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Differential regulation of Ca^{2+}-dependent Cl^- currents by FP prostanoid receptor isoforms in *Xenopus* oocytes

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Abstract

The FP_A and FP_B prostanoid receptor isoforms are G-protein-coupled receptors that are activated by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Differences in their carboxyl termini prompted us to examine the intracellular calcium (Ca^{2+}) signaling of these receptor isoforms using the *Xenopus* oocyte expression system. Protein expression was determined by immunofluorescence microscopy and whole cell binding with [^{3}H]$PGF_{2\alpha}$. Positive immunolabeling was observed on the outer membranes of oocytes expressing FLAG-tagged FP receptor isoforms, but not on control (water-injected) oocytes. Intracellular signaling was examined using a two-electrode voltage clamp. Specific whole-cell binding was also detected for both receptor isoforms. Bath application of 10 μM $PGF_{2\alpha}$ to FP_A-expressing oocytes produced a chloride (Cl^-) current response similar to that of an injection of inositol 1,4,5-trisphosphate ($InsP_3$) (5.76 ± 0.6 μA, peak current; $N = 23$) that returned to control levels within 25 min. In FP_B-expressing oocytes the activation of the Cl^- current was delayed or completely absent (1.38 ± 0.2 μA, peak current; $N = 18$). Control oocytes were not responsive to the application of $PGF_{2\alpha}$ (0.87 ± 0.1 μA, peak current; $N = 10$). Activation of Cl^- currents for both FP receptor isoforms was dependent upon intracellular Ca^{2+} stores as a 30-min pretreatment with thapsigargin (1 μM; $N = 5$) blocked the $PGF_{2\alpha}$ induction of the Cl^- current. These data indicate that the FP prostanoid receptor isoforms differ in their ability to activate Ca^{2+}-dependent Cl^- channels when expressed in *Xenopus* oocytes. The difference appears to be in the ability of the two FP prostanoid receptor isoforms to mobilize intracellular calcium. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: *Xenopus*; Prostaglandin; $PGF_{2\alpha}$; Calcium; Chloride channel

1. Introduction

Intracellular calcium is an important signaling molecule in a variety of cells. Increases in the levels of intracellular calcium regulate responses including secretion, receptor internalization, cell division, migration, and cellular contraction [1]. Cytosolic calcium can be increased either by calcium influx from extracellular fluid or through the release of stored calcium from intracellular compartments [2]. Many GPCRs and tyrosine kinase receptors stimulate PLC and $InsP_3$, resulting in a transient increase in the levels of cytosolic calcium. Depletion of intracellular calcium stores then stimulates calcium entry through plasma membrane SOCs via a process known as SOCE [3,4].

In mammalian cells, $PGF_{2\alpha}$ acts on a subfamily of GPCRs, known as the FP prostanoid receptors, to stimulate the release of intracellular calcium. There are two FP receptor isoforms generated by alternative mRNA splicing, designated FP_A and FP_B. The two FP receptor isoforms are identical except that the FP_A isoform has an additional 46 amino acids at its carboxyl terminus. Both receptor isoforms are coupled to the $InsP_3$/DAG second messenger pathway through G_q [5]. When heterologously expressed in mammalian cells, the two FP receptor isoforms have similar binding affinities for $PGF_{2\alpha}$ and stimulate the formation of total inositol phosphates to a similar extent and with similar EC_{50} values [5]. However, basal levels of $InsP_3$ are higher in mammalian cells expressing the FP_B

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Abbreviations: GPCR, G-protein coupled receptor; PLC, phospholipase C; $InsP_3$, inositol 1,4,5-trisphosphate; SOC, store-operated calcium channel; SOCE, store-operated calcium entry; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; DAG, diacylglycerol; and PKC, protein kinase C.

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isoform than in cells expressing the FP_A isoform. Recently, we demonstrated that pretreatment with bisindolylmaleimide I, a PKC inhibitor, enhanced $PGF_{2\alpha}$ stimulation of total inositol phosphate accumulation in HEK cells stably expressing the FP_A receptor isoform; however, this increase was not observed in FP_B-expressing cells [6].

Xenopus oocytes provide a useful model system for studying receptor-mediated activation of intracellular calcium signaling. They endogenously express Ca^{2+}-activated Cl^- channels that respond rapidly to changes in intracellular calcium [7,8]. The pathways leading to Ca^{2+} activation of Cl^- channels in *Xenopus* oocytes have been well characterized, and distinct components of the chloride conductance have been described [9,10]. Machaca and Hartzell [11] identified two Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes that are selectively activated by calcium release from intracellular stores or by calcium influx. The Cl^- current activated by calcium release ($I_{Cl1\text{-}S}$) shows an outwardly rectifying steady-state current voltage relationship, whereas the Cl^- current activated by the influx of calcium (I_{Cl2}) shows an inwardly rectifying steady-state current voltage relationship [12]. In this study, we expressed the FP_A and FP_B prostanoid receptor isoforms in *Xenopus* oocytes and examined the ability of $PGF_{2\alpha}$ to stimulate these two components of the Ca^{2+}-activated Cl^- currents using a two-electrode voltage clamp.

2. Materials and methods

2.1. In vitro transcription of FP_A and FP_B RNA

The DNA coding for ovine FP_A and FP_B receptor isoforms (with and without a FLAG tag) was subcloned into a *Xenopus* expression vector (pXβGev). Plasmid DNA was linearized with *Bam*HI (37°), and capped cRNA transcripts were synthesized *in vitro* with T3 RNA polymerase. The DNA template was digested with RQ1 DNase (Sigma), and the cRNA was resuspended in diethyl pyrocarbonate (DEPC)-treated water before injection into oocytes.

2.2. Oocyte preparation and RNA injection

Oocytes were surgically removed from anesthetized, adult female *Xenopus laevis*. The follicular cell layer was removed by incubation in nominally calcium-free medium (OR-2: 104 mM NaCl, 3.3 mM KCl, 1.3 mM $MgCl_2$, 6.3 mM HEPES, 100 U/mL of penicillin, and 100 μg/mL of streptomycin, pH 7.6) containing collagenase (type I, 1.5 mg/mL; Worthington Biochemical) and trypsin inhibitor (type III-O chicken egg white, 0.5 mg/mL, Sigma) for approximately 2 hr. Oocytes were injected with 50 nL of sterile water as a control or 50 nL of sterile water containing FP_A RNA (0.2, 20, or 200 ng/μL) or FP_B RNA (0.2, 20, or 200 ng/μL) with the use of a positive displacement pipette (Drummond Scientific) and incubated for 2–5 days at 18° in culture medium (ND96: 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM sodium pyruvate, 100 U/mL of penicillin, and 100 μg/mL of streptomycin, pH 7.6).

