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Misoprostol elevates intracellular calcium in Neuro-2a cells via protein kinase A

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ABSTRACT

Misoprostol, a prostaglandin type E analogue, has been implicated in a number of neurodevelopmental disorders. However, its mode of action in the nervous system is not well understood. Misoprostol acts on the same receptors as prostaglandin E_2 (PGE_2), a natural lipid-derived compound, which mediates important physiological functions in the nervous system via activation of four EP receptors (EP1-4). In this study we use a ratiometric calcium imaging with fura-2 AM as a calcium indicator to show that misoprostol alters intracellular calcium levels in mouse neuroblastoma (Neuro-2a) cells via similar mechanisms as PGE_2. We demonstrate that the misoprostol-induced increase in calcium is mediated by a protein kinase A (PKA)-dependent mechanism and that the EP4 receptor signaling pathway may play an inhibitory role on calcium regulation. Overall, this study provides further support for the involvement of PGE_2 signaling in calcium homeostasis and suggests its important role in the nervous system.

1. Introduction

Misoprostol is a drug that is structurally similar to the naturally occurring family of lipid-derived prostaglandin type E [1]. It has been shown that misoprostol can activate the same receptors as the endogenous compound prostaglandin E_2 (PGE_2) [2–5]. PGE_2 is a 20-carbon fatty acid derived from plasma membrane arachidonic acid. PGE_2 exerts its diverse effects in the nervous system through four G-protein coupled EP receptors: EP1-EP4 [6,7]. Generally, EP1 receptor activation is associated with increase in intracellular calcium concentration, $[Ca^{2+}]_i$, mediated by the action of molecules such as phospholipase C and inositol 1,4,5-trisphosphate (IP_3) [2,8,9]. EP2 and EP4 receptors are both known to couple to stimulatory G-protein (G_s) and mediate activation of protein kinase A (PKA) through the action of cyclic adenosine monophosphate (cAMP) [2,10]. In the recent years additional EP4 signaling pathways have been proposed via G proteins other than G_s [10–13]. The EP3 receptor has multiple isoforms formed through alternative splicing, which further diversify the action of PGE_2 [8,14]. It has been shown that EP3 can increase or decrease cAMP concentration and intracellular calcium levels via coupling to G_s, G_i, or G_q proteins [15–19].

Misoprostol, the prostaglandin E analogue, has a broad array of therapeutic applications such as prevention and treatment of gastric ulcers [20–23] induction of uterine contractions, and medical termination of pregnancy [24–26]. Misuse of misoprostol in some cases of self-attempted termination of pregnancy has linked this drug to development of neurodevelopmental conditions such as Möbius syndrome and autism related disorders [27–30] suggesting that the drug might have neurotoxic effects in the developing nervous system. Interestingly, our previous study has shown that misoprostol elevates the amplitude of calcium fluctuations in growth cones of mouse neuroblastoma (Neuro-2a) cells and induces neurite retraction demonstrating for the first time its effects on cell function [31].

Since the molecular mechanisms of misoprostol action are not clearly understood this study investigates the effects of the drug on the intracellular level of calcium in Neuro-2a cells and describes the potential signaling pathways involved. We provide evidence that misoprostol alters calcium level in the cytosol via a PKA-mediated pathway with a novel role carried out by EP4 receptor signaling. Our study furthers our understanding of the molecular mechanisms of misoprostol action and shows that it may interfere with PGE_2 signaling pathway in Neuro-2a cells.

2. Materials and methods

2.1. Cell culture

Mouse Neuro-2a cells were maintained in an incubator containing 5% CO_2, 95% humidified environment and at 37 °C. Cells were obtained from American Tissue Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (Invitrogen).

