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PGE_2 inhibits basolateral 50 pS potassium channels in the thick ascending limb of the rat kidney

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Abstract

To study the inhibition of the inwardly rectifying basolateral 50 pS potassium channels by PGE_2 we performed patch-clamp studies on the basolateral membrane of the rat kidney thick ascending limb. PGE_2's effect was mimicked by the selective EP1- and EP3-receptor agonist, sulprostone, but was prevented by inhibiting protein kinase-C with calphostin-C. The mitogen-activated protein kinase inhibitor PD98059 (ERK) or SB203580 (p38) increased basal channel activity; however, while neither alone prevented the inhibitory effect of PGE_2, but using both of them together completely abolished PGE_2's effect on channel activity. Treatment with PGE_2 stimulated phosphorylation of both p38 and ERK in primary cultures of medullary thick ascending limb cells. The PGE_2-mediated mitogen-activated protein kinase activation was not affected by indomethacin, but was completely blocked by calphostin-C. These studies show that inhibition of basolateral 50 pS potassium channels by PGE_2 is mediated by protein kinase-C, which in turn stimulates mitogen-activated protein kinases in the thick ascending limb of the rat kidney.

Keywords

cyclooxygenase; arachidonic acid; p38; ERK; inwardly rectifying; K channel

The thick ascending limb (TAL) is responsible for absorption of 20–25% of the filtered NaCl load and plays a key role in the ability of the kidney to concentrate urine.[1–3] Potassium (K) channels in the TAL are an important component for maintaining the function of TAL: apical K channels are essential for K recycling across the luminal membrane,[4] while basolateral K channels play a key role in generating the cell membrane potential in the TAL.[5] Several types of basolateral K channels have been identified with patch-clamp experiments.[6,7] We and others have confirmed that a 50 pS inwardly rectifying K channel was highly active in the basolateral membrane. However, the regulatory mechanism of the 50 pS K channels is not completely understood. We have demonstrated previously that arachidonic acid inhibited basolateral 50 pS K channels through a cytochrome P450-dependent pathway,[6] and that cAMP stimulated the 50 pS K channels.[8]

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Prostaglandins such as prostaglandin-E_2 (PGE_2) have been shown to inhibit Cl transport by antagonizing vasopressin's effect in the TAL.[9,10] Cl transport in the TAL is a two-step process: Cl enters the cells through an Na/K/Cl co-transporter and leaves the cells across the basolateral membrane via Cl channels.[1,11] As Cl exit across the basolateral membrane is electrogenic, alteration of basolateral K channel activity is expected to affect the Cl exit and indirectly the activity of the Na/Cl/K co-transporter in the apical membrane. Thus, it is likely that the PGE_2-induced inhibition of Cl transport in the TAL is partially mediated by regulation of the basolateral K channels. The main goal of this study is to examine whether PGE_2 regulates the basolateral 50 pS K channels in the TAL and to investigate the pathway by which PGE_2 regulates the K channels.

Results

We first examined the effect of PGE_2 on the 50 pS K channels in the basolateral membrane of the TAL. Figure 1 is a channel recording showing that application of 10 μM PGE_2 inhibited the 50 pS K channels in the basolateral membrane of the TAL and reduced NP_o from 0.30 ± 0.05 to 0.04 ± 0.03 (n = 5, P < 0.01). As prostaglandin E receptor 3 (EP3) has been shown to be a major type of PGE_2 receptor expressed in the TAL,[12] we next examined the effect of sulprostone (a specific EP1- and EP3-receptor agonist)[13] on the 50 pS K channels in the basolateral membrane of the TAL. Figure 2 is a recording demonstrating that sulprostone (10 μM) mimicked the effect of PGE_2 and decreased NP_o from 0.32 ± 0.06 to 0.05 ± 0.03 (n = 5, P < 0.01). This suggests that the inhibitory effect of PGE_2 on the 50 pS K channels was mediated by stimulating either EP1 or EP3 receptors in the TAL. Stimulation of EP3 receptor has been shown to activate pertussis toxin-sensitive G-protein and decrease cAMP generation.[14] We have demonstrated previously that stimulation of protein kinase-A (PKA) with the membrane-permeable cAMP analog increased the activity of the basolateral 50 pS K channels.[8] If the inhibitory effect of PGE_2 on the basolateral 50 pS K channels was due to decrease of cAMP and inhibition of PKA, inhibiting PKA should mimic the effect of PGE_2. Thus, we examined the effect of H8 (inhibitor of PKA) on the 50 pS K channels in the TAL. We confirmed the previous finding that inhibition of PKA had no significant effect on the basolateral 50 pS K channels in the TAL (data not shown),[8] suggesting that PKA did not play a role in determining the basal activity of the basolateral K channels. In addition to decreasing cAMP, stimulation of EP3 receptor has also been reported to activate PKC and Rho-kinase.[15,16] Thus, we examined whether PKC was responsible for mediating the inhibitory effect of PGE_2 on the basolateral 50 pS K channels in the medullary thick ascending limb (mTAL). We first tested the effect of 300 nM calphostin-C on the 50 pS K channel in the absence of PGE_2. Figure 3 is a representative recording showing that application of calphostin-C had no significant effect on the basal level of channel activity (control, 0.24 ± 0.06 and calphostin-C, 0.26 ± 0.06) (n = 3), suggesting that PKC is not involved in determining the basal level of the 50 pS K channel activity. However, blocking PKC with 300 nM calphostin-C was restored the channel activity inhibited by PGE_2 (Figure 4). Data summarized in Figure 5 demonstrate that inhibition of PKC with 300 nM calphostin-C restored channel activity to 0.26 ± 0.03 (n = 4), a value which was not significantly different from that in the control. This strongly suggests that the inhibitory effect of PGE_2 on the basolateral 50 pS K channel was mediated by stimulation of PKC in the TAL. Figure 5 also shows that stimulation of PKC with 5 μM phorbol 12-myristate 13-acetate significantly decreased activity of the 50 pS K channels to 0.06 ± 0.02 (n = 4, P < 0.01).