2.3. Binding assays

Oocytes (7–10 per sample) expressing either FP_A or FP_B prostanoid receptors were transferred to glass tubes (Kimax-51, VWR) in ND96 culture medium. The oocytes were rinsed in ND96 and resuspended in volumes of 360 μL (total binding) and 320 μL (non-specific binding) of the same medium. Forty microliters of unlabeled $PGF_{2\alpha}$ (10^{-4} M final concentration) was added to the tubes in which non-specific binding was measured. Next, 40 μL of [^{3}H]$PGF_{2\alpha}$ was added to each sample (2.5 nM, final concentration) and incubated at room temperature for 1 hr. Then each sample was rinsed gently three times with 500 μL ND96 at room temperature. ND96 was carefully aspirated, scintillation fluid was added, and the intact oocytes were transferred to scintillation vials. Radioactivity was determined by liquid scintillation counting.

2.4. Confocal microscopy

Oocytes were injected with either 50 nL of sterile water or 50 nL of sterile water containing either FLAG-tagged FP_A RNA (20 ng/μL) or FLAG-tagged FP_B RNA (20 ng/μL) and incubated for 2–5 days at 18° in culture medium. Oocytes were fixed in 4% paraformaldehyde for 2 min and washed twice in ND96. After overnight incubation at 18° with the primary antibody (ANTI-FLAG® M1; 1:1000; Sigma), the oocytes were washed with ND96 and incubated for 1 hr at room temperature with the secondary antibody (goat anti-mouse-fluorescein isothiocyanate; 1:1000; Sigma). Oocytes were transferred to 4-chamber slides (Fisher Scientific) containing ND96 for viewing. Images were captured using a 10× objective (pinhole size 100) on a Leica TCS-4D laser scanning confocal microscope.

2.5. Electrophysiological methods

Oocytes were voltage clamped with two microelectrodes (GeneClamp 500, Axon Instruments), and traces were filtered at 2 kHz and digitized at 50–2000 μsec to obtain the fastest possible voltage clamp. Recordings were performed at room temperature with electrodes (1.2 to 3 MΩ) filled with 3 M KCl. The bath was grounded with a Ag/AgCl pellet. Oocyte resting membrane potentials ranged between −25 and −40 mV. The voltage protocol was adapted from Kuruma and Hartzell [10]; the membrane potential was held at −35 mV, stepped up to +40 mV for 1 sec, back to −140 mV for 1 sec, and then to +40 mV for 1 sec before returning to the holding potential. The steps were repeated every 10 sec. Stimulation and data acquisition were controlled by pCLAMP6 (Axon Instruments) via

a Digidata analog-to-digital-to-analog converter (Axon Instruments).

2.6. Solutions

Normal recording solution consisted of: 123 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1.8 mM $MgCl_2$, and 10 mM HEPES, with pH adjusted to 7.4 with NaOH. Zero-Ca^{2+} recording solution was the same as normal recording solution, except that $CaCl_2$ was omitted, $MgCl_2$ was increased to 5 mM, and 0.1 mM EGTA was added. Stock solutions of $InsP_3$ (Sigma) were made at 10 mM in H_2O, kept at -20°, and diluted to a final concentration of 1 mM using recording saline. BAPTA-AM (10 mM in DMSO) was diluted to a final concentration of 100 μM using normal recording solution.

3. Results

3.1. Stimulation of calcium-activated chloride currents by $PGF_{2\alpha}$ in Xenopus oocytes

The current responses to $PGF_{2\alpha}$ application for oocytes expressing the FP prostanoid receptor isoforms and control oocytes are shown in Fig. 1. *Xenopus* oocytes expressing either the FP_A or FP_B prostanoid receptor isoforms were initially examined using voltage protocols designed to monitor the effects of SOC ($I_{Cl1\text{-}S}$) and SOCE ($I_{Cl1\text{-}T}$) activation of the Ca^{2+}-activated Cl^- channels. The $I_{Cl1\text{-}S}$ (activated by release of Ca^{2+} from stores) current was measured at the end of the first 1-sec +40 mV(1) step. The $I_{Cl1\text{-}T}$ (dependent upon Ca^{2+} entry) current was measured as the peak time-dependent outward current during the second 1-sec +40 mV(2) step, evoked after the −140 mV step, which served to activate the I_{Cl2} current (Fig. 1; trace). Bath application of $PGF_{2\alpha}$ (10 μM, arrow) produced rapid outward current responses that returned to baseline within 25 min in oocytes expressing the FP_A prostanoid receptor isoform (Fig. 1A, $I_{Cl1\text{-}S}$). The SOCE elicited an outward current that was sustained and gradually increased over the sampling period (Fig. 1A; $I_{Cl1\text{-}T}$). In the absence of extracellular calcium, SOCE is prevented; $PGF_{2\alpha}$ (10 μM, arrow) produced a calcium-release-dependent outward current response in oocytes expressing the FP_A prostanoid receptor isoform (Fig. 1B), although the SOCE response was blocked, as expected. Identical sets of experiments were performed on oocytes expressing the FP_B prostanoid receptor isoform; bath application of $PGF_{2\alpha}$ (10 μM, arrow) produced a slow $I_{Cl1\text{-}T}$ current response that lacked the rapid outward current (Fig. 1C; $I_{Cl1\text{-}S}$). However, the SOCE response in FP_B-expressing oocytes was similar in both time course and peak amplitude to those seen in the FP_A-expressing oocytes (Fig. 1C; $I_{Cl1\text{-}T}$). In the absence of extracellular calcium, the SOCE response was blocked (Fig. 1D). Identical sets of experiments performed using oocytes expressing the FLAG-FP_A and FLAG-FP_B receptor isoforms produced identical responses (data not shown). Control oocytes did not respond to the application of $PGF_{2\alpha}$ (10 μM, arrow) with or without extracellular calcium (Fig. 1, E and F, respectively). These results demonstrate that the Ca^{2+}-activated Cl^- channel conductance is the result of $PGF_{2\alpha}$ stimulation of heterologously expressed FP_A and FP_B receptors.