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2.2. Effects of prostaglandin E_2 and misoprostol treatments on cytosolic calcium level in Neuro-2a cells

PGE_2- and misoprostol-dependent measurement of intracellular calcium level ($[Ca^{2+}]_i$) in Neuro-2a cells was determined using the ratiometric fura-2AM calcium indicator as we previously described [31,32]. Changes in $[Ca^{2+}]_i$ were determined in Neuro-2a cells in response to 1.0 and 10.0 μM PGE2 and misoprostol following the incubation of the cells with fura-2AM as described before [31]. First, the basal R value was measured before adding the drug to establish a stable baseline of calcium level. After addition of the drug, the 340/380 ratio (R) was monitored for a total of 10 min. The fluorescence ratio of 340/380 in fura-2AM loaded Neuro-2a cells was measured in 100–400 cells and then $[Ca^{2+}]_i$ was calculated [32]. All the concentrations in nM were converted to a percentage, with the basal $[Ca^{2+}]_i$ set as 100%. The cells were also incubated with 20 μM SC19220 (2-acetylhydrazide 10(11H)-carboxylic acid, 8-chlorodibenz[b,f][1,4]oxazepine-10(11H)-carboxylic acid, EP1 antagonist), AH6809 (6-isopropoxy-9-10oxoxanthene-2-carboxylic acid, EP1/2 antagonist), AH23848 hemicalcium salt hydrate ((4Z)-7-[(rel-1S,2S,5R)-5-((1,1′-biphenyl-4-yl)methoxy)-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid hemicalcium salt hydrate, EP4 antagonist), 100 nM wortmannin (phosphoinositide 3-kinases blocker, PI3K) or 10 μM H89 (PKA blocker) for 20 min during the de-esterification process. The controls for the dose–response conditions include HBSS and ethanol ($HBSS_{EtOH}$) at the concentration used in PGE_2 and misoprostol solutions. Our results were normalized to EP receptor antagonists and kinase blockers alone in HBSS used as controls for the respective experiments. All the reagents were purchased from Sigma–Aldrich.

2.3. Data analysis and statistics

Statistical analysis was performed with Student's *t*-test and a value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of misoprostol and PGE_2 treatment on intercellular free calcium concentration in Neuro-2a Cells

Ratiometric real-time calcium imaging was used to investigate whether misoprostol can alter calcium homeostasis in Neuro-2a cells and mimic PGE_2's action and whether the changes in $[Ca^{2+}]_i$ are concentration-dependent. Previous studies in CHO or HEK-293 cells overexpressing EP receptors reported the affinity of PGE_2 for the EP receptors in the range of concentrations below 1 μM [3,33]. Since Neuro-2a cells express adequate levels of the EP receptors [31] in this study we used untransfected cells to measure the PGE_2- and misoprostol-dependent changes in the intracellular calcium level. The basal intracellular calcium level for Neuro-2a cells was found to be 100 ± 40 nM. We did not observe any significant increase in $[Ca^{2+}]_i$ when Neura-2a cells were treated with concentrations lower than 0.1 μM (data not shown). Exposure to 1.0 and 10.0 μM misoprostol induced 10% ($N = 230$) and 20% ($N = 246$, $p = 0.022$) increase in $[Ca^{2+}]_i$ (Fig. 1A). Moreover, treatment with the same concentrations of PGE_2 resulted in $[Ca^{2+}]_i$ increase by 20% ($N = 284$, $p = 0.028$) and 31% ($N = 280$, $p = 0.002$) (Fig. 1B). In summary, these results have shown that the drug misoprostol increases $[Ca^{2+}]_i$ in Neuro-2a cells and mimics the action of the endogenous compound PGE_2. Calcium increases due to misoprostol seem to be generally lower than that of PGE_2. We have determined, by using an IP3 receptor (IP_3R) blocker (2-APB), that the source of calcium increase in response to misoprostol and PGE_2 is from intracellular calcium stores, such as the ER (data not shown).