Stimulation of PKC has been shown to activate mitogen-activated protein kinase (MAPK) such as p38 and extracellular signal-regulated kinase (ERK) in the colleting duct.[17] To test whether p38 and ERK are also involved in mediating the effect of PGE_2 on the basolateral 50 pS K channels in the TAL, we examined the effect of PD98059, an inhibitor of ERK,[18] on the basolateral 50 pS K channels in the TAL. Figure 6 is a recording demonstrating that inhibiting ERK with 50 μM PD98059 increased the 50 pS K channel activity from 0.18 ± 0.04 to 0.59 ±

0.09 ($n = 4$, $P < 0.01$). This suggests that ERK is involved in determining the basal activity of the basolateral 50 pS K channels in the TAL. Alternatively, basolateral 50 pS K channels were inhibited by ERK under control conditions and inhibition of ERK reactivated the 50 pS K channels. We next examined the effect of PGE_2 on the basolateral 50 pS K channels in the TAL treated with 50 μM PD98059. Although inhibition of ERK increased the activity of the basolateral 50 pS K channels, treatment of the TAL with PD98059 did not abolish the 10 μM PGE_2-induced inhibition of the 50 pS K channels and decreased NP_o from 0.59 ± 0.09 to 0.3 ± 0.07 ($n = 4$, $P < 0.01$) (Figure 6). However, upon examination of the time course of the experiments, it is apparent that the onset time of PGE_2 effect on the 50 pS K channels was also increased. Thus, inhibition of ERK could not completely abolish the effect of PGE_2 on the basolateral 50 pS K channels in the TAL. We then examined the effect of inhibiting p38 on the basolateral 50 pS K channels in the TAL. Similar to PD98059, inhibiting p38 with 5 μM SB203580[19] also increased basolateral 50 pS K channel activity from 0.21 ± 0.06 to 0.49 ± 0.08 (Figure 7) ($n = 4$, $P < 0.01$). This suggests that p38 MAPK inhibited the 50 pS K channels under control conditions. Moreover, in the presence of SB203580, PGE_2 was still able to inhibit the 50 pS K channels in the TAL and decreased NP_o from 0.49 ± 0.08 to 0.29 ± 0.08 ($n = 4$, $P < 0.01$). Thus, similar to PD98059, inhibition of p38 alone was not sufficient to abolish the PGE_2-induced inhibition of the basolateral 50 pS K channels. We then examined the possibility that the effect of PGE_2 was the result of stimulating both ERK and p38 MAPKs. Data summarized in Figure 7 demonstrate that inhibition of both p38 and ERK not only significantly increased the activity of the basolateral 50 pS K channels from 0.18 ± 0.05 to 0.70 ± 0.09 ($n = 4$, $P < 0.001$), but also abolished the PGE_2-induced inhibition of the basolateral 50 pS K channels completely. Thus, it is suggested that PGE_2 inhibits basolateral 50 pS K channels in the TAL through activation of PKC–MAPK pathways.

We next used the mTAL cell line, which has been shown to possess the properties of the native mTAL,[20] to study the effect of PGE_2 on ERK and p38 phosphorylation. We treated mTAL cell with 10 μM PGE_2 for 15 min and examined the phosphorylation of p38 and ERK. Figure 8 is a western blot showing that application of 10 μM PGE_2 stimulates phosphorylation of both p38 and ERK by 85 ± 10% ($n = 4$, $P < 0.05$) and 50 ± 8% ($n = 4$), respectively. Inhibition of cyclooxygenase with 10 μM indomethacin did not significantly affect the basal level of MAPK phosphorylation, suggesting that endogenous PGE_2 was not involved in regulating p38 and ERK phosphorylation. Moreover, in the presence of indomethacin, application of PGE_2 still stimulates p38 and ERK phosphorylation by 70 ± 10% ($n = 4$, $P < 0.05$) and 50 ± 8% ($n = 4$, $P < 0.05$), respectively. Thus, our results indicate that PGE_2 stimulates ERK and p38 MAPK. As the inhibitory effect of PGE_2 on the 50 pS K channels was abolished by blockade of PKC, we examined whether calphostin-C could abolish the effect of PGE_2 on the MAPK phosphorylation. We treated mTAL cells with 300 nM calphostin-C or the vehicle for 20 min, followed by addition of 10 μM PGE_2 for 5 min in the presence or absence of calphostin-C. As shown in Figure 9, inhibiting PKC did not significantly affect the basal level of MAPK phosphorylation, but it abolished the effect of PGE_2 on both ERK and p38 phosphorylation. This suggests that PKC is involved in mediating the effect of PGE_2 on MAPK phosphorylation.