3.2. Characterization of FP_A and FP_B prostanoid receptor expression in Xenopus oocytes

FP prostanoid receptor isoform expression was confirmed using immunocytochemistry and radioligand binding assays, each done in parallel with the same batch of oocytes (Fig. 2). Immunoreactivity was observed on the outer membrane of oocytes injected with FLAG-FP_A and FLAG-FP_B receptor cRNA (20 ng/μL) but was not observed in control oocytes (Fig. 2A). The same concentration of cRNA was injected into oocytes for each FP receptor isoform to determine the specific radioligand binding of [^{3}H]$PGF_{2\alpha}$. These experiments were performed on the same day, using the same population of injected oocytes. Both sets of expressing oocytes demonstrated levels of specific radioligand binding of [^{3}H]$PGF_{2\alpha}$ that were not significantly different (Fig. 2B). These data demonstrate that the difference in the activation of the Ca^{2+}-activated Cl^- channels by $PGF_{2\alpha}$ is *not* due to differences in the levels of plasma membrane expression between the two FP prostanoid receptor subtypes.

The dramatic differences in coupling to chloride currents seen between the two FP prostanoid receptor isoforms led us to examine the concentration-dependent effects of $PGF_{2\alpha}$ on chloride current activation in *Xenopus* oocytes. Fig. 3 shows the relationship between the peak outward current ($I_{Cl1\text{-}S}$) and the concentration of $PGF_{2\alpha}$ in oocytes expressing either the FP_A or FP_B prostanoid receptor isoforms. A concentration-dependent increase in peak current amplitude was observed in oocytes expressing both the FP_A and FP_B prostanoid receptor isoforms (Fig. 3A); however, the magnitude of the current evoked in oocytes expressing the FP_B prostanoid receptor isoform was significantly lower (Fig. 3A: open squares). Only at the highest concentration of $PGF_{2\alpha}$ (10 μM) examined was an outward current observed in FP_B-expressing oocytes. In contrast, FP_A receptors were capable of eliciting Cl^- currents at 100 nM $PGF_{2\alpha}$. Summaries of the peak current amplitudes ($I_{Cl1\text{-}S}$) to 10 μM $PGF_{2\alpha}$ are shown in Fig. 3B. These data suggest that the two FP prostanoid receptor isoforms differ in their abilities to mobilize calcium from intracellular stores.

3.3. Stimulation of thapsigargin-sensitive calcium stores by FP_A receptor agonist

We performed a series of experiments to assess $PGF_{2\alpha}$-induced responses after intracellular calcium stores had