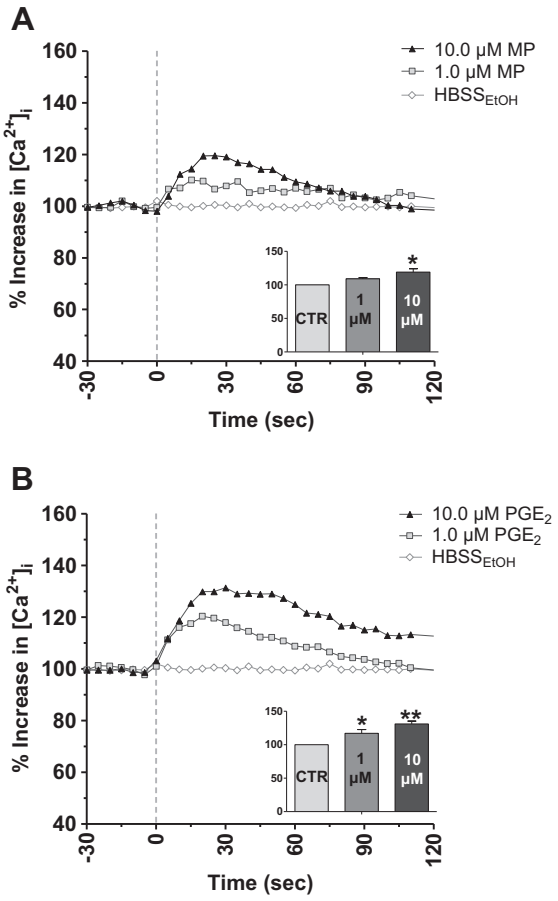

Fig. 1. Concentration-dependent increase in $[Ca^{2+}]_i$ in response to misoprostol and PGE_2 treatments. (A) Treatments with 1.0 and 10.0 μM of misoprostol elevated $[Ca^{2+}]_i$ by 10% and 20% and (B) PGE_2 induced an increase of 20% and 31%, respectively. The *y*-axes represent percentage of $[Ca^{2+}]_i$ and *x*-axes depict the time in seconds with time 0 representing time of addition of the drug (dashed line). Results represent a minimum of three independent experiments. MP = misoprostol.

3.2. Effects of blocking EP2 signaling pathway on misoprostol- and PGE_2-induced changes in intracellular calcium concentration

To characterize the mechanism involved in the $[Ca^{2+}]_i$ elevation commonly used EP receptor antagonists and downstream kinase blockers were used in conjunction with misoprostol or PGE_2. To ensure that the responses were drug specific, antagonists or blockers alone were applied to the cells and no changes in the $[Ca^{2+}]_i$ was observed. The results were normalized to these controls. Addition of SC19220, an antagonist for EP1 receptor, did not result in a reduction in the intracellular calcium increase following the drug treatments (data not shown). AH6809 is a species-specific EP1/2 receptor antagonist which shows no binding affinity for EP1 receptor in mouse and only binds to EP2 receptor [3]. Misoprostol treatment alone induced an increase of 24% in $[Ca^{2+}]_i$ ($N = 307$, $p < 0.0001$) and misoprostol with AH6809 caused a similar increase of 27% ($N = 304$, $p = 0.001$) (Fig. 2A). When the cells were incubated with AH6809 and treated with PGE_2, $[Ca^{2+}]_i$ increased by 25% ($N = 257$, $p = 0.004$), which was significantly lower ($p = 0.006$) than the 40% increase caused by the PGE_2 alone ($N = 201$, $p = 0.001$) (Fig. 2B). These results indicate that activation of EP2 may contribute to the increase of cytosolic calcium level in Neuro-2a cells in response to PGE_2 but not misoprostol.

Since there is no specific EP3 receptor antagonist available commercially we tested a potential involvement of PKA, the downstream activator of EP2 and EP3 receptors. PKA activation has

