Full Paper

Pharmacological Evidence for Involvement of Phospholipase D, Protein Kinase C, and Sodium-Calcium Exchanger in α-Adrenoceptor-Mediated Negative Inotropy in Adult Mouse Ventricle

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Abstract. The intracellular signalling pathway for α-adrenoceptor-mediated negative inotropy was studied pharmacologically in isolated adult mouse ventricle. The negative inotropy was inhibited by GF-109203X, a nonselective protein kinase C inhibitor. Phorbol 12-myristate 13-acetate also produced sustained negative inotropy, which was inhibited by KB-R7943, a Na+/Ca2+ exchanger inhibitor. The α-adrenoceptor-mediated negative inotropy was augmented by RHC-80267, a diacylglycerol lipase inhibitor, but was inhibited either by C2-ceramide, a phospholipase D inhibitor, and high concentration of propranolol (50 μM), which inhibits phosphatidate phosphohydrolase. The inotropy was not affected by U-73122, a phospholipase C inhibitor. Lavendustin-A, a tyrosine kinase inhibitor, also inhibited the negative inotropy. These findings suggest that α-adrenoceptor-mediated negative inotropy in adult mouse ventricle is mediated by activation of tyrosine kinase, the phospholipase D-phosphatidate phosphohydrolase pathway, and protein kinase C.

Keywords: α-adrenoceptor, inotropy, phospholipase D, protein kinase C

Introduction

The sympathetic neurotransmitter, norepinephrine, is a major regulator of myocardial contractile force. Stimulation of β-adrenoceptors enhances Ca2+ influx through L-type calcium channels and Ca2+ handling by the sarcoplasmic reticulum through increase in intracellular cAMP. However, α-adrenoceptors are also present in myocardial tissue, and their stimulation is known to affect cardiac rhythm and contractility through mechanisms different from those of β-adrenoceptor stimulation (1–3). Mechanisms such as increase in transsarcolemmal Ca2+ influx through the L-type Ca2+ channel due to inhibition of transient outward K+ current and increase in Ca2+ sensitivity of the myofibrils have been postulated to underlie the α-adrenoceptor-mediated positive inotropy. Inotropic response to α-adrenoceptor stimulation is known to vary among experimental animal species. A positive response has been observed in the human, rabbit, bovine, feline, and porcine myocardia, while no response was observed in the canine myocardium (1, 4). A biphasic response is observed in the rat ventricular myocardium; a transient negative response is followed by a positive response (5).

In the case of mouse ventricle, we found that α-adrenoceptor stimulation produces sustained negative inotropy in the adult (6–9). Interestingly, the inotropy is positive in the neonate and is developmentally converted to negative. Similar developmental conversion of inotropy from positive to negative was observed with endothelin I and angiotensin II (10–12). The sustained nature of the negative response in the adult mouse enables us to clarify the mechanisms underlying α-adrenoceptor-mediated negative inotropy. The mouse myocardium has properties different from myocardia from other experimental animal species such as short action potential duration (13, 14), high dependence on sarcoplasmic reticulum function (14, 15), and positive inotropic response to acetylcholine (16–18). As for the ionic mechanisms underlying α-adrenoceptor-mediated negative inotropy, we performed electrophysiological
experiments and showed that it results from enhancement of Ca\(^{2+}\) extrusion by the Na\(^+\)/Ca\(^{2+}\) exchanger, leading to decreased Ca\(^{2+}\) release from the sarcoplasmic reticulum (8, 9). The Na\(^+\)/Ca\(^{2+}\) exchanger is not only involved in the basic excitation-contraction mechanisms, but is also known to be related to pathological changes of the myocardium (19, 20). Thus, the exchanger has received much attention as a therapeutic target for the treatment of various cardiovascular disorders (21 – 25). However, the intracellular signalling pathway for the regulation of the exchanger has not yet been fully clarified.

The present study was undertaken to clarify the intracellular signal transduction pathway underlying \(\alpha\)-adrenoceptor-mediated negative inotropy and activation of the Na\(^+\)/Ca\(^{2+}\) exchanger. We obtained pharmacological evidence for the involvement of protein kinase C (PKC) activation through the phospholipase (PL) D-phosphatidate phosphohydrolase (PPH) pathway.

**Materials and Methods**

*Measurement of force of contraction*

All experiments were performed in accordance to the “Guiding Principles for the Care and Use of Laboratory Animals” approved by the Japanese Pharmacological Society. Right ventricular free wall strips were rapidly isolated from adult (4- to 5-week-old, 16 – 25 g) ddY strain mice. Preparations were placed horizontally in a 20-ml organ bath containing modified Ringer solution of the following composition: 135 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 15 mM NaHCO\(_3\), and 5.5 mM glucose (pH 7.4 at 36°C). The solution was gassed with 95% O\(_2\) : 5% CO\(_2\) and maintained at 35 – 36°C. The preparations were driven by a pair of platinum plate electrodes (field stimulation) with rectangular current pulse (1 Hz, 2 ms, 1.5 \(\times\) threshold voltage) generated from an electronic stimulator. Developed tension was recorded isometrically with a force-displacement transducer connected to a minipolygraph.

