Prostanoid F receptors elicit an inotropic effect in rat left ventricle by enhancing myosin light chain phosphorylation

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Aims The aims of this study were to determine if the prostanoid F receptor (FPR)-mediated inotropic effect in rat ventricle is mediated by increased phosphorylation of myosin light chain-2 (MLC-2) and to elucidate the signalling pathway(s) activated by FPRs to regulate MLC-2 phosphorylation.

Methods and results Contractility was measured in left ventricular strips from adult male rats. Strips were also snap-frozen, and changes in the phosphorylation level of both MLC-2 and myosin phosphatase targeting subunit-2 (MYPT-2) were quantified. FPR stimulation with fluprostenol increased contractility by 100% above basal and increased phosphorylation of both MLC-2 (by 30%) and MYPT-2 (by 50%). The FPR-mediated inotropic effect and MLC-2 phosphorylation were reduced by a similar magnitude in the presence of the myosin light chain kinase (MLCK) inhibitor ML-7 (60–70%) and an inhibitor of Ca2+ /calmodulin, W-7 (35%). Inhibition of Rho-associated kinase by Y-27632 reduced the FPR-mediated inotropic effect and MLC-2 phosphorylation by 40–45% and MYPT-2 phosphorylation by 70%. ML-7 and Y-27632 together reduced contractility and MLC-2 phosphorylation by 70–80%. The FPR-mediated inotropic effect was only modestly affected by high concentrations of the inositol tris-phosphate (IP3) receptor blocker 2-APB, but not by the protein kinase C (PKC) inhibitor bisindolylmaleimide. Conclusion The FPR-evoked inotropic effect is mediated by increasing the phosphorylation of MLC-2 through regulation of both MLCK and myosin light chain phosphatase activities. The second messenger IP3 and PKC are unlikely to be involved in the signalling cascade of the FPR-mediated positive inotropic effect. Therefore, FPR signalling mechanism(s) regulating MLC-2 phosphorylation likely extend beyond those classically established for Gq/11-coupled receptors.

1. Introduction
Prostaglandins mediate a wide array of effects in different tissues. In the heart, prostaglandins have effects upon inflammation and cardiomyocyte hypertrophy. Prostaglandins have been shown to elicit inotropic effects in myocardium of various species. In human heart, protacynol increases cardiac output, but it is difficult to establish if this is a direct effect, or an effect of increased sympathetic drive secondary to the vasodilatory effect of protacynol. In rat ventricle, stimulation of the FPR evokes a marked inotropic effect. The cardiac inotropic effects of Gq/11-coupled receptors, such as the α1-adrenoceptor (α1-AR) and endothelin-1 receptor, occur at least partly through myofilament Ca2+ sensitization by increased phosphorylation of MLC-2. In smooth and cardiac muscle, the level of MLC-2 phosphorylation is regulated through both Ca2+/calmodulin-dependent activation of myosin light chain kinase (MLCK) and Rho-associated kinase (ROCK)-mediated inhibition of myosin light chain phosphatase (MLCP). Stimulation of the Gq/11-coupled FPR was reported to elicit an inotropic effect in rat left ventricle and increase levels of the second messengers IP3 and diacylglycerol (DAG). Although several mechanisms have been proposed, the mechanism underlying the FPR-mediated inotropic effect remains unknown. Since the FPR is coupled to Gq/11 as is the α1-AR, it seems likely that FPR stimulation utilizes the same mechanism of enhancing MLC-2 phosphorylation for inotropic support. Increased phosphorylation of myosin light chain enhances Ca2+ sensitivity of the myofilaments in vascular smooth muscle during PGF2α-evoked contractions. Therefore, the primary objective of these studies was to determine if increasing MLC-2 phosphorylation was a requisite for FPR-mediated inotropic effects. We also wanted to determine...
if both MLCK and MLCP activities could be regulated by FPR stimulation. Lastly, we evaluated the role of the classical \( G_{\text{q/11}} \)-coupled second messengers DAG and IP\(_3\).

Our data show that the FPR stimulation increases MLC-2 phosphorylation through both activating MLCK and inhibiting MLCP. The classical second messengers of \( G_{\text{q/11}} \)-coupled receptors are unlikely signalling candidates for the FPR-mediated inotropic effect.

