an intracellular Ca²⁺ antagonist) and A23187 (an intracellular Ca²⁺ agonist) on NE (1 μM)-induced IP₃. One and 10 μM TMB-8 and A23187 did not significantly affect NE-induced IP₃ production. NE (1 μM) significantly stimulated mobilization of intracellular Ca²⁺ by 78%. Ten μM TMB-8 significantly blocked mobilization of intracellular Ca²⁺ by 67% and 10 μM A23187 significantly stimulated mobilization of intracellular Ca²⁺ by 92% ($P < 0.001$).

To study the effect of PKC on NE-induced IP₃ production, we studied the effects of PMA (a PKC agonist) and staurosporine (a PKC antagonist) on NE-induced IP₃ production. PMA alone or staurosporine alone had no effect on IP₃ production. The ratio of membrane activity/cytosolic activity plus membrane activity changed from 0.38 ± 0.04 of control to 0.77 ± 0.09 after 10 nM PMA

Figure 1. Effect of extracellular calcium on NE-induced IP₃ production. Cardiomyocytes were incubated for 60 minutes at 37°C in the presence of extracellular calcium (0, 0.25, 0.5, 1 and 5 mM). IP₃ production was measured after NE (1 μM) stimulation for 20 seconds as described in the Methods. Data are mean ± SEM. $n = 6$. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ vs NE-induced IP₃ production in absence of extracellular calcium. #: $P < 0.05$, vs basal.

administration, 0.27 ± 0.02 after 10 nM staurosporine administration, and 0.54 ± 0.08 after 1 μ M NE administration. One and 10 nM PMA significantly decreased NE-induced IP₃ production from 344.7 ± 19.4 to 267.3 ± 19.4 and 221.8 ± 14.8 pmol mg protein⁻¹. One and 10 nM staurosporine significantly stimulated IP₃ production from 344.7 ± 19.4 to 411.3 ± 28.3 and 482.5 ± 25.7 pmol mg protein⁻¹ (Figure 2).

We studied the effect of W7, a calmodulin antagonist, on NE-induced IP₃ production. One μ M W7 alone had no effect on IP₃ production. One hundred nM and 1 μ M W7 significantly increased NE-induced IP₃ from 344.7 ± 13.0 to 410.3 ± 19.3 ($P < 0.05$) and 523.5 ± 34.7 ($P < 0.01$) pmol mg protein⁻¹.

ANP secretion from atrial cells increased from 0.63 ± 0.6 pmol mL⁻¹ of control to 5.22 ± 0.75 pmol mL⁻¹ after administration of 1 μ M NE, 2.58 ± 0.31 pmol mL⁻¹ after administration of 0.5 μ M extracellular calcium, 2.01 ± 0.64 pmol mL⁻¹ after administration of 10 μ M A23187, 3.60 ± 0.38 pmol mL⁻¹ after administration of 10 nM PMA or 2.37 ± 0.39 pmol mL⁻¹ after administration of 1 μ M W7.

Figure 2. Effect of phorbol 12 myristate 13 acetate (PMA)(a PKC agonist) and staurosporine (a PKC antagonist) on NE-induced IP₃ production. The cultures were incubated at 37°C in the presence of 1 and 10 nM PMA for 30 minutes and 1 and 10 nM staurosporin for 30 minutes. IP₃ production was measured after NE (1 μ M) stimulation for 20 seconds as described in the Methods. The concentration of extracellular calcium in the culture was 0.5 μ M. Data are mean $\pm$ SEM. $n = 6$. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, vs NE-induced IP₃ production. ###: $P < 0.001$, vs Basal.

DISCUSSION

We found that NE-induced IP₃ production in atrial myocytes was stimulated by extracellular Ca²⁺ in a dose-dependent manner. G-protein activated by extracellular Ca²⁺ stimulates PLC to activate mobilization of intracellular Ca²⁺ stores and stimulate Ca²⁺ entry across the plasma membrane depending on phosphoinositide turnover.⁷⁾ Extracellular Ca²⁺ is reported to activate G-protein via calcium ion sensing receptors.⁸⁾ NE increased the intracellular Ca²⁺ concentration. The increase appears to be due to both activation of mobilization of intracellular Ca²⁺ stores and stimulation of Ca²⁺ entry across the plasma membrane. Thus, IP₃ production stimulated by extracellular Ca²⁺ appears to be associated with calcium ion sensing receptors. TMB-8 (an intracellular calcium antagonist) and A23187 (an intracellular calcium agonist) could not significantly affect the NE-induced IP₃ production. TMB-8 inhibits Ca²⁺ influx from plasma membrane and IP₃-induced Ca²⁺ release from sarcoplasmic reticulum (SR).⁹⁾ A23187 selectively acts on voltage-dependent Ca²⁺ channels, resulting in elevation of the intracellular calcium concentration. NE-induced IP₃ production is regulated by extracellular Ca²⁺, but not by intracellular Ca²⁺ in atrial myocytes. These results support the finding that ANP released by elevation of extracellular Ca²⁺ is associated with elevation of NE-induced IP₃ production.¹⁾ However, ANP release by elevation of intracellular Ca²⁺ appears to involve a different pathway from that of NE-induced IP₃ production.