When phenylephrine (PE) was used, 0.3 \(\mu\)M propranolol was present in the organ bath to prevent \(\beta\)-stimulation by the agent. For the experiments with C\(_2\)–ceramide and dihydro-C\(_2\)–ceramide, the ventricular tissue was incubated with modified Ringer solution supplemented with 100 \(\mu\)M ceramide and equimolar bovine serum albumin (BSA). After 4-h incubation, PE was applied. To prevent the accumulation of foam in the organ bath due to bubbling of the BSA containing solution, anti-foam (Sigma Chemical, St. Louis, MO, USA) was used. Other inhibitors were applied to the bath solution 30 min before PE or phorbol 12-myristate 13-acetate (PMA) application.

**Chemicals**

The following drugs were used (sources in parentheses): \(l\)-phenylephrine hydrochloride (Wako Pure Chemicals Ltd., Osaka); \(dl\)-propranolol hydrochloride, PMA (Sigma); C\(_2\)–ceramide, dihydro-C\(_2\)–ceramide, RHC-80267 (Biomol, Plymouth Meeting, PA, USA); GF-109203X, Gö6976, U-73122, lavendustin-A and lavendustin-B (Calbiochem, LA Jolla, CA, USA). KB-R7943 was generously supplied to us by Kanebo Co., Ltd. (Osaka).

**Evaluation of results**

All values are expressed as means ± S.E.M. Statistical significance of difference between means was evaluated either by one-way analysis of variance (ANOVA) or by the unpaired t-test.

**Results**

*Effect of PKC inhibitors on PE-induced negative inotropy*

In the presence of 0.3 \(\mu\)M propranolol, PE (10 \(\mu\)M) produced a negative inotropic response (Fig. 1); the contractile force was decreased by about 40%, which was the same as the results in our previous study (8, 9). This negative inotropic response to \(\alpha\)-adrenoceptor stimulation was inhibited by GF-109203X, an isoform nonselective PKC inhibitor, which inhibits PKC\(\alpha\), \(\beta I\), \(\gamma\), \(\delta\), and \(\epsilon\) (26). The inhibitory effect was concentration-dependent and 10 \(\mu\)M GF-109203X almost abolished PE-induced negative inotropy. On the other hand, Gö6976 (10 \(\mu\)M), a Ca\(^{2+}\)-dependent isoform selective PKC inhibitor, which inhibits PKC\(\alpha\) and \(\beta I\) (Ca\(^{2+}\)-dependent isozymes) but not Ca\(^{2+}\)-independent PKC\(\delta\), \(\epsilon\), and \(\zeta\) isozymes (27), did not affect the PE-induced negative inotropy. The basal contractile force in the presence of 10 \(\mu\)M GF-109203X and 10 \(\mu\)M Gö6976 was 134 ± 8% and 127 ± 5% of the value in the absence of drugs, respectively.

*PMA-induced negative inotropy and effect of KB-R7943 and GF-109203X*

A potent PKC activator PMA (100 nM) produced negative inotropy (Fig. 2); the time course was slower, but the magnitude was similar to that by PE. PMA-induced negative inotropy was inhibited by 30 \(\mu\)M KB-R7943, a Na\(^+\)/Ca\(^{2+}\) exchange inhibitor which was shown to completely inhibit the PE-induced negative inotropy at this concentration (9). The PMA-induced negative inotropy was inhibited by 10 \(\mu\)M GF-109203X.
Effect of RHC-80267 on PE- and PMA-induced negative inotropy

RHC-80267 (50 μM), an inhibitor of DAG lipase, significantly augmented PE-induced negative inotropy (Fig. 3). In contrast, PMA-induced negative inotropy was not affected by RHC-80267. RHC-80267 increased the basal contractile force (122 ± 7% of the value in the absence of drug).

Effect of phospholipase inhibitors on PE- and PMA-induced negative inotropy

U-73122 (10 μM), a PI-PLC inhibitor, did not affect the basal contractile force and PE-induced negative inotropy (Fig. 4). PE-induced negative inotropy in tissue preincubated with C_2-ceramide (100 μM), a membrane permeable ceramide reported to inhibit PLD (28, 29), was significantly smaller than that in tissue preincubated with bovine serum albumin (Fig. 4). On the other hand, preincubation with dihydro-C_2-ceramide (100 μM), an inactive form of C_2-ceramide, had no significant effect. PMA-induced negative inotropy was not affected by C_2-ceramide. The basal contractile force after preincubation with C_2-ceramide, dihydro-C_2-ceramide, and albumin were not significantly different.

Effect of propranolol on PE- and PMA-induced negative inotropy

High concentration of propranolol (50 μM), which inhibits PPH, significantly attenuated PE-induced, but not PMA-induced, negative inotropy (Fig. 5). Propranolol also decreased basal contractile force to 37 ± 3% of the value in the absence of drug.