2. Materials and methods

2.1 Animals

Animal care was according to the Norwegian Animal Welfare Act, which conforms to the European Convention for the protection of Vertebrate animals used for Experimental and other Scientific Purposes (Council of Europe no. 123, Strasbourg 1985) and were approved by the Norwegian National Animal Research Committee. Male Wistar rats of approximately 250–350 g were anesthetized (2–3% isoflurane in air) and subsequently euthanized, and the hearts were harvested.

2.2 Isolated papillary muscles and ventricular strips

Left ventricular strips and posterior papillary muscles were prepared and the contraction-relaxation cycles recorded and analysed as described previously.\(^\text{18}\) Maximal development of force \((dF/dt)_{\text{max}}\) was measured and inotropic responses to agonists were expressed by increases in \((dF/dt)_{\text{max}}\). The contractile parameters at the end of the equilibration period were used as basal (control) values. The experiments were performed in the presence of blockers (added 90 min prior to agonist) of adrenergic (prazosin 1 \(\mu\)M, timolol 1 \(\mu\)M) and muscarinic cholinergic (atropine 1 \(\mu\)M) receptors. Other inhibitors, when used, were added to the muscles 45 min before the agonist. The values after agonist responses were expressed as a percentage of the values in the control period (100%) before the addition of inhibitors. Agonist was added cumulatively (concentration–response curves) or as a single bolus in the presence or absence of the inhibitors until the maximal response was obtained. Following force measurements, papillary muscles were immediately frozen in liquid nitrogen.

2.3 Sample preparations

Frozen tissue samples (10–20 mg) were homogenized in 700 \(\mu\)L ice-cold buffer containing 10% trichloroacetic acid, 90% acetone, 10 mM dithiothreitol (DTT) and phosphatase inhibitors (20 mM NaF and 0.1 mM calyculin A). The resulting homogenate with the precipitated proteins was mixed with an equal volume of cold 1 M KCl and incubated for 10 min at 4°C to prevent the aggregation of the actin and myosin myofilaments. In our experience, this step results in a more equal loading of protein amongst the samples. The homogenate was then centrifuged (20000 g, 4°C, 10 min) and washed twice with 10 mM DTT, 0.1 mM calyculin A, and 20 mM NaF. The vacuum dried pellets were dissolved in ice-cold urea sample buffer at pH 8.6 at room temperature (8 M urea, 20 mM Tris–HCl, 22 mM glycine, and 10 mM DTT). The samples were then sonicated on ice and left for 2 h at 4°C while being rotated. The sonication step was repeated. The samples were sheared through ice-cold needles before they were spun down for 1 min (20 000 g at 4°C). Total protein concentration of tissue homogenate supernatant was determined according to Bradford\(^\text{19}\) and the samples were diluted with urea sample buffer to equalize total protein between samples. In addition, 10% of 0.0125% bromophenol blue with 10% glycerc was added to the MLC-2 samples and 1X SDS-loading buffer (50 mM Tris–HCl, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 10% \(\beta\)-mercaptoethanol, pH 6.8 at room temperature) was added to the MYPT samples. Samples for electrophoresis were snap-frozen in liquid nitrogen and stored at −80°C until use.

2.4 MLC-2 phosphorylation

The level of MLC-2 phosphorylation was analysed using glycerol polyacrylamide gel electrophoresis (40% glycerol/12.5% acrylamide) separating proteins by charge-to-mass ratio.\(^\text{20}\) This method separates the phosphorylated and non-phosphorylated MLC-2 on the same gel, allowing simultaneous probing of phosphorylated and total MLC-2. The gels were pre-run for 90 min at 400 V before sample application (6 \(\mu\)g/lane) and then run for 90 min at 100 V and 100 min at 500 V. The proteins were blotted to polyvinylidene difluoride (PVDF) membranes, blocked with 5% non-fat dry milk in phosphate buffered saline with 0.05% Tween-20, and hybridized with mouse anti-ventricular MLC-2 monoclonal antibody (Cat 1150-S, 1:1000, GE Healthcare), followed by a horseradish peroxidase-conjugated secondary anti-mouse antibody (GE Healthcare). Protein bands were visualized by chemiluminescence and quantified by densitometry. The data are reported as phosphorylated MLC-2 in percent of total MLC-2. The effect of fluprostanol in the presence of inhibitors was calculated as the percent change relative to the inhibitor alone in ventricular strips taken from the same heart.