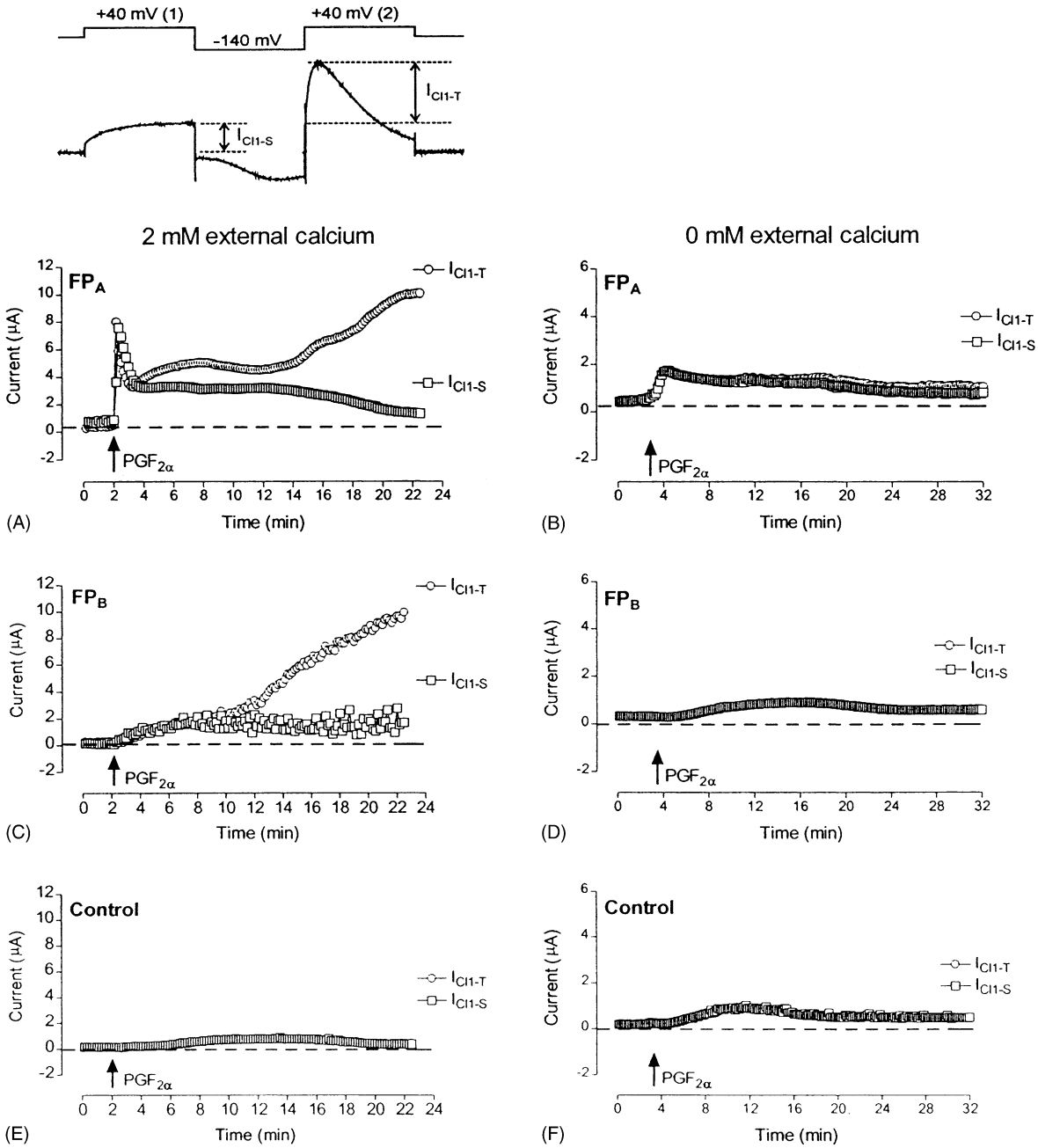

Fig. 1. Characterization of the Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes expressing the FP_A and FP_B prostanoid receptor isoforms. Bath application of $PGF_{2\alpha}$ (10 μM; arrow) produced a rapid outward current response at steps up to +40 mV ($I_{Cl1\text{-}S}$; open squares) and a slower developing outward current ($I_{Cl1\text{-}T}$; open circles) in oocytes expressing the FP_A prostanoid receptor isoform (panel A; N = 23). In the absence of extracellular calcium, $PGF_{2\alpha}$ was still capable of producing an outward current ($I_{Cl1\text{-}S}$; open squares) but failed to produce the slow outward current ($I_{Cl1\text{-}T}$; open circles) in oocytes expressing the FP_A prostanoid receptor isoform (panel B; N = 10). Bath application of $PGF_{2\alpha}$ (10 μM; arrow) failed to produce the rapid outward current response ($I_{Cl1\text{-}S}$; open squares) but did produce the slower outward current ($I_{Cl1\text{-}T}$; open circles) in oocytes expressing the FP_B prostanoid receptor isoform (panel C; N = 18). In the absence of extracellular calcium, $PGF_{2\alpha}$ failed to produce either response (panel D; N = 10). Control oocytes showed no response to $PGF_{2\alpha}$ in either situation (panels E and F; N = 10). An example of how $I_{Cl1\text{-}S}$ and $I_{Cl1\text{-}T}$ current values were measured is shown at the top of the figure. See text for an explanation of the voltage protocols.

been depleted with thapsigargin in order to identify the component of the Cl^- current response that was dependent upon Ca^{2+} release from intracellular stores. Oocytes expressing either FP_A or FP_B receptor isoforms were pretreated with 1 μM thapsigargin for 20 min in the absence of extracellular calcium. Treated oocytes were placed in the recording chamber with continued exposure to 1 μM thapsigargin in Ca^{2+}-free saline. Bath application of $PGF_{2\alpha}$ (10 μM) produced a rapid outward current ($I_{Cl1\text{-}S}$) in oocytes expressing the FP_A prostanoid receptor (Fig. 4A; top trace). Pretreatment with thapsigargin completely blocked the response to 10 μM $PGF_{2\alpha}$ in FP_A-expressing

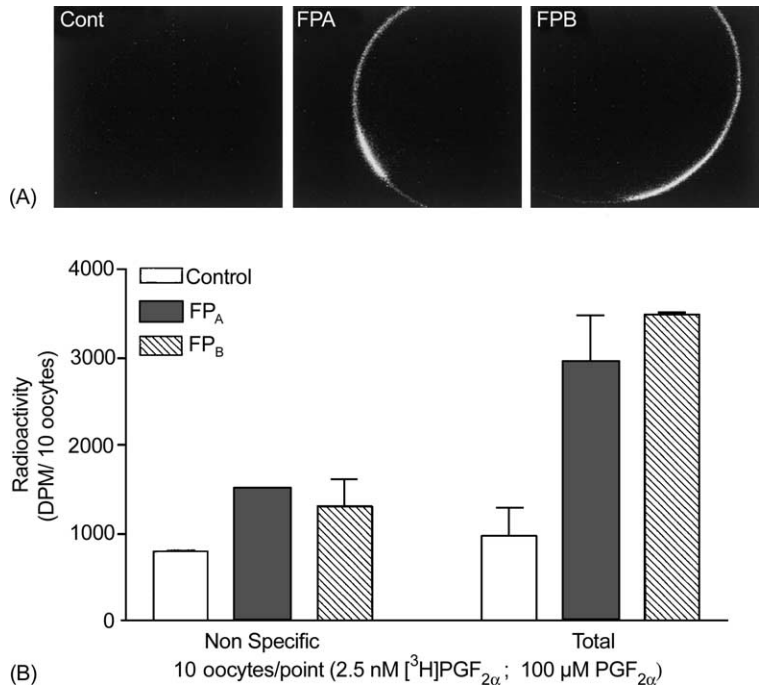

Fig. 2. Characterization of prostanoid FP_A and FP_B receptor isoforms expressed in *Xenopus* oocytes by confocal microscopy and radioligand binding assays. (A) Representative figures of control (water) oocytes and oocytes expressing the FLAG-tagged FP_A (FPA) and the FLAG-tagged FP_B (FPB) after labeling with primary ANTI-FLAG® M1 antibody. The secondary antibody was fluorescein isothiocyanate-goat anti-mouse immunoglobulin G. Immunofluorescence was visualized using a fluorescein filter (495 nm). (B) Radioligand binding assays in *Xenopus* oocytes expressing the FP prostanoid receptor isoforms. No significant differences were observed between the FP_A- and FP_B-expressing oocytes (N = 3). Values are means ± SEM. Data for both assays were obtained from the same population of oocytes and performed on the same day. Whole cell binding was performed as described in Section 2.

oocytes (Fig. 4A; bottom trace). An identical set of experiments using oocytes expressing the FP_B receptor isoform (Fig. 4B) showed that bath application of 10 μM $PGF_{2\alpha}$ failed to produce a current response with or without thapsigargin treatment. Summaries of the peak current amplitudes ($I_{Cl1\text{-}S}$) to 10 μM $PGF_{2\alpha}$ with and without thapsigargin treatment are shown (Fig. 4C). These data are consistent with the hypothesis that FP_A and FP_B prostanoid receptor isoforms differentially stimulate the release of intracellular calcium, and show that FP_A but not FP_B receptors induce calcium release from intracellular stores.

3.4. Depletion of intracellular calcium stores by the FP_A prostanoid receptor isoform

The extent of intracellular calcium mobilization by $PGF_{2\alpha}$ was tested in oocytes expressing either the FP_A or FP_B prostanoid receptor isoform by direct injection of $InsP_3$ (50 nL, 10 mM). Both FP_A- and FP_B-expressing oocytes produced rapid outward current ($I_{Cl1\text{-}S}$) responses to intracellular application of $InsP_3$ (data not shown). These data suggest that exogenous expression of the FP prostanoid receptor isoforms did not interfere with the normal mechanisms of intracellular signaling in the oocytes.