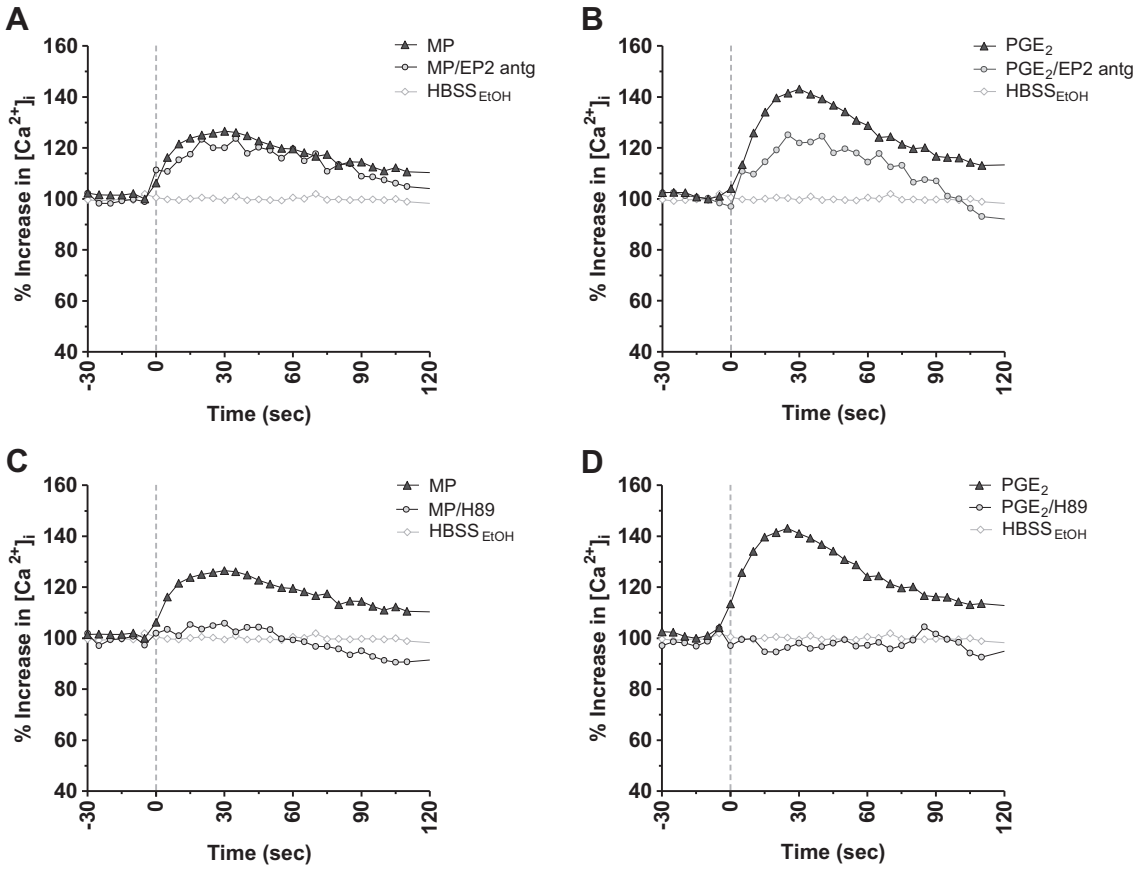

Fig. 2. Misoprostol- and PGE_2-induced changes in $[Ca^{2+}]_i$ in presence of EP2 receptor antagonist and PKA blocker. (A and B) Treatment with misoprostol or PGE_2 along with EP2 antagonist. (A) In response to misoprostol $[Ca^{2+}]_i$ increased by 24% in presence and 27% in absence of EP2 antagonist. (B) PGE_2 increased $[Ca^{2+}]_i$ by 25% and 43% in presence and absence of EP2 antagonist. (C and D) With H89 $[Ca^{2+}]_i$ remained at the baseline in response to misoprostol or PGE_2. Results represent a minimum of three independent experiments. MP = misoprostol, antg = antagonist.

been shown to play a role in increase of $[Ca^{2+}]_i$ in various cell types via a number of mechanisms [34–37]. Our results show that misoprostol- ($N = 138$) and PGE_2-induced ($N = 159$) elevations of $[Ca^{2+}]_i$ were attenuated in the presence of 10 μM H89, a commonly used PKA blocker (Fig. 2C and D) suggesting the involvement of PKA-dependent responses to the drugs.

3.3. Effects of blocking EP4 signaling pathway on misoprostol- and PGE_2-induced changes in intracellular calcium concentration

To assess whether activation of EP4 receptor had an effect on the changes in $[Ca^{2+}]_i$ in response to PGE_2 and misoprostol, AH23848 hemicalcium salt hydrate was used as an EP4 receptor antagonist. Our results show that the addition of 20 μM AH23848 resulted in an unexpected 76% ($N = 384$, $p = 0.001$) and 73% ($N = 284$, $p = 0.003$) increase of $[Ca^{2+}]_i$ in response to misoprostol and PGE_2, respectively (Fig. 3A and B). These percent increases were significantly higher than the typical $[Ca^{2+}]_i$ increases induced by misoprostol ($p < 0.0001$) and PGE_2 ($p < 0.0001$). These results suggest that EP4 receptor pathway might be involved in the inhibition of the intracellular calcium level in the Neuro-2a cells.