Effect of lavendustin-A on PE- and PMA-induced negative inotropy

Lavendustin-A (10 μM), a tyrosine kinase inhibitor, significantly inhibited PE-induced, but not PMA-induced, negative inotropy (Fig. 6). The basal contractile
force was decreased by the drug to 70 ± 4% of the value in its absence. Lavendustin-B (10 μM), an inactive analogue of lavendustin-A, did not affect the basal contractile force and PE-induced negative inotropy.

Discussion

We have shown that α-adrenoceptor stimulation produces sustained negative inotropy through enhancement of the Na⁺/Ca²⁺ exchanger (6 – 9). The present study was undertaken to pharmacologically clarify the intracellular signalling pathway for this phenomenon. PE-induced negative inotropy was inhibited by GF-109203X, indicating the involvement of PKC (Fig. 1). Activation of PKC by PMA produced negative inotropy similar in amplitude to that by α-adrenergic stimulation, and the response was inhibited by KB-R7943 and GF-109203X (Fig. 2), which was the same as in the case with PE-induced negative inotropy. These results suggest that the α-adrenoceptor stimulation-induced negative inotropy and enhancement of Na⁺/Ca²⁺ exchanger activity are mediated by activation of PKC. This appears to be consistent with earlier observations that α-adrenoceptor-mediated negative inotropy is mediated by PKC and that cardiac Na⁺/Ca²⁺ exchanger is activated by PKC (30 – 32). PE-induced negative inotropic response was inhibited by GF-109203X, which inhibits PKCα, βI, βII, γ, δ, and ε, but not by Gö6976, which inhibits PKCα and βI (Ca²⁺-dependent isozymes)
but not Ca\(^{2+}\)-independent PKC\(\delta\), \(\varepsilon\), and \(\zeta\) isoforms (Fig. 1). This indicates that the PKC involved is the Ca\(^{2+}\)-independent type. This agrees with the fact that the Ca\(^{2+}\)-independent isoforms of PKC expressed in cardiomyocytes are the \(\varepsilon\) and \(\sigma\) isoforms (33, 34) and that these isoforms can be activated by DAG. It was also reported that the Ca\(^{2+}\)-independent PKC, but not the Ca\(^{2+}\)-dependent isoforms, are activated by \(\alpha\)-adrenoceptor stimulant and phorbol ester (33, 34). GF-109203X and Gö6976 alone produced positive inotropy, which indicates constitutive PKC-mediated reduction of contractile force. Although our present results show that only the Ca\(^{2+}\)-independent isoform is involved in \(\alpha\)-adrenoceptor-mediated negative inotropy, both Ca\(^{2+}\)-dependent and independent isoforms are present in the mouse heart and can activate sodium-calcium exchanger (35).

PE-induced, but not PMA-induced negative inotropy was augmented by the DAG lipase inhibitor RHC-80267 (Fig. 3), indicating involvement of DAG as the activator of PKC. Although breakdown of phospholipids by phospholipase C is generally thought to be the major source of DAG, successive breakdown by PLD and PPH is also known as an alternative pathway for DAG production. In the present study, PE- and PMA-induced negative inotropy was inhibited by C2-ceramide (Fig. 4), which inhibits PLD activation, and 50 \(\mu\)M propranolol (Fig. 5), which inhibits PPH at this concentration. Although earlier reports indicate that \(\alpha\)-adrenoceptor stimulation increases the PLC activity (1, 36), U-73122 did not affect the PE- and PMA-induced negative inotropic response. These results suggest that the negative inotropic response to \(\alpha\)-adrenergic stimulation is mediated by PLD and PPH rather than PLC. As for the mechanisms leading to activation of PLD, there is evidence for involvement of soluble tyrosine kinase following stimulation of heterotrimeric G protein-coupled receptors (37). In the present study, lavendustin-A inhibited the PE-induced, but not PMA-induced, negative inotropic effect (Fig. 6), which suggests that tyrosine kinase is involved at a point upstream of PKC activation.
Myocardial and vascular PLD is known to be activated not only by $\alpha$-adrenoceptor stimulation (38, 39) but also by various agonists (37, 40, 41) including PE, endothelin-1, and angiotensin II. These agonists are known to produce sustained negative inotropy in adult mouse ventricle (6 – 12). While generation of DAG by PI-PLC is generally transient, that by the PLD-PPH pathway is considered to be long lasting (42). Thus, involvement of the PLD-PPH pathway in the $\alpha$-adrenoceptor-mediated negative inotropy may be related to its sustained nature. It is known that cardiac $\alpha$-adrenoceptor-mediated inotropy is altered under conditions such as hypertrophy and ischemia. It is also suggested that PLD activity is altered under such conditions (43, 44). Thus, the present study raises the possibility that changes in PLD activity contribute to the changes in myocardial response to $\alpha$-adrenoceptor stimulation under certain pathophysiological conditions.

In summary, we pharmacologically studied the intracellular signalling pathway for the $\alpha$-adrenoceptor-mediated negative inotropy in mouse ventricle and obtained results suggesting the involvement of activation of tyrosine kinase, PLD, PPH, and Ca$^{2+}$-independent PKC (Fig. 7). This is the first evidence for involvement of the PLD/PPH pathway in regulation of cardiac contractile function by neurotransmitters.

References


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