Fig. 5 demonstrates that FPA receptor activation by $PGF_{2\alpha}$ is capable of exhausting stored calcium. In Ca^{2+}-free recording saline, bath application of $PGF_{2\alpha}$ (10 μM) produced the rapid outward current ($I_{Cl1\text{-}S}$) response seen previously (Fig. 5A; arrow). After 20 min, the current response returned to baseline, and the oocyte was given time to equilibrate. During equilibration, a microinjection pipette containing 10 mM $InsP_3$ was inserted into the oocyte. The step protocol was continued, and 50 nL of $InsP_3$ solution was injected into the oocyte (arrowhead). Subsequent injection of $InsP_3$ was not able to produce an outward current response, suggesting that the intracellular stores had been depleted from the initial application of $PGF_{2\alpha}$. Furthermore, application of $PGF_{2\alpha}$ at the same time point failed to elicit a similar response (data not shown). Bath application of $PGF_{2\alpha}$ (10 μM) to FP_B-expressing oocytes produced a blunted outward current ($I_{Cl1\text{-}S}$) response that had a much longer time course (Fig. 5B; arrow). However, subsequent intracellular injection of $InsP_3$ (50 nL, 10 mM) produced a robust outward current with a peak amplitude similar to that seen in the FP_A-expressing oocytes after bath application of $PGF_{2\alpha}$ (Fig. 5B; arrowhead). A summary of the peak current ($I_{Cl1\text{-}S}$) amplitudes evoked in response to 10 μM $PGF_{2\alpha}$ and subsequent $InsP_3$ injection is shown (Fig. 5C). These data demonstrate that FP_A receptor activation produced a rapid release of intracellular calcium resulting in the depletion of the stores, whereas FP_B receptor activation resulted in an attenuated release from intracellular stores that could be stimulated further by $InsP_3$ injection.

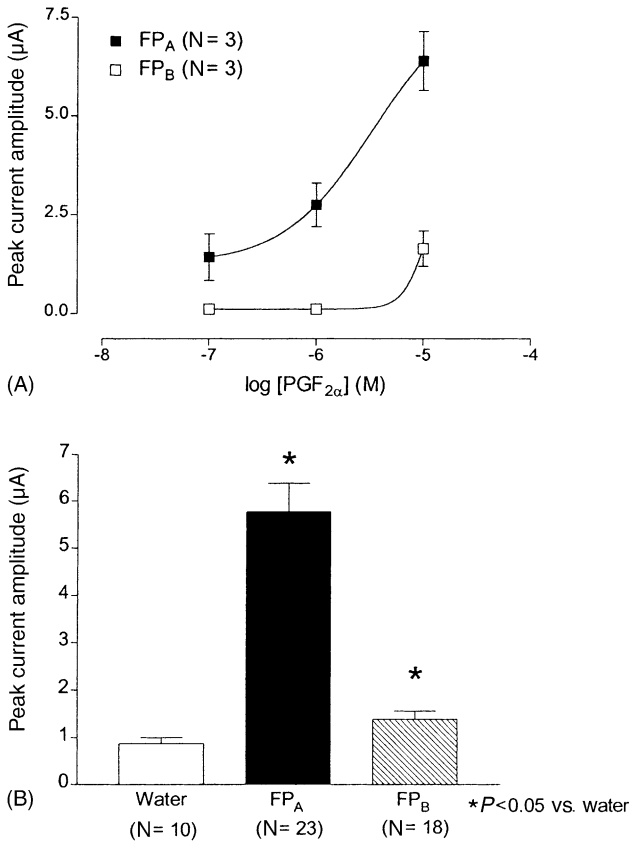

Fig. 3. Concentration–response relationship of the peak amplitude ($I_{Cl1\text{-}S}$) of the Ca^{2+}-activated Cl^- current in *Xenopus* oocytes expressing the FP prostanoid receptor isoforms. (A) $PGF_{2\alpha}$ concentration-dependently stimulated the Ca^{2+}-activated Cl^- current in both the FP_A (filled squares) and the FP_B (open squares) receptor isoforms with relative EC_{50} values of 3 and 9 μM, respectively. Data points are means ± SEM (N = 3). (B) Summary of the peak current amplitudes ($I_{Cl1\text{-}S}$) elicited by $PGF_{2\alpha}$ (10 μM). The peak current of FP_A-expressing oocytes (5.76 ± 0.6 μA, N = 23, $P < 0.001$) and FP_B-expressing oocytes (1.38 ± 0.2 μA, N = 18, $P = 0.023$) was significantly greater compared with the control (0.87 ± 0.1 μA, N = 10). Values are means ± SEM. Statistical differences were determined using Student's *t*-test.

3.5. Effect of a protein phosphatase inhibitor on $PGF_{2\alpha}$-mediated stimulation of calcium-activated chloride currents

Previous studies in our laboratory have demonstrated that the two FP prostanoid receptor isoforms are differentially phosphorylated [6]. We hypothesized that the difference in phosphorylation state may explain, in part, the differences in the ability of FP receptor isoforms to release intracellular calcium stores. The effects of okadaic acid (an inhibitor of protein phosphatases-1 and -2A) were investigated in calcium-free recording saline to determine what effect inhibiting phosphatases have on the ability of $PGF_{2\alpha}$ to stimulate calcium-activated chloride channels. Oocytes expressing the FP_A prostanoid receptor isoform were given an initial application of $PGF_{2\alpha}$ (10 μM) that produced a rapid outward current response (Fig. 6A, top trace). Pretreatment with okadaic acid (30 min, 100 nM) reduced the response to $PGF_{2\alpha}$ application (Fig. 6A, bottom trace).