2.5 MYPT-2 phosphorylation

MYPT-2 phosphorylation was determined by 6% SDS–polyacrylamide gel electrophoresis (SDS–PAGE), separating proteins based on size.\(^\text{15}\) After electrophoresis, proteins (6.75 \(\mu\)g/lane) were blotted onto a PVDF membrane, blocked with 5% non-fat dry milk in phosphate-buffered saline with 0.05% Tween-20, and incubated with anti-phospho MYPT recognizing Thr696 of rat MYPT-1 and a corresponding site on rat MYPT-2 (Cat 07-251, 1:2000, Upstate) followed by a horseradish peroxidase-linked secondary antibody (GE Healthcare) and visualization by chemiluminescence using a UCP Sensicam (UVP Inc., CA, USA). Membranes incubated initially with anti-phospho MYPT antibody were stripped with 0.5 M NaOH, re-blocked and re-probed with anti-MYPT-1/MYPT-2 (Cat 1488-1, 1:4000, Epitomics) for the determination of total MYPT. Changes in MYPT-2 phosphorylation were reported as relative changes to its own control value (non-stimulated). In studies conducted in the presence of inhibitors, the effect of fluprostanol upon MYPT-2 phosphorylation in the presence of inhibitors was calculated according to the following formula and reported as the percentage of the effect of fluprostanol in the absence of inhibitors: (fluprostanol in the presence of the inhibitor/inhibitor alone)/(fluprostanol alone/no drug).

2.6 Statistics

Data are expressed as mean ± SEM from \(n\) animals. \(p < 0.05\) was considered statistically significant (student’s \(t\)-test and ANOVA). When appropriate, Bonferroni corrections were made to control for multiple comparisons.

3. Results

3.1 FPR stimulation elicits a large inotropic effect qualitatively similar to \(\alpha_1\)-AR stimulation

FPR stimulation with the selective agonist fluprostanol first induced a small transient negative, followed by a large and sustained positive, inotropic response similar to phenylephrine stimulation of the \(\alpha_1\)-AR (Figure 1A and B). The transient negative inotropic component was \(11 ± 1\% (n = 25)\) and \(29 ± 2\% (n = 23)\) below basal for FPR and \(\alpha_1\)-AR, respectively. The maximal positive inotropic response evoked by fluprostanol (100 nM) was \(102 ± 4\% (n = 30)\) above basal and \(67 ± 5\% (n = 23)\) for phenylephrine (100 \(\mu\)M) (Figure 1C). The positive inotropic response elicited by fluprostanol and phenylephrine, approximated 75
and 49%, respectively, of the maximal inotropic effect of 10 \( \mu \)M isoproterenol (139 ± 9% above basal, \( n = 12 \)) (Figure 1C). Fluprostenol concentration–response curves without and with 3.9 \( \mu \)M or 11.1 \( \mu \)M of the FPR antagonist AL-8810 were nearly parallel with \(-\log EC_{50} (M)\) values of 8.7 ± 0.1, 8.1 ± 0.1 and 7.6 ± 0.1, respectively (Figure 2). The inhibition constant (\( K_b \)) calculated for AL-8810 was 1.0–1.3 \( \mu \)M (\( pK_b \) = 6.0–5.9), consistent with the reported affinity of AL-8810 at FPRs in vascular smooth muscle cells.21 Concentrations of AL-8810 up to 10 \( \mu \)M are selective for the FPR.21 The positive inotropic response evoked by 100 nM fluprostenol is likely through FPR activation due to its high selectivity for FPR.22 AL-8810 caused no significant change in basal or maximal fluprostenol-induced positive inotropic response (42 ± 6% above baseline without and 36 ± 3% with AL-8810). The lower maximal positive inotropic response in these experiments compared to a single concentration (100 nM fluprostenol) likely reflects desensitization. The maximal positive inotropic response evoked by phenylephrine did not differ significantly between a single saturating concentration and that obtained in concentration–response curves (66 ± 4.5 vs. 59.5 ± 3.8% above baseline).