Discussion

In this study, we have shown that PGE_2 inhibited the basolateral 50 pS K channels in the TAL and that inhibiting PKC abolished the effect of PGE_2 on the 50 pS K channel activity. As the effect of PGE_2 on the basolateral K channels was mimicked by sulprostone, results strongly suggest that the effect of PGE_2 is mediated by stimulation of either EP1 or EP3 receptor. Both EP1 and EP3 receptors are expressed in the kidney.[12,21] A large body of evidence indicates that EP1 receptors are mainly located in the distal nephron segment such as the connecting tubule and collecting duct,[22,23] whereas EP3 receptor is a major PG receptor in the TAL.[12] It is well demonstrated that EP3 receptor is a G-protein-coupled receptor and that stimulating

EP3 receptor inhibits adenylate cyclase and decreases cAMP generation. However, the observation that inhibiting PKA had no significant effect on the basolateral 50 pS K channel, suggests that PKA is not involved in determining the basal activity of the 50 pS K channels. Thus, it is unlikely that the inhibitory effect of PGE_2 on the 50 pS K channels was the result of inhibition of PKA, although activation of PKA stimulated the basolateral 50 pS K channel activity in the TAL.[8] In addition to decreasing camp generation, stimulation of EP3 receptor has also been reported to activate PKC, MAPK, and Rho-kinase,[15,16] and to increase inositol triphosphate/Ca^{2+} release.[24] The finding that inhibiting PKC restored the channel activity inhibited by PGE_2, strongly suggests that PKC was responsible for mediating the effect of PGE_2 on the basolateral K channels. The role of PKC in inhibiting the basolateral 50 pS K channels is also suggested by the observation that stimulation of PKC with phorbol 12-myristate 13-acetate inhibited the basolateral K channels. Relevant to our observation is that PGE_2 inhibited vasopressin-induced stimulation of bicarbonate transport in the mTAL by activating PKC-δ.[25,26] Also, we have demonstrated that PGE_2 inhibits the apical 70 pS K channels by a PKC-dependent signaling mechanism in the TAL.[27] However, we could not completely exclude the possibility that EP1 receptor is responsible for the inhibitory effect of PGE_2 on the basolateral K channels in the TAL.

This study has also demonstrated that p38 and ERK were involved in mediating the effect of PGE_2 on the basolateral 50 pS K channels in the TAL. This view is supported by two lines of evidence: (1) PGE_2 stimulated phosphorylation of p38 and ERK in the mTAL cells and (2) inhibition of p38 and ERK abolished the inhibitory effect of PGE_2 on the 50 pS K channels. Because inhibition of PKC abolished the effect of PGE_2 on the phosphorylation of both p38 and ERK, MAPK may be a downstream signaling molecule, which mediates the effect of PGE_2 on the basolateral K channels. MAPK plays an important role in the regulation of renal K channels. We have demonstrated previously that p38 and ERK inhibited the big conductance K channels and renal outer-modullary K channel (Kirl.1) in the CCD.[28,29] The observation that inhibiting p38 or ERK stimulated the basolateral 50 pS K channel activity suggests that MAPKs were involved in determining the basal activity of the basolateral 50 pS K channels. This is in contrast to PKC, which is not involved in determining the basal activity of the 50 pS K channels because inhibition of PKC did not significantly alter basolateral K channel activity. We speculate that MAPK is activated under control conditions (without PGE_2) and application of PGE_2 causes further stimulation of MAPK by a PKC-dependent mechanism. PKC has been shown to stimulate MAPK in different types of cells.[30,31] The mechanism by which PKC stimulates MAPK in response to PGE_2 is not clear. PKC has been shown to stimulate NADPH oxidase[32] and stimulation of NADPH oxidase increases superoxide production, which is a potent stimulator for p38 and ERK in a variety of tissues, including kidney.[33]

The physiological significance of this study is to illustrate the mechanism by which PGE_2 inhibits Cl absorption in the TAL through inhibiting basolateral K channels. Figure 10 is a cell model illustrating the way PGE_2 inhibits basolateral K channels in the TAL. Stimulation of EP1 or EP3 receptor activates PKC, which further increases p38 and ERK activity. As a consequence, PGE_2 inhibits basolateral K channels. PGE_2-induced inhibition of the basolateral K channel activity is expected to depolarize the basolateral cell membrane potential and decrease the driving force for Cl exit across the basolateral membrane. Decreasing Cl exit would raise intracellular Cl concentrations, which leads to inhibition of the Na/K/Cl co-transporter.[34] A large body of evidence indicates that PGE_2 inhibits Cl absorption in the TAL.[10,35,36] The inhibitory effect of PGE_2 on Cl transport may be partially mediated by inhibiting the Na/K/Cl co-transporter.[36,37] However, it has been reported that PGE_2 had no effect on the basal level of Cl transport in the absence of vasopressin, although it abolished vasopressin-induced stimulation of Cl transport in the mouse medullary TAL.[10] We have now demonstrated that PGE_2 may inhibit Cl absorption by inhibiting basolateral K channel activity in the absence of vasopressin. The discrepancy may be due to the different concentrations of PGE_2 and