To further confirm these results wortmannin was used to inhibit activation of PI3K, one of the downstream regulators of the EP4 receptor pathway [10–12]. Blocking PI3K with 100 nM wortmannin resulted in similar calcium responses as seen with EP4 receptor antagonist. In the presence of wortmannin $[Ca^{2+}]_i$ increased by 67% ($N = 408$, $p < 0.0001$) and 90% ($N = 312$, $p < 0.0001$) in response to misoprostol and PGE_2 treatments, respectively (Fig. 3C and D). Calcium responses were significantly higher ($p < 0.0001$) than those mediated by misoprostol (27%, $N = 109$) and PGE_2 (55%, $N = 124$) treatments only. These results were in agreement with the EP4 antagonist data suggesting a unique involvement of the EP4 pathways in the inhibition of $[Ca^{2+}]_i$ in Neuro-2a cells.

4. Discussion

In the present study we show that the exogenous drug misoprostol, a prostaglandin type E agonist, and the endogenous compound PGE_2, increases $[Ca^{2+}]_i$ in Neuro-2a cells through similar mechanisms to PGE_2. We provide evidence that misoprostol and PGE_2 elevate $[Ca^{2+}]_i$ via a PKA-mediated pathway and describe a novel inhibitory role carried out by the EP4 receptor signaling pathway.

Our imaging results show that both misoprostol and PGE_2 elevate $[Ca^{2+}]_i$. We observed significant elevation of $[Ca^{2+}]_i$ in Neuro-2a in response to 1.0 and 10.0 μM. Previous studies in HEK-293 and CHO cells stably overexpressing the EP receptors have shown cellular responses to concentrations lower than 1.0 μM [3,4,33]. Our study uses untransfected Neuro-2a cells expressing endogenous levels of EP receptors to measure $[Ca^{2+}]_i$, which potentially explains responses to higher concentrations. Generally, the elevation of $[Ca^{2+}]_i$ were more profound in response to PGE_2 compared to misoprostol, which could be attributed to the higher affinity of PGE_2 for the EP receptors [3,38]. It has been shown previously that PGE_2 acts on all four EP receptors with varying affinities and predominantly on EP3 and EP4 receptors (EP3 > EP4 ≫ EP2 > EP1)