### 3.2 Characteristics of the contraction–relaxation cycle

The contraction–relaxation cycle was not shortened by fluprostenol or phenylephrine indicating a lack of a lusitropic effect (Figures 1A and B, inset graphs). As expected, \( \beta \)-adrenergic receptor stimulation with isoproterenol produced a large lusitropic effect [reduced time to peak force (TPF) and relaxation time (RT)], a characteristic of activation of the cAMP/PKA pathway (Figure 1D).18 The lack of a lusitropic effect of fluprostenol is shared with agonists acting on \( G_{q} \)-coupled receptors such as the \( \alpha_1 \)-adrenergic and 5-HT_{2A} serotonin receptors.12,18

### 3.3 FPR stimulation increases phosphorylation of MLC-2 and MYPT-2

In the absence of agonist stimulation (basal) 21.0 ± 0.8% (\( n = 19 \)) of the total MLC-2 was phosphorylated. Fluprostenol (100 nM) increased the percentage of MLC-2 phosphorylated to 27.4 ± 1.0% (\( n = 19 \)), representing a 29.4% increase in phosphorylated MLC-2 above basal (Figure 3A and B). Fluprostenol (100 nM) increased MYPT-2 phosphorylation by 49 ± 6% above basal (\( n = 18 \); Figure 3A and B). The absolute
phosphorylation level of MYPT-2 in percent of total MYPT-2 could not be determined since estimates of non-phosphorylated and phosphorylated MYPT-2 levels were done separately using different antibodies.

3.4 Effect of myosin light chain kinase inhibition
MLCK inhibition attenuates the α1-AR inotropic effect and phosphorylation of MLC-2 in rat heart. Thus, we hypothesized that FPR activation enhanced MLC-2 phosphorylation through similar mechanisms. The selective MLCK inhibitor ML-7 (20 μM) significantly reduced the FPR-mediated inotropic effect by ~70% [35 ± 4% (n = 9) above basal with ML-7 vs. 102 ± 5% (n = 30) without; P < 0.05] (Figure 4A). ML-7 reduced basal contractility by 15 ± 4% (n = 19). ML-7 reduced the fluprostenol-induced increase of MLC-2 phosphorylation by 57 ± 5% (Figure 4B). Phosphorylation of MYPT-2 trended lower (~18% decrease), but was not significantly reduced by ML-7 (Figure 4F). These data indicate that FPR-stimulated activation of MLCK contributes to both enhanced MLC-2 phosphorylation and the inotropic effect.

3.5 Effect of calmodulin inhibition
The Ca²⁺-binding protein calmodulin (CaM) activates MLCK in smooth muscle, inducing contraction through enhancing MLC phosphorylation. Therefore, we evaluated the effect of inhibiting CaM (with 50 μM W-7) upon the FPR-mediated inotropic effect. In the presence of W-7, the fluprostenol-induced inotropic effect was reduced by ~33% [69 ± 5% above basal with W-7 (n = 10) vs. 102 ± 5% without (n = 30); P < 0.05; Figure 4A]. W-7 reduced basal contractility by 17 ± 1% (n = 20). W-7 reduced the fluprostenol-induced increase of MLC-2 phosphorylation by 35 ± 10% (Figure 4C). Phosphorylation of MYPT-2 was not affected by W-7 (Figure 4F). These data suggest a possible role for CaM in the activation of MLCK.

3.6 Effect of Rho-associated kinase inhibition
ROCK inhibition attenuates the α1-AR inotropic effect in rat heart. The phosphorylation status of MLC-2 is regulated by both MLCK and MLCP. The ability of MLCP to dephosphorylate MLC-2 is decreased by ROCK-mediated phosphorylation of MYPT. Therefore, we evaluated the effect of the ROCK inhibitor Y-27632 upon the FPR-mediated effects. Inhibition of ROCK by Y-27632 (50 μM) significantly reduced the FPR-mediated inotropic effect by ~45% [46 ± 4% above basal with Y-27632 (n = 12) vs. 102 ± 5% without (n = 30); P < 0.05; Figure 4A]. Y-27632 reduced basal contractility by 17 ± 2% (n = 22). Y-27632 reduced the fluprostenol-induced increase of MLC-2 phosphorylation by 40 ± 9% (Figure 4D). Phosphorylation of MYPT-2 was reduced by ~72% by Y-27632.
The inhibition profile of Y-27632 indicates that the phosphatase is also involved, i.e. decreased MLCP activity likely contributes to the increased phosphorylation of MLC-2 and subsequent inotropic effect of FPR stimulation.