different part of TAL because we used high concentrations of PGE_2 and cortical TAL in our experiments. The finding that PGE_2 at high concentrations may affect the activity of epithelial transport in the TAL, has also been reported in our previous study.[27] We have shown that PGE_2 at low concentrations (<1 μM) abolished the stimulatory effect of vasopressin on the apical K channels in the TAL, while 5–10 μM PGE_2 inhibited apical K channels by PKC. PGE_2-induced inhibition of basolateral K channels may be partially responsible for the inhibition of salt transport in the TAL during K-restriction, which is known to cause impairment of the epithelial transport in the Henle's loop. Because K-restriction increases cyclooxygenase-2 expression and PGE_2 generation in the kidney,[17] increasing PGE_2 production could suppress the salt transport by inhibiting apical K channels and the Na/K/Cl co-transporter and basolateral K channels. We conclude that PGE_2 inhibits the basolateral 50 pS K channels and that the effect of PGE_2 is mediated by stimulating the PKC–MAPK-dependent pathway.

Materials and Methods

Preparation of the TAL

Pathogen-free Sprague–Dawley rats of either sex (40–50 g) were purchased from the animal facility of the Second Affiliated Hospital of Harbin Medical University. The rats were fed normal rat chow and had free access to water for 7 days before experiments. Rats were killed by cervical dislocation (the weight of rats was less than 90 g) and kidneys were removed immediately. Thin coronal sections were cut with a razor blade and kidney slices were incubated in a HEPES buffer solution containing collagenase type-1A (1 mg/ml; Sigma, St Louis, MO, USA) at 37 °C for 45–60 min. After collagenase treatment, the kidney slices were rinsed with a solution containing (in mM) 140 NaCl, 5 KCl, 1.5 $MgCl_2$, 1.8 $CaCl_2$, and 10 HEPES (pH = 7.4) at 4 °C. The cortical TAL was dissected and the isolated tubule was transferred onto a 5 × 5-mm cover glass coated with polylysine (Sigma) to immobilize the tubule. The cover glass was placed in a chamber mounted on an inverted microscope (Nikon, Melville, NY, USA). The TAL tubules were superfused with HEPES-buffered NaCl solution plus 5 mM glucose. The basolateral membrane of cells, which had no sign of swelling and a clear board to their neighbor cells, was then patched. The animal protocol was approved by the animal use committee of Harbin Medical University.

Patch-clamp technique

The patch pipette was pulled with Narishege PP-81 electrode puller and the pipette solution contained (in mM) 140 KCl, 1.8 $MgCl_2$, and 10HEPES (pH = 7.4). We used an Axon 200A patch-clamp amplifier to record channel current. The current was low-pass filtered at 0.5 kHz and digitized with an Axon interface (Digidata 1200). Data were collected with an IBM-compatible Pentium computer at a rate of 2 kHz and analyzed using pClamp software system 7.0 (Axon Instruments, Burlingame, CA, USA). Channel activities were defined as NP_o, a product of channel open probability (P_o) and channel number (N). NP_o was calculated from data samples of 60-s duration in the steady state as follows:

$$NP_o = \sum (1t_1 + 2t_2 + \ldots it_i)$$

where t_i is the fractional open time spent at each of the observed current levels. We selected a 60-s-long trace at the steady state after the onset of PGE_2 effect to calculate the channel activity. The channel conductance was determined by measuring K current at several different holding potentials.

Cell culture

The method used to culture mTAL cell has been described previously.[20] Briefly, mTAL cells were grown in Dulbecco's modified Eagle's medium/F12(1:1) medium with 2.5 mM L-glutamine, 15 mM HEPES, 0.5 mM sodium pyruvate, and 1.2 g/l sodium bicarbonate supplemented with 0.005 mM dexamethasone and 5% fetal bovine serum. mTAL cells were treated with PGE_2 for 5 min. After treatment with PGE_2, cells were washed with ice-cold phosphate-buffered saline twice and incubated for 30 min in radio-immunoprecipitation assay lysis buffer (1 × phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonyl fluoride, 10 μl/ml Cocktail, 100 mM sodium orthovanadate).

Western blot

Proteins homogenized from mTAL cells were separated by electrophoresis on 8–10% SDS–polyacrylamide gels and transferred to Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with Odyssey blocking buffer for fluorescent western blotting (Rockland, Gilbertsville, PA, USA) and incubated with the primary antibody at 4 °C for 12 h. The membrane was washed four times (5 min for each wash) with phosphate-buffered saline containing 0.1% Tween-20, followed by incubation with the secondary antibody for an additional 30 min. The membrane was then washed three times (10 min for each wash) with phosphate-buffered saline and scanned by Odyssey infrared imaging system (LI-COR, Lincoln, NE, USA) at wavelength of 680 or 800 nm.