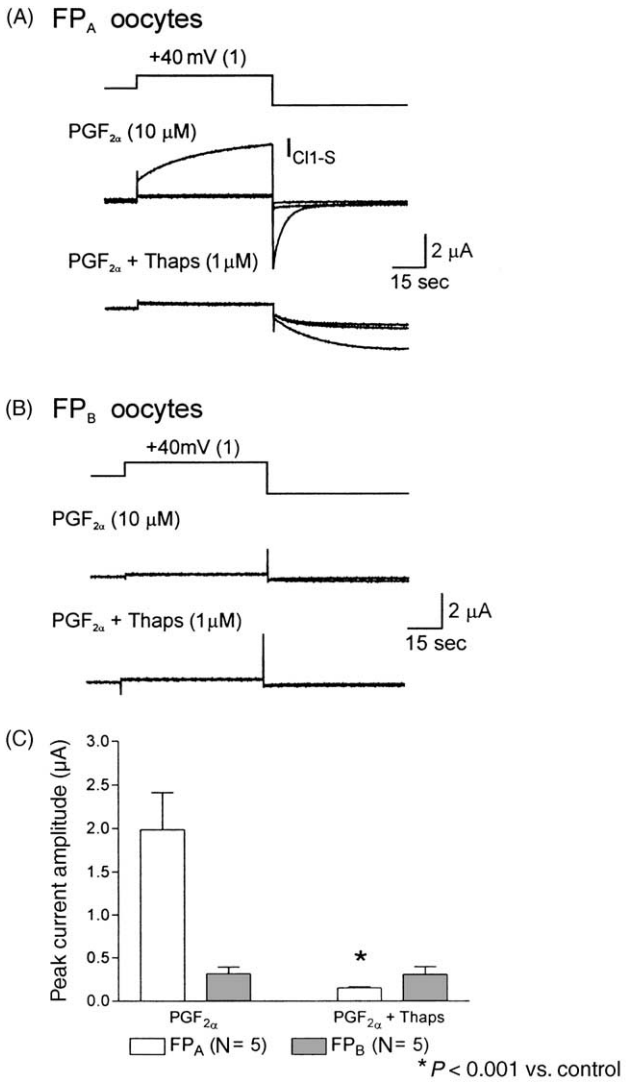

Fig. 4. Contribution of intracellular calcium release to the stimulation of Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes expressing the FP_A and FP_B prostanoid receptor isoforms in Ca^{2+}-free recording saline. (A and B) The initial +40 mV step with three representative current traces. Bath application of $PGF_{2\alpha}$ (10 μM) produced a rapid outward current ($I_{Cl1\text{-}S}$) response in FP_A (1.98 ± 0.36 μA; N = 5) expressing oocytes that were sensitive to 1 μM thapsigargin pretreatment (0.32 ± 0.03 μA; N = 5, panel A). Bath application of $PGF_{2\alpha}$ (10 μM) failed to produce a rapid outward current response in FP_B-expressing oocytes in the absence (0.15 ± 0.01 μA, N = 5) or presence of 1 μM thapsigargin (0.31 ± 0.06 μA, N = 5, panel B). (C) Summary of the peak current ($I_{Cl1\text{-}S}$) amplitudes to 10 μM $PGF_{2\alpha}$ in the presence and absence of thapsigargin. Values are means ± SEM. Statistical differences were determined using Student's *t*-test.

$PGF_{2\alpha}$ application (Fig. 6B; top trace) or $PGF_{2\alpha}$ application after a 30-min pretreatment with okadaic acid (Fig. 6B; bottom trace) did not produce a response from FP_B-expressing oocytes. A summary of the peak current amplitudes ($I_{Cl1\text{-}S}$) evoked by a 10 μM $PGF_{2\alpha}$ application with and without pretreatment with okadaic acid is shown in Fig. 6C. These data suggest that protein phosphatases are involved in coupling the intracellular calcium signaling activated by the FP_A prostanoid receptor isoform to the Ca^{2+}-dependent Cl^- channels in *Xenopus* oocytes.

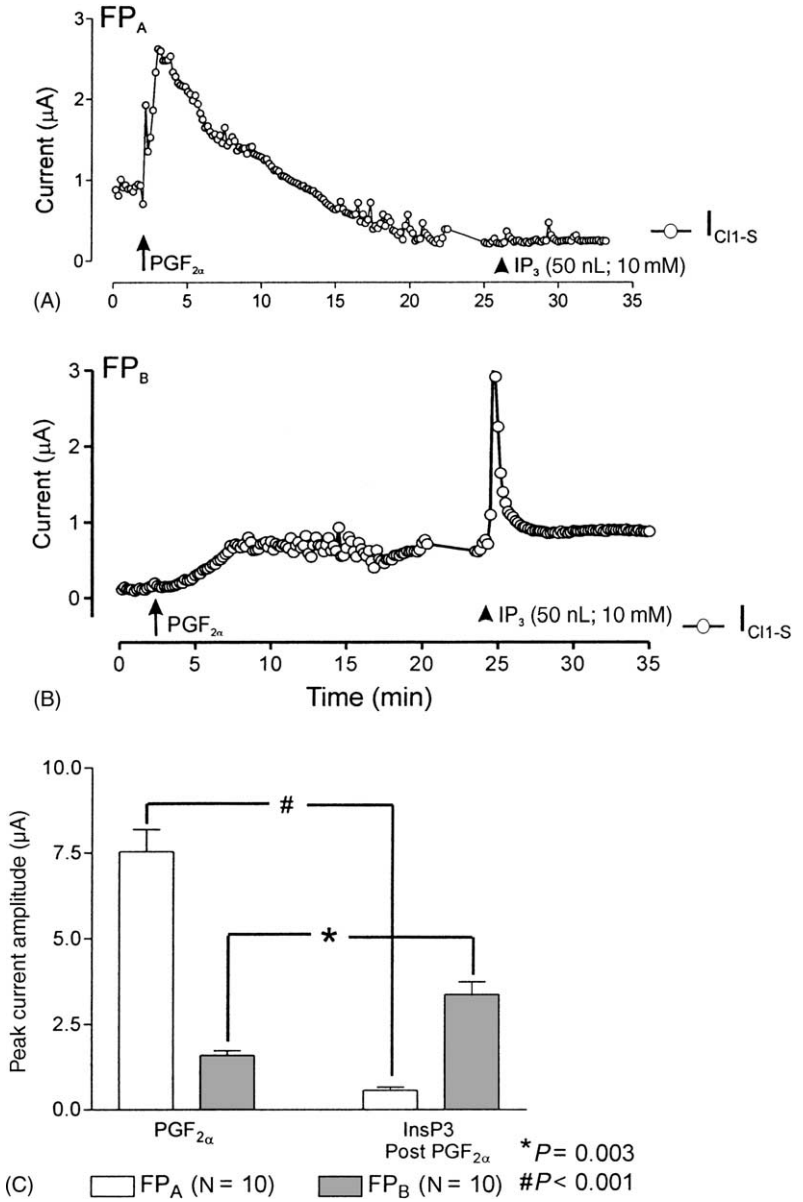

Fig. 5. Depletion of intracellular calcium stores during $PGF_{2\alpha}$ stimulation by FP_A prostanoid receptor agonist. (A) Bath application of $PGF_{2\alpha}$ (10 μM; arrow) produced a rapidly activating outward current in FP_A-expressing oocytes that returned to baseline after 20 min. Subsequent intracellular injection of $InsP_3$ (IP_3 arrowhead) failed to produce a response. (B) Bath application of $PGF_{2\alpha}$ (10 μM; arrow) produced a slowly developing outward current with a long time course in FP_B-expressing oocytes. However, the subsequent intracellular injection of $InsP_3$ (IP_3 arrowhead) produced a rapid outward current response with an amplitude similar to that seen after $PGF_{2\alpha}$ application in FP_A-expressing oocytes (panel B). (C) Summary of the peak current ($I_{Cl1\text{-}S}$) amplitudes to 10 μM $PGF_{2\alpha}$ (FP_A, 7.54 ± 0.64 μA; and FP_B, 1.58 ± 0.14 μA) and peak current amplitudes to $InsP_3$ injection post $PGF_{2\alpha}$ application (FP_A, 0.56 ± 0.09 μA; and FP_B, 3.37 ± 0.36 μA). Values are means ± SEM. Statistical differences were determined using Student's *t*-test.