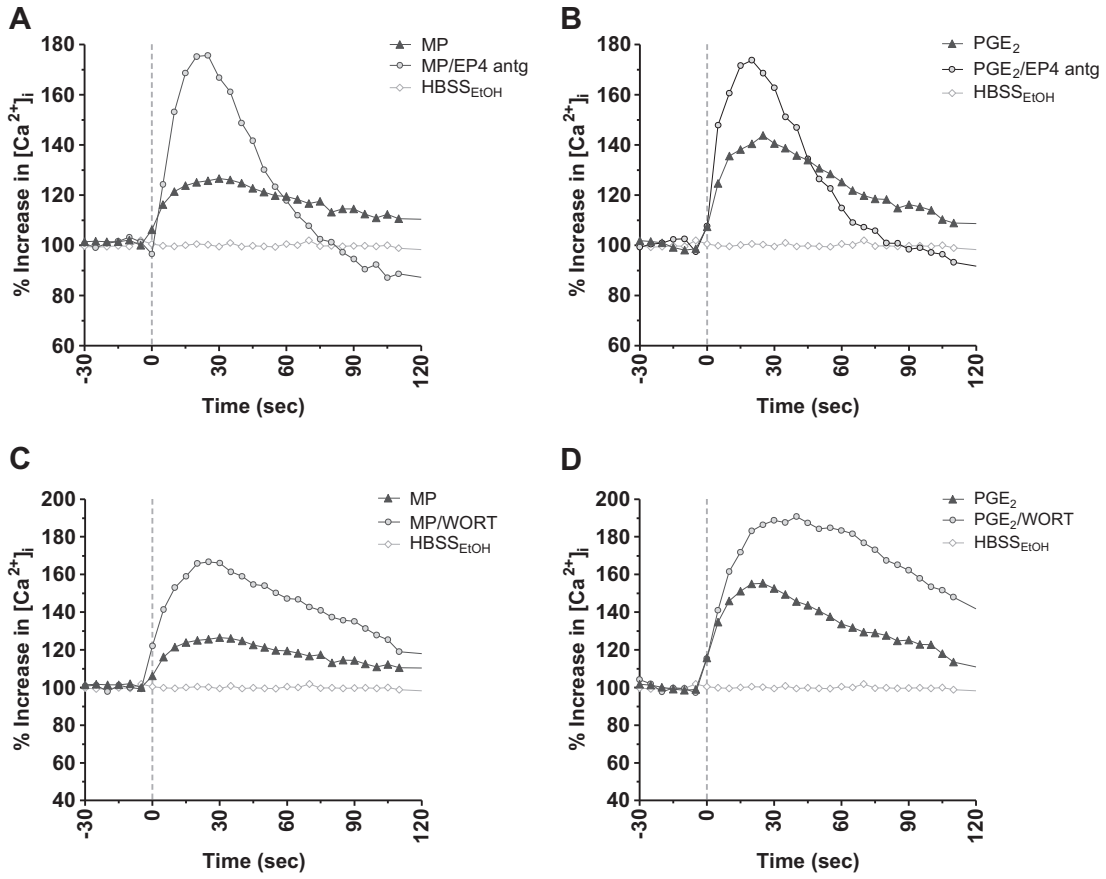

Fig. 3. $[Ca^{2+}]_i$ traces in response to misoprostol and PGE_2 in presence of EP4 receptor antagonist and PI3K blocker. In the presence of EP4 antagonist $[Ca^{2+}]_i$ increased by 76% and 73% in response to (A) misoprostol and (B) PGE_2, respectively, and by 26% and 41% in response to (A) misoprostol and (B) PGE_2 alone. In the presence of wortmannin $[Ca^{2+}]_i$ increased by 67% in response to (C) misoprostol and 90% in response to (D) PGE_2. Results represent a minimum of three independent experiments. WORT = wortmannin. MP = misoprostol.

[8,38]. According to previous studies misoprostol also acts on EP receptors but with much lower affinities than PGE_2 and mainly on the EP3 receptor [3].

Current literature from various tissues and cell lines typically links EP1 receptor activation to calcium mobilization in cells. Our results show that the EP1 receptor did not contribute to changes in the calcium during misoprostol or PGE_2 treatments in Neuro-2a cells, likely due to its low expression in these cells [31]. Moreover, EP1 has a much lower affinity for PGE_2 (compared to other EP receptors) and it is not activated by misoprostol [3,8], therefore the possibility of it mediating the $[Ca^{2+}]_i$ increase in Neuro-2a cells seems to be unlikely. When the EP2 receptor pathway was blocked, we observed a smaller calcium elevation in response to PGE_2 treatment, whereas misoprostol-induced calcium increase was not affected indicating that the receptor likely contributes to the increase in $[Ca^{2+}]_i$ in response to PGE_2, but not misoprostol in Neuro-2a cells. The EP2 receptor is typically associated with an increase of cAMP concentration via activation of adenylyl cyclase (AC). Evidence linking EP2 receptors to increases in $[Ca^{2+}]_i$ comes from rat primary astrocytes [39]. In this study we provide further evidence for the possibility of this novel role carried out by the EP2 signaling pathway.

Various studies have confirmed the existence of three splice variants of EP3 receptor in mouse: $EP3_\alpha$, $EP3_ß$ and $EP3_\gamma$, which all can couple to G_i, and $EP3_\gamma$ can also bind to G_s [15,40–42]. The existence of different splice variants and coupling to multiple G proteins (G_i, G_s and G_q) diversifies the action of EP3 signaling in the cells. Previous research has linked EP3 receptor to calcium mobilization in different cell types such as EP3-transfected canine dorsal root, COS-7 and HEK-293 cells [16,17,43]. Since the affinity of PGE_2 and misoprostol for the EP3 receptor seems to be the highest [3,4,8,38], and because Neuro-2a cells express high levels of $EP3_\gamma$ [31] its contribution to PGE_2- and misoprostol-induced elevation of $[Ca^{2+}]_i$ is a likely possibility. It has been previously reported that PGE_2–dependent stimulation of EP2, EP4 and G_s-coupled EP3 receptors may lead to activation of AC and subsequent production of cAMP and PKA [10,42,44]. It is also shown that PKA activation can mediate release of calcium from intracellular calcium stores via different mechanisms [39,45,46]. In this study we demonstrated that the calcium increases in response to PGE_2 and misoprostol in Neuro-2a cells are PKA-dependent.