3.7 Effect of simultaneous inhibition of MLCK and ROCK activity

We evaluated the effect of blocking both MLCK and ROCK activity simultaneously. In the presence of both ML-7 (20 µM) and Y-27632 (50 µM), the fluprostenol-induced inotropic effect was reduced by ~89% [10 ± 3% above basal with both inhibitors (n = 13) vs. 102 ± 5% (n = 30) without inhibitors, Figure 4A], a significantly larger decrease compared with ML-7 alone (~70%). Combining ML-7 and Y-27632 further reduced the fluprostenol-induced increase of MLC-2 phosphorylation by 70 ± 9% compared to the effect of either ML-7 (57 ± 5%) or Y-27632 (40 ± 9%) alone (Figures 4B, D, and E). In the presence of both W-7 (50 µM) and Y-27632 (50 µM), the fluprostenol-induced inotropic effect was reduced by ~64% [38 ± 5% above basal with both inhibitors (n = 13) vs. 102 ± 5% (n = 30) without inhibitors, Figure 4A], a lesser decrease than observed after combining ML-7 and Y-27632. Combining W-7 and Y-27632 further reduced the fluprostenol-induced increase of MLC-2 phosphorylation (55 ± 8%) although the effect was not significantly different from Y-27632 (40 ± 9%) alone (Figures 4B, D, and E). Basal contractility was

(Figure 4F). The inhibition profile of Y-27632 indicates that the phosphatase is also involved, i.e. decreased MLCP activity likely contributes to the increased phosphorylation of MLC-2 and subsequent inotropic effect of FPR stimulation.
reduced by \(\sim 26\%\) in the combined presence of ML-7 and Y-27632 and by \(\sim 33\%\) in the combined presence of W-7 and Y-27632, slightly greater reductions than by ML-7 or Y-27632 alone (15 and 17\%, respectively). These data further support that both MLCK and MLCP are involved in the FPR-mediated increase of MLC-2 phosphorylation.

### 3.8 Effects of inhibiting the G_q second messengers DAG and IP_3

The FPR signals through G_q \(^{24}\) and FPR stimulation in rat ventricular cardiomyocytes increases the formation of IP_3 and DAG. Their role in mediating the FPR inotropic effect, however, remains unknown. Therefore, we evaluated the effects of inhibiting the downstream targets of IP_3 and DAG, the IP_3 receptor and PKC, respectively. The FPR-mediated inotropic effect was slightly, but significantly reduced (\(\sim 23\%)\) in the presence of the IP_3 receptor blocker 2-aminoethoxydiphenylborate (2-APB; 20 \(\mu M\)) (76 \(\pm 4\%\) above basal with 2-APB vs. 99 \(\pm 7\%\) without, \(n = 9\); \(P < 0.05\); Figure 5A). Although 20 \(\mu M\) 2-APB produced a modest, but significant rightward shift (\(\sim 0.2\) log unit) in the concentration–response curve of fluprostenol, the shift was not significantly greater than that produced by 2 \(\mu M\) 2-APB (Figure 5B). The maximum positive inotropic response developed in the concentration–response experiments was not significantly different between the three groups (42.6 \(\pm 4.9\%\) and 50.2 \(\pm 4.3\%\) above basal with 2 \(\mu M\) and 20 \(\mu M\) 2-APB, respectively, and 44.4 \(\pm 4\%\) without inhibitors). Basal contractility was reduced by \(\sim 17\%\) in the presence of 2-APB. The \(\alpha_1\)-AR-mediated inotropic effect was also attenuated by 2-APB by a similar magnitude (23 \(\pm 11\%\); \(n = 9\)) To determine if activation of CaM is dependent upon Ca^{2+} released from IP_3 receptor sensitive intracellular stores, we evaluated the effect of blocking simultaneously the IP_3 receptor by 2-APB and CaM directly by W-7. The fluprostenol-induced inotropic effect was reduced by 57 \(\pm 6\%\) when combining W-7 and 2-APB, a value significantly greater than the individual effect of each inhibitor and similar to the sum of the individual effects of W-7 and 2-APB that is 62 \(\pm 5\%\). These data indicate that the inhibitory effect of 2-APB occurs independently of the activation of CaM, reducing the likelihood that CaM is dependent upon IP_3 receptor-mediated Ca^{2+} release. The non-selective PKC inhibitor bisindolylmaleimide (BIM) abolished the transient negative inotropic effect of fluprostenol or phenylephrine, as previously found for the 5-HT_2A receptor. However, BIM did not reduce the positive inotropic effect induced by either fluprostenol or phenylephrine (Figure 5A, data not shown for phenylephrine) in agreement with previous data.