4. Discussion

Calcium-activated chloride currents have been studied extensively in *Xenopus* oocytes. The properties of the chloride current activation have been used as rapid indicators of cytosolic calcium concentration [13]. We utilized the voltage protocols that distinguish Ca^{2+}-activated Cl^- currents activated by intracellular calcium release and calcium entry established by Kuruma and Hartzell [10] to examine the pathways involved in intracellular calcium signaling of the ovine FP prostanoid receptor isoforms expressed in *Xenopus* oocytes. We found that $PGF_{2\alpha}$ stimulation of oocytes expressing the ovine FP_A and FP_B prostanoid receptor isoforms produces dramatic differences in the Ca^{2+}-activated Cl^- channel activity. The difference appears to be regulated at the level of receptor-activated calcium release.

FP prostanoid receptors are G-protein-coupled receptors that are activated by $PGF_{2\alpha}$, resulting in the stimulation of inositol phosphate turnover. The activation of these receptors can also cause induction of actin stress fibers in HEK cells through a pathway involving the G_q-independent

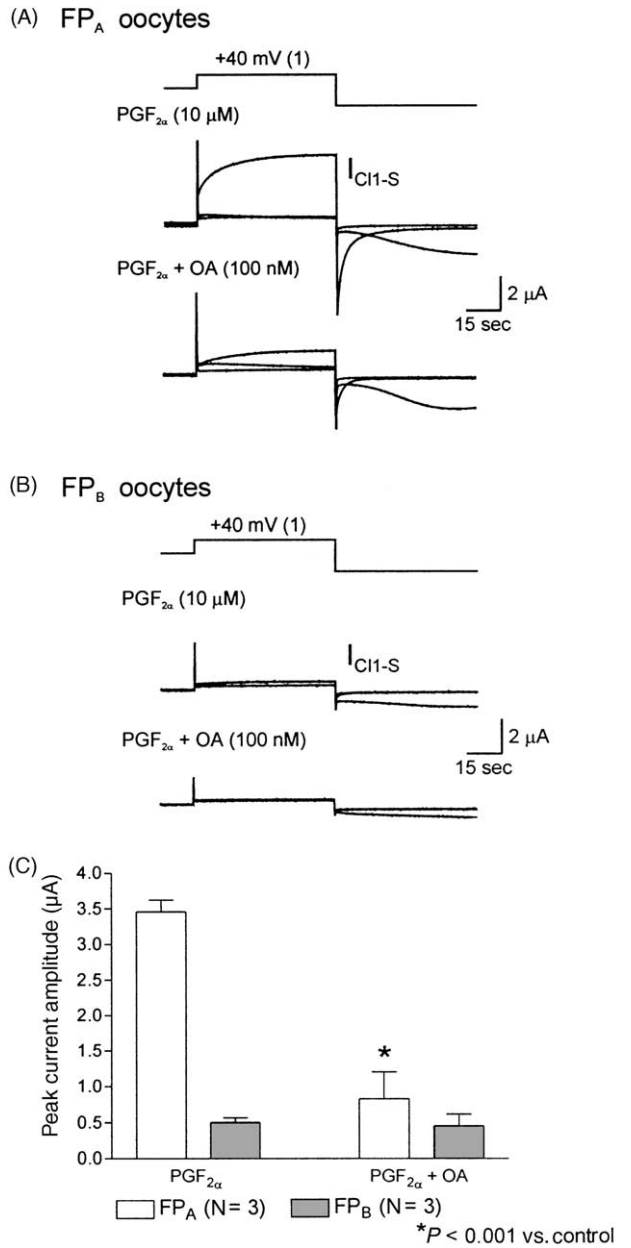

Fig. 6. Attenuation of the $PGF_{2\alpha}$ response in FP_A-expressing oocytes in calcium-free saline by protein phosphatase inhibition. (A) Bath application of $PGF_{2\alpha}$ (10 μM) produced a rapid outward current ($I_{Cl1\text{-}S}$) response in FP_A-expressing oocytes that was attenuated after a 30-min pretreatment of okadaic acid (OA, 100 nM). (B) Bath application of $PGF_{2\alpha}$ (10 μM) failed to produce a response in FP_B-expressing oocytes in either condition. (C) Summary of the peak current ($I_{Cl1\text{-}S}$) amplitudes to 10 μM $PGF_{2\alpha}$ (FP_A, 3.35 ± 0.38 μA; and FP_B, 0.51 ± 0.04 μA; $N = 3$) and peak current amplitudes of $PGF_{2\alpha}$ in the presence of okadaic acid (FP_A, 0.69 ± 0.14 μA; and FP_B, 0.47 ± 0.09 μA; $N = 3$). Values are means ± SEM. Statistical differences were determined using Student's *t*-test.

activation of Rho [14]. However, little is known about the calcium-signaling abilities of these two FP receptor isoforms. In this study, we demonstrated differences in the ability of ovine FP prostanoid receptor isoforms expressed in *Xenopus* oocytes to mobilize intracellular calcium. The present findings show that the FP_A isoform depletes the intracellular calcium stores after receptor activation, whereas calcium depletion is not seen after FP_B receptor activation.

The difference in calcium signaling between the FP_A and FP_B isoforms could be the result of constitutive activity of the FP_B receptor isoform. We recently observed higher levels of basal phosphatidylinositol 3-kinase activity in HEK cells stably expressing the FP_B receptor isoform as compared with the FP_A receptor isoform (unpublished observations). This is also consistent with the higher levels of inositol phosphates in FP_B-expressing cells that we have reported previously [5]. A low level of constitutive FP_B receptor activity could result in a slow release of intracellular calcium that alters the subplasmalemmal calcium concentration. It has been suggested that if the release of calcium is slow enough, then the concentration of calcium in the subplasmalemmal space may not reach sufficient levels to drive the Ca^{2+}-activated Cl^- channels [11]. However, in the data presented, we did not observe any basal activity of SOC currents in FP_B-expressing oocytes, suggesting that a low level of constitutive activity is not responsible for the observed differences in calcium signaling.