We investigated whether PMA (a PKC agonist) or staurosporine (a PKC antagonist) affect NE-induced IP₃ formation. NE activated PKC and the treatment with PMA inhibited NE-induced IP₃ formation and the treatment with staurosporine stimulated NE-induced IP₃ formation, indicating that there is feedback regulation of PKC to IP₃. Langendorff perfusion in guinea pig hearts with PMA causes an impairment of contraction of the left ventricle and the down-regulation by activation of PKC may be associated with a decrease in inositol phosphates.¹⁰⁾ Barr and Watson reported that a PKC inhibitor potentiated agonist-stimulated accumulation of inositol phosphates in astrocytoma cells.¹¹⁾ They suggested that the inhibitory feedback by PKC may mediate phosphorylation of PLC. Thus, the inhibitory effect of PKC on NE-induced IP₃ formation appears to be associated with both receptor desensitization and down-regulation in atrial myocytes.

The IP₃-induced Ca²⁺ release requires calmodulin-dependent IP₃ receptor phosphorylation.¹²⁾ W7 interferes with the binding between the calmodulin receptor and Ca²⁺-calmodulin complex in cardiomyocytes.¹³⁾ We found that W7 has a positive effect on NE-induced IP₃ formation. Wolf, *et al* reported that W7 caused Ca²⁺ release from SR and potentiated IP₃-induced Ca²⁺ mobilization.¹⁴⁾ These results indicate that W7 causes Ca²⁺ release from SR through elevation of IP₃. IP₃ is metabolized by a IP₃-3-kinase to IP₄. The IP₃-3-kinase is activated in

a calmodulin dependent manner.¹⁵⁾ This phenomenon autocatalytically enhances the Ca^{2+} signal, as IP_3 promotes the Ca^{2+} -mobilizing function of IP_3 and inhibits IP_3 dephosphorylation. Thus, it is possible that IP_3 formation stimulated by the calmodulin antagonist is mediated partly by a negative feedback mechanism that controls the IP_3 response to NE.

Signaling pathway NE-induced IP_3 formation in atrial cells is shown in Figure 3. NE activates PLC, resulting in the rapid formation of IP_3 and DAG. IP_3 stimulates the release of Ca^{2+} from sarcoplasmic reticulum. The intracellular calcium binds to calmodulin. The calcium-calmodulin complex activates Ca^{2+} /CaM-dependent protein kinase and contributes to phosphorylation of myosin light chain. DAG produces PKC, which also plays an important role in phosphorylation of myosin light chain. Extracellular Ca^{2+} increases NE-induced IP_3 formation through activation of G-protein via calcium ion sensing receptors. PMA significantly decreased and staurosporine significantly stimulated NE-induced IP_3 production. W7 (a calmodulin antagonist) significantly increased the NE-induced IP_3 .

Figure 3. NE activates PLC, resulting in the rapid formation of IP_3 and DAG. IP_3 stimulates the release of Ca^{2+} from sarcoplasmic reticulum. The intracellular calcium binds to calmodulin. The calcium-calmodulin complex activates Ca^{2+} /CaM-dependent protein kinase and contributes to phosphorylation of myosin light chain. DAG produces PKC, which also plays an important role in phosphorylation of myosin light chains. Extracellular Ca^{2+} increases NE-induced IP_3 formation through activation of G-protein via calcium ion sensing receptors. PMA (a PKC agonist) significantly decreased and staurosporine (a PKC antagonist) significantly stimulated NE-induced IP_3 production. W7 (a calmodulin antagonist) significantly increased the NE-induced IP_3 .

Neonatal myocardium is more sensitive to extracellular calcium concentrations than is adult myocardium. This is related to the poorly developed sarcoplasmic reticulum of neonatal myocardium.¹⁶⁾ It is unclear whether the intracellular changes associated with maturation of the atrial myocytes affect NE-induced IP₃ formation. PMA, staurosporine, TMB-8, and W7 have been used for many years as pharmacologic tools. PMA and staurosporine are known as a PKC agonist and antagonist, respectively. We used 1 and 10 nM PMA and staurosporine for preventing several actions at high concentrations. TMB-8 inhibits the release of intracellularly stored Ca²⁺.¹⁶⁾ Two μ M TMB-8 for 15 minutes significantly decreased the concentration of intracellular calcium in atrial muscle. As high concentrations of TMB-8 have several actions, including depression of protein kinase C and tyrosine kinase,¹⁷⁾ the culture was exposed for 30 minutes at a concentration of 1 μ M TMB-8 in this study. W-7 has been reported to increase the release from the SR by interfering with the binding between the calmodulin receptor and Ca²⁺-calmodulin complex,¹⁸⁾ but is ineffective as an inhibitor of PKC or protein kinase A.¹⁹⁾ Thus, PMA, staurosporine, TMB-8, or W7 at the concentration used in this study appear to be specific.

In conclusion, elevation of extracellular Ca²⁺ concentrations affects NE-induced IP₃ formation in neonatal rat atrial myocytes. PKC and calmodulin are involved in regulation of the IP₃ response to NE.

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