**Figure 5** (A) Effect of the IP_3 receptor blocker 2-APB (20 \(\mu M\); \(n = 9\)) or the PKC inhibitor BIM (10 \(\mu M\); \(n = 8\)) upon the fluprostenol (100 nM)-mediated inotropic effect. Data are mean \(\pm\) SEM presented as percent increase above basal. *\(P < 0.05\) vs. fluprostenol alone. (B) Concentration–response curves for fluprostenol in left ventricular strips without (\(n = 15\)) or in the presence of 2 \(\mu M\) (\(n = 6\)) or 20 \(\mu M\) 2-APB (\(n = 13\)). The inotropic responses are expressed in percent of maximum in each experiment. *\(P < 0.05\) vs. fluprostenol in the absence of inhibitor.

### 4. Discussion

These data indicate that FPR stimulation evokes a robust positive inotropic effect in rat left ventricle through increasing the phosphorylation level of MLC-2. Both MLCK and MLCP are involved in the FPR-mediated increase of MLC-2 phosphorylation. FPR stimulation increases phosphorylation of MYPT-2 by activating ROCK, decreasing MLCP activity. We propose that the FPR increases myofilament sensitivity to Ca^{2+} through enhancing MLC-2 phosphorylation, analogous to that proposed for the \(\alpha_1\)-AR and endothelin receptors in normal myocardium and the 5-HT_2A receptors in failing myocardium. This study did not support a role for the G_q/11-coupled second messenger IP_3 or PKC in regulating the phosphorylation of MLC-2.

FPR stimulation elicits an inotropic response qualitatively similar to that of \(\alpha_1\)-AR, composed first of a relatively fast transient negative followed by a sustained positive effect (Figure 1). The FPR-mediated inotropic response develops relatively slowly, and is characterized by a symmetrical change in the contraction–relaxation cycle with unchanged or slightly prolonged TPF and RT (Figure 1D), qualitative characteristics typical of cardiac G_q-coupled receptors.

The MLCK inhibitor ML-9 selectively inhibits cAMP- independent inotropic effects in heart tissue indicating that these effects are mediated via increased MLC-2 phosphorylation. In the present study, the ventricular FPR-mediated
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inotropic response was significantly attenuated by ML-7, another potent selective inhibitor of MLCK. Likewise, ML-7 reduced the FPR-mediated increase of phosphorylated MLC-2 by a similar percentage as the inotropic effect. In smooth muscle, MLC is activated by the Ca^{2+}-binding protein CaM. Although inhibition of CaM with W-7 attenuated the FPR-mediated inotropic effect and phosphorylation of MLC-2 by similar magnitudes, it was significantly less efficacious than ML-7 (~35 vs. ~60–70% decrease, respectively). Possibly, 50 μM W-7 does not block CaM as efficiently in ventricular strips as in bovine smooth muscle. This seems likely, since the FPR-mediated inotropic effect was further reduced (~55% decrease) in the presence of 100 μM W-7 (data not shown). Alternatively, a cardiac specific isoform of MLCK that is not regulated by CaM may contribute to the FPR-mediated increase of MLC-2 phosphorylation. The slightly larger inhibitory effect of ML-7 compared to 100 μM W-7 is most likely due to partial blockade of ROCK in addition to MLCK. Twenty micromolar ML-7 reportedly inhibited ROCK activity by ~26%,28 a value corresponding with the modest reduction (~18%) of FPR-mediated MYPT-2 phosphorylation (Figure 4E). As expected, MYPT-2 phosphorylation was not reduced by blockade of CaM by W-7, consistent with its reported specificity for CaM.

There is increasing evidence that the RhoA/ROCK pathway is involved in agonist induced inotropic effects.10,12 In the present study, the FPR-mediated inotropic effect was significantly attenuated by Y-27632, a selective ROCK inhibitor,29 when added prior to or during the steady-state response to fluprostenol. Furthermore, Y-27632 reduced the fluprostenol-stimulated increase in MLC-2 phosphorylation by a similar magnitude. These data indicate that FPR activation also signals through the RhoA/ROCK pathway, similar to the stimulation of the 5-HT_{2A}12 and α_{1}-AR.10,11.