Other possibilities for the observed differences in Ca^{2+}-activated signaling could be that either the receptor proteins were not expressed on the membrane surface or there were different levels of receptor protein expression; however, our data make these possibilities unlikely. Immunofluorescent data demonstrated that both receptor proteins were expressed on the outer membrane of the *Xenopus* oocytes (Fig. 2A). The level of receptor expression also appeared to be equivalent based on the radioligand binding data. The two FP receptor isoforms bound the $[^3H]PGF_{2\alpha}$ at comparable levels. In the present study, we demonstrated that inhibition of protein phosphatases affected FP_A-mediated calcium signaling, suggesting that phosphorylation does contribute to the inhibition of current activation. The FP_A receptor isoform, but not the FP_B receptor isoform, is subject to a rapid negative feedback regulation by PKC [6], which probably results from phosphorylation at one or more of three PKC phosphorylation consensus sites present at the carboxyl terminus of the FP_A receptor isoform. Additionally, HEK cells stably expressing the ovine FP_B receptor isoform showed a greater degree of functional desensitization of agonist-stimulated calcium release than FP_A-expressing cells [15]. This difference was attributed to a slower resensitization of the FP_B receptor isoform compared to the FP_A receptor isoform. The data presented support differences in intracellular calcium signaling between the two FP receptor isoforms and for the role of phosphorylation in this difference.

Regulation of calcium entry from the activation of G-proteins in *Xenopus* oocytes has been suggested to occur early in the signaling cascade. Activation of PKC or cyclic-AMP-dependent protein kinase was shown to have profound effects on capacitative calcium entry in *Xenopus* oocytes [16]. In the latter study, a direct effect of G-proteins

on capacitative calcium entry could not be excluded, but suggested that a protein kinase was involved in the response. In *Xenopus* oocytes, overexpression of $G_{\alpha 16}$ or $G_{\alpha 15}$ causes a greater than 10-fold inhibition of receptor-mediated calcium activation [17]. The inhibitory effect was attributed to desensitization of calcium release from internal stores, due to prolonged activation of PLCβ by free $G_{\alpha 16}$ subunits. The G_α subunits that are involved in the downstream signaling of the FP prostanoid receptor isoforms remain to be identified.

The release of intracellular calcium by the direct injection of $InsP_3$ into oocytes expressing either FP receptor isoform produced identical rapid outward current ($I_{Cl1\text{-}S}$) responses (data not shown). This observation demonstrated that the exogenous expression of the FP receptor isoforms did not interfere with the normal signaling cascade at the level of the $InsP_3$ receptor. Exogenous expression of membrane receptor proteins in *Xenopus* oocytes can recruit additional proteins to the plasma membrane [18]. Therefore, we also examined the intracellular signaling of the endogenous lysophosphatidic acid (LPA) receptors in oocytes expressing exogenous FP_A or FP_B receptors. The LPA receptor is coupled to G_q, and because it is expressed endogenously in *Xenopus* oocytes, it has been used to examine the effects of exogenously expressed proteins on this Ca^{2+}-mediated signaling pathway [19]. We determined that the exogenous expression of the FP_A and FP_B receptor isoforms did not interfere with or enhance the Ca^{2+}-activated Cl^- currents produced by activation of endogenous LPA receptors (data not shown). Taken together, these data suggest that the observed difference in calcium signaling mediated through FP_A and FP_B receptor activation in *Xenopus* oocytes may be at the level of the receptors themselves or at some point prior to the level of the intracellular calcium stores.

Although we have demonstrated that the FP_A and FP_B prostanoid receptor isoforms appear to be differentially coupled to the release of intracellular calcium, the basis of the difference remains unknown. We demonstrated that $PGF_{2\alpha}$ stimulation of FP_A-expressing oocytes exhibited a distinct activation of the Ca^{2+}-activated Cl^- channels in the presence of extracellular calcium. We found that in the absence of extracellular calcium the initial rapid spike was attenuated and the $I_{Cl1\text{-}T}$ current activation was abolished, similar to previously published observations [10]. These data support the conclusion that the $I_{Cl1\text{-}T}$ does not require Ca^{2+}-induced Ca^{2+} release and that the initial rapid spike response seen in FP_A-expressing oocytes is the result of the release of stored calcium. In addition, part of the amplitude of the initial spike response appears to be an additive effect of calcium release and influx of calcium from the extracellular space.

In contrast, FP_B-expressing oocytes were unable to produce the initial rapid response to $PGF_{2\alpha}$ stimulation seen in FP_A-expressing oocytes. However, the development of the $I_{Cl1\text{-}T}$ in FP_B-expressing oocytes was similar in time course and amplitude to that of FP_A-expressing oocytes, indicating that the FP_B response did stimulate the response component that was dependent upon direct calcium entry. In the absence of extracellular calcium the $I_{Cl1\text{-}T}$ current was abolished, similar to the response in FP_A-expressing oocytes. These data suggest that $PGF_{2\alpha}$ stimulation of the FP_A and FP_B receptor isoforms is capable of activating SOCE to similar levels in the *Xenopus* oocytes. This, in turn, suggests that in the absence of a robust initial calcium response, as seen with FP_A receptor activation, there is enough intracellular calcium mobilization to stimulate SOCE in the FP_B-expressing oocytes, or that the FP_B receptor can modulate the SOCE channels through a mechanism that is independent of calcium release from intracellular stores.

Differential intracellular calcium mobilizations have been reported in *Xenopus* oocytes from the activation of expressed m2 and m3 muscarinic acetylcholine receptors. Stimulation of the m2 receptors produced a calcium increase, which was slow to onset, oscillatory, focal, and correlated well with activation of oscillatory chloride currents. In contrast, the m3-mediated calcium response was large, rapid, and usually transient [20]. Although we did not directly measure intracellular calcium in our experiments, we did observe similar responses in Cl^- current activation. In our studies, stimulation of the FP_B prostanoid receptor isoform lacked the rapid outward current response. However, FP_B receptor activation did produce a slowly developing outward current ($I_{Cl1\text{-}T}$) similar to the current response reported for m2 receptor activation. Differences in intracellular calcium release have also been reported after direct injections of $InsP_3$ or the inositol phosphate analogue adenophostin A [11]. Both $InsP_3$ and adenophostin A were capable of activating SOCE, but had different kinetics of intracellular calcium release. We have observed a comparable distinction between the intracellular calcium responses activated by the FP_A and FP_B prostanoid receptor isoforms.

We have reported a difference in the intracellular calcium signaling of heterologously expressed prostanoid FP receptor isoforms in *Xenopus* oocytes based on the distinct differences in Ca^{2+}-activated Cl^- currents. The difference appears to be regulated at the level of receptor activation and may be a result of receptor phosphorylation.

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