Experimental solutions and statistics

Phorbol 12-myristate 13-acetate, PGE_2, sulprostone, calphostin-C, SB203580, and PD98059 were purchased from Biomol (Plymouth Meeting, PA, USA) and dissolved in either ethanol or dimethyl-sulfoxide solution. The final concentration of ethanol or dimethyl-sulfoxide was less than 0.1% and had no effect on channel activity. Data are shown as mean ± s.e.m. We used either Student's *t*-test or one-way analysis of variance to determine the significance between the two groups. $P < 0.05$ was considered statistically significant.

Acknowledgments

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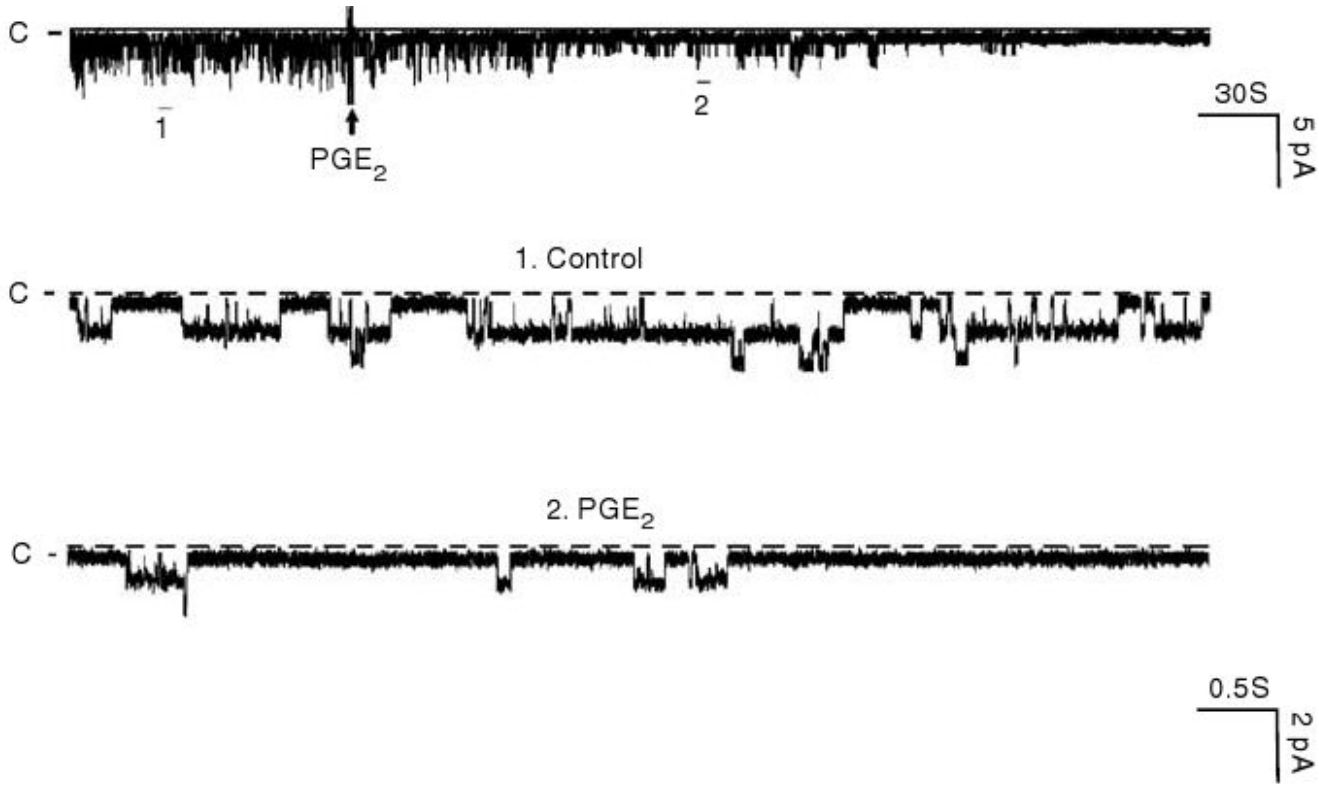

Figure 1. A channel recording demonstrating the effect of 10 μM PGE_2 on the basolateral 50 pS K channels

The experiment was performed on a cell-attached patch and the holding potential was −20 mV (depolarization). The channel close level is indicated by a dotted line and C. Two parts of the record indicated by numbers are extended to show the fast time resolution.