In additions, our study also shows that EP4 receptor might be involved in the inhibition of the $[Ca^{2+}]_i$ in Neuro-2a cells. EP4 receptor has been often associated with increase in cAMP in cells, however with much less efficiency than EP2 [10]. Emerging evidence has established an additional pathway mediated by the EP4 receptor via activation of PI3K, which is thought to be the predominant EP4 pathway [10,11]. In our study, we showed that blocking EP4 receptor or PI3K resulted in greater elevation of intracellular calcium in response to PGE_2 and misoprostol. A similar EP4-dependent inhibition of cAMP response element binding protein (CREB) activation has been previously reported in EP4-transfected HEK-293 cells [11,12]. In this study, we show for the first time that the inhibitory role of EP4 receptor may regulate the $[Ca^{2+}]_i$ in Neuro-2a cells. We propose a possible mechanism by which EP4 receptor signaling pathway contributes to the calcium regulation in those cells (Fig. 4).

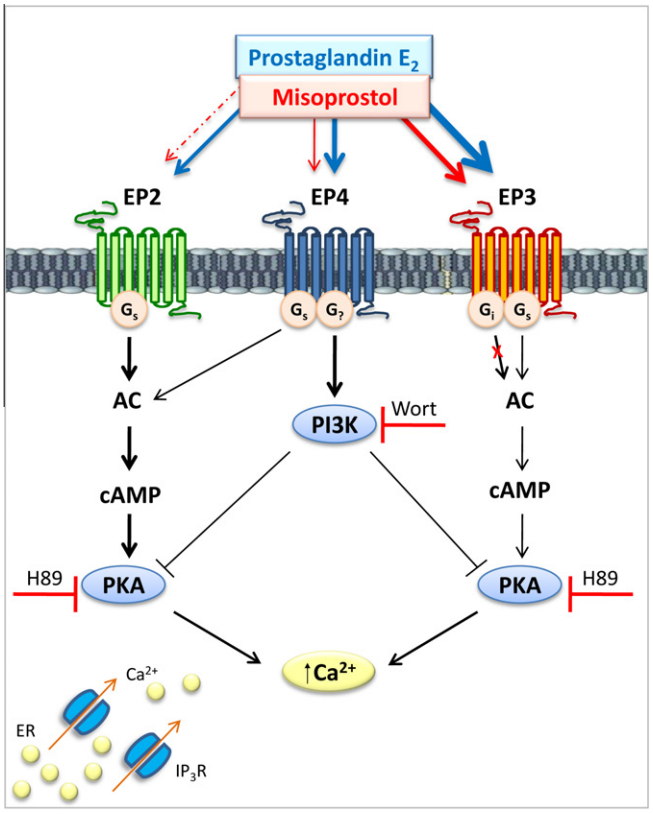

Fig. 4. Model for misoprostol- and PGE_2-mediated intracellular calcium increase in Neuro-2a cells. Misoprostol and PGE_2 act on EP2, EP3 and EP4 receptors to alter $[Ca^{2+}]_i$ to various degrees via PKA-dependent mechanism. EP4 receptor activation of PI3K results in inhibition of PKA and reduction of $[Ca^{2+}]_i$. The increases in calcium levels are due to release of calcium from intracellular stores through the activation of IP_3R.

This study shows that treatment with misoprostol and PGE_2 significantly increases $[Ca^{2+}]_i$ in Neuro-2a cells. Although further studies on primary cells or *in vivo* are needed, the present study and our previous findings [31] suggest that PGE_2 and misoprostol can alter calcium homeostasis in Neuro-2a cells and contribute to abnormal cell function. Calcium signaling is essential for elementary forms of neuronal communication and many aspects of neuronal development, including gene expression, neuronal migration and differentiation [47,48]. Interestingly, we have previously shown that the expression of EP1, EP2, $EP3_\beta$ and EP4 receptors is significantly higher during the neurogenesis period in mouse [31] suggesting the importance of the PGE_2 signaling pathway in the developing nervous system. Therefore, imbalances in calcium homeostasis can potentially affect early neuronal development. In fact, it has been documented by various studies that alterations in calcium homeostasis play a role in the pathogenesis of autism spectrum disorders [49,50].

In summary, our study shows that misoprostol and PGE_2 work via similar mechanisms to induce an elevation of $[Ca^{2+}]_i$ level in neuronal-type cells. The responses are PKA-dependent with the EP4 receptor playing a novel inhibitory role. This study furthers our understanding the molecular mechanisms of misoprostol signaling and an important role of PGE2 signaling in the nervous system.

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