To the best of our knowledge, these data are the first to demonstrate in intact cardiac tissue that inotropic effects are mediated in part through activation of ROCK increasing phosphorylation of the binding protein MYPT, possibly resulting in functional inhibition of MLCP. These findings are compatible with an earlier study reporting a similar reduction of agonist-induced MYPT phosphorylation by Y-27632 in normal rat ventricular cardiomyocytes.15 Furthermore, Okamoto et al.30 reported that ROCK-mediated phosphorylation of MYPT nearly abolished the activity of MLCK-phosphatase (PP1c) in virus-transfected rat cardiomyocytes. Many reports have documented a connection between RhoA signalling and cardiac MLC-2 phosphorylation. Rajashree et al.15 reported that an increased Ca^{2+} sensitivity of tension in myocytes is dependent on ROCK activation. In addition, the pattern of MYPT phosphorylation in unstimulated heart correlated with the spatial gradient of MLC-2 phosphorylation levels.15 Although our data are consistent with a role for MYPT phosphorylation in mediation of the FPR-evoked inotropic effect, it is not certain if changes in MLCP activity are involved, since phosphatase 1 (PP1) activity was not measured in the current study. However, it seems likely since phosphorylation of the inhibitory site (Thr696) in MYPT by ROCK promotes dissociation of the trimeric holoenzyme (MLCP), inhibiting the translocation of PP1 to the myofilaments.15,30,31 Further, increased phosphorylation of MYPT is associated with decreased PP1 activity and increased MLC-2 phosphorylation in smooth muscle and rat ventricular cardiomyocytes.15,32

The FPR couples to G_{q/11}24 and FPR activation increases the formation of IP_{3} and DAG in rat ventricle,9 the latter activating most PKC isoforms.33 In this study, the FPR- and α_{1}-AR-mediated inotropic effects were not reduced by inhibition of DAG-activation of most PKC isoforms using the non-subtype selective PKC inhibitor BIM.28,34 These data do not support a role for PKC in either the FPR- or α_{1}-AR-mediated positive inotropic effect. The latter finding is in agreement with the study of Andersen et al.,10 and both findings correspond to that reported at the 5-HT_{2A} receptor.12 However, in mouse atrium, inhibition of PKCs enhanced G_{q}-coupled α_{1}-AR responses.36 In contrast, the transient negative inotropic effect induced by both fluprostenol and phenylephrine was abolished by PKC inactivation with BIM. These data are in agreement with the observed PKC-dependent α_{1}-AR and 5-HT_{2A} receptor-mediated negative inotropic response in rat myocardium.12,37 BIM also increased basal contractility by 52 ± 5% (n = 22) 20–30 min after administration (data not shown), consistent with reports indicating that PKCα mediates a negative inotropic effect in the heart.38–40 Furthermore, both fluprostenol- and phenylephrine-induced inotropic effects were of a similar magnitude in the absence or presence of BIM, despite the large increase in basal contractility, highlighting the dissociation of the effect of PKC inhibition upon basal contractility as opposed to the FPR-mediated positive inotropic effect.

It seems unlikely that IP_{3} plays a pronounced role in both the FPR- and α_{1}-AR-mediated inotropic effects, since the magnitude of inhibition by blockade of the IP_{3} receptor was modest and significantly less than that observed by inhibition of either MLCK or ROCK (Figures 4 and 5). The effects of 2-APB are difficult to explain since IP_{3} receptors are not present in the sarclemma or in the sarcoplasmic reticulum in rat ventricular cardiomyocytes.41 In contrast, IP_{3} receptors are localized to the junctional sarcoplasmic reticulum in rat atrial myocytes42 and may participate in arrhythmogenesis.42,43 Possibly, the modest effect of 2-APB in the present study could result from inhibition of IP_{3} receptors residing in the nuclear envelope,41 known to connect with the sarcoplasmic reticulum.44 Since increasing the dose of 2-APB from 2 to 20 μM did not produce an expected further rightward shift in the fluprostenol concentration-response curve (Figure 5B), the effect of 2-APB is more likely a non-specific effect upon contractility, possibly through an inhibitory effect on store-operated Ca^{2+} channels.45

Taken together, our data indicate that the signal transduction pathway underlying the FPR-mediated inotropic effect likely extends beyond the classical pathway established for G_{q/11}-coupled receptors. Recently, it has been shown that G_{12/13} can activate ROCK through the signalling pathway of leukaemia-associated Rho guanine nucleotide exchange factor (LARG) activation of Rho.46 In vascular smooth muscle cells, the G_{q}-coupled endothelin and angiotensin receptors also couple to G_{12/13}, inducing cell contraction in a Rho/ROCK-dependent manner.47 It remains to be determined how FPR activation of G_{q/11} modulates ROCK and MLCK activity or whether the FPR also initiates downstream signalling by coupling to G_{12/13}.
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