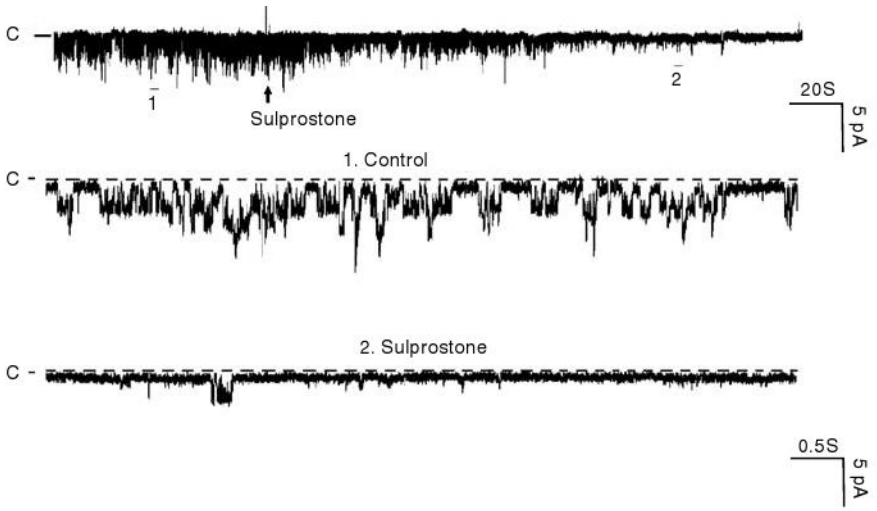

Figure 2. A channel recording showing the effect of 10 μM sulprostone on the basolateral 50 pS K channels

The experiment was performed on a cell-attached patch and the holding potential was 0 mV. The channel close level is indicated by a dotted line and C. Two parts of the record indicated by numbers are extended to show the fast time resolution.

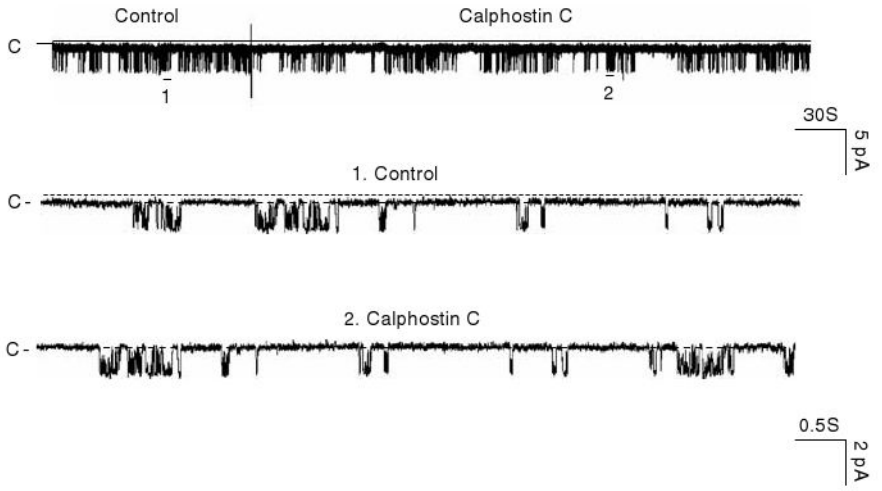

Figure 3. A channel recording showing the effect of calphostin-C on the 50 pS K channels in the TAL

The experiment was performed on a cell-attached patch and the holding potential was −20 mV (depolarization). The channel close level is indicated by a dotted line and C. Two parts of the record indicated by numbers are extended to show the fast time resolution.

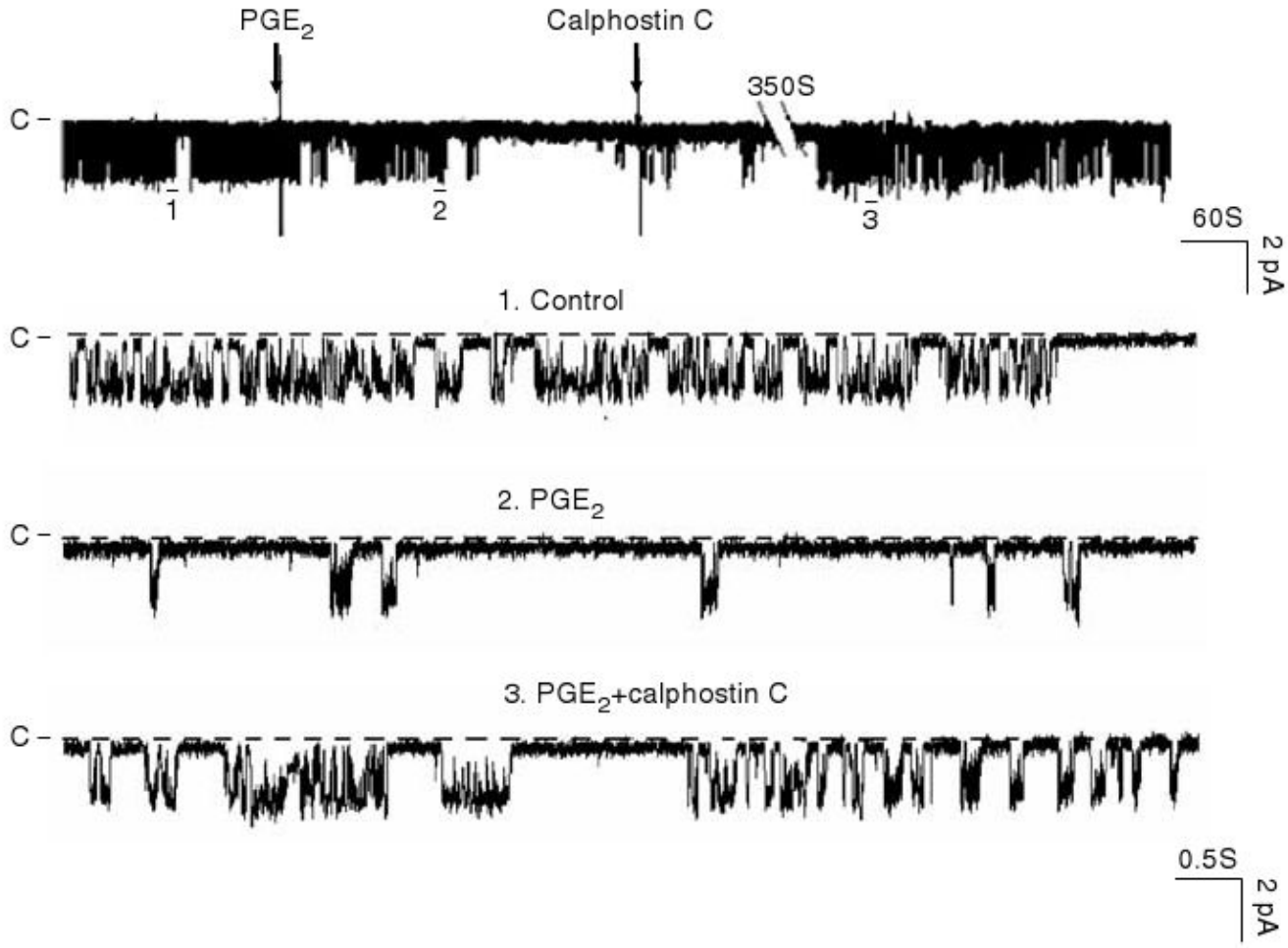

Figure 4. A channel recording demonstrating the effect of 10 μM PGE_2 on the basolateral 50 pS K channels in the absence and presence of 300 nM calphostin-C

The experiment was performed on a cell-attached patch and the holding potential was −20 mV. The channel close level is indicated by a dotted line and C. Three parts of the record indicated by numbers are extended to show the fast time resolution.

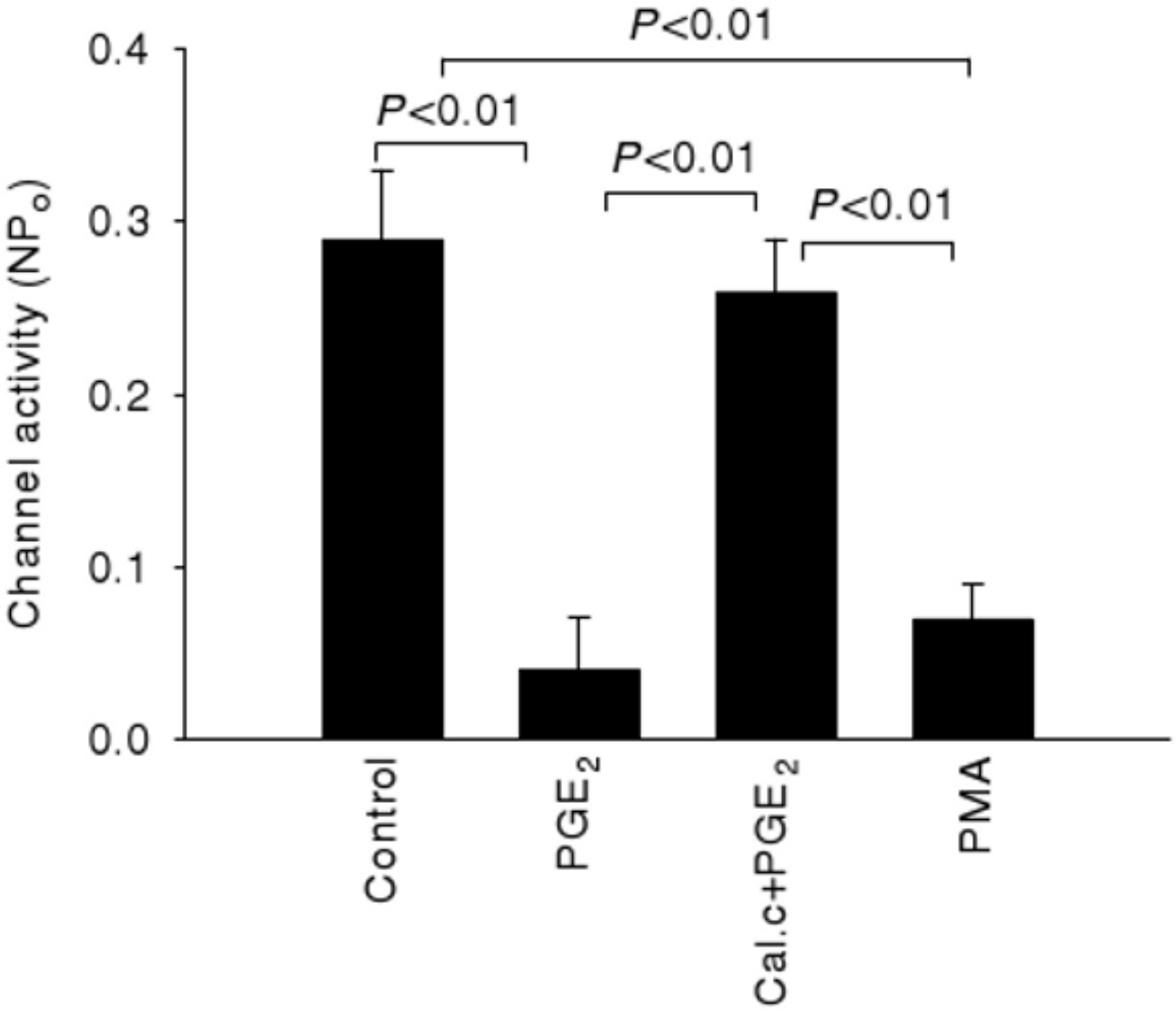

Figure 5. Effect of PGE_2 (10 μM), PGE_2 + calphostin-C (cal.c) (300 nM), and phorbol 12-myristate 13-acetate (5 μM) on the basolateral 50 pS K channels

The experiments were carried out on cell-attached patches.

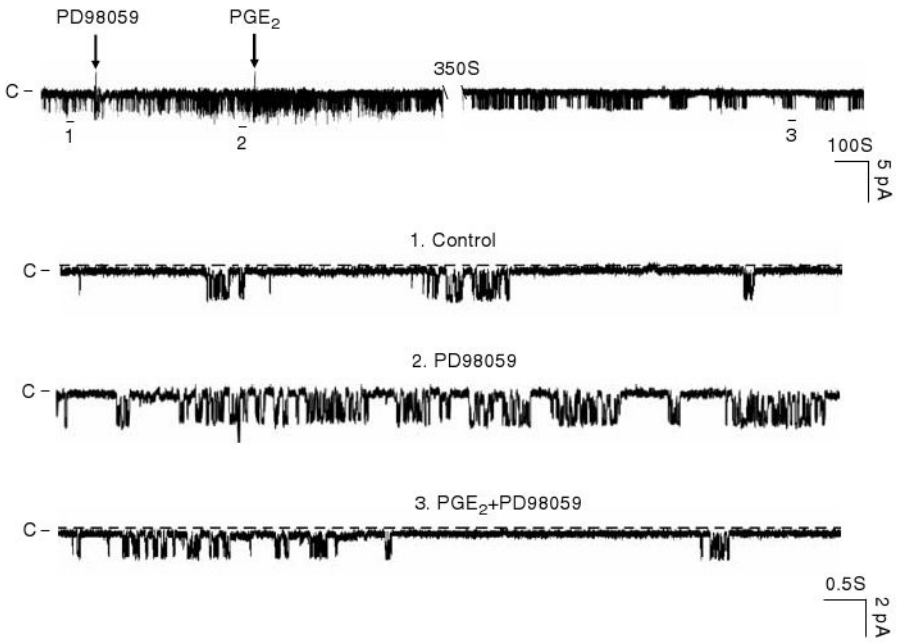

Figure 6. A channel recording demonstrating the effect of PD98059 (50 μM) and 10 μM PGE_2 + PD98059 on the basolateral 50 pS K channels in the TAL

The experiment was performed on a cell-attached patch and the holding potential was −10 mV (depolarization). The channel close level is indicated by a dotted line and C. Three parts of the record indicated by numbers are extended to show the fast time resolution.

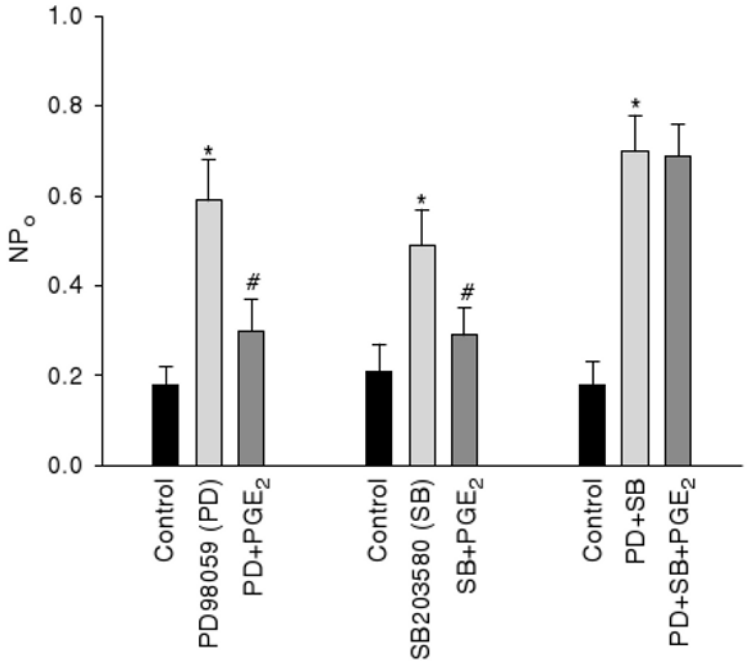

Figure 7. Effect of 10 μM PGE_2 on the basolateral 50 pS K channels in the TAL treated with PD98059, SB203580 or both agents

The experiments were performed on cell-attached patches. The asterisk indicates the significant difference between the MAPK inhibitor and the corresponding control. # Indicates a significant difference between the PD group and PD + PGE_2 group or between the SB and SB + PGE_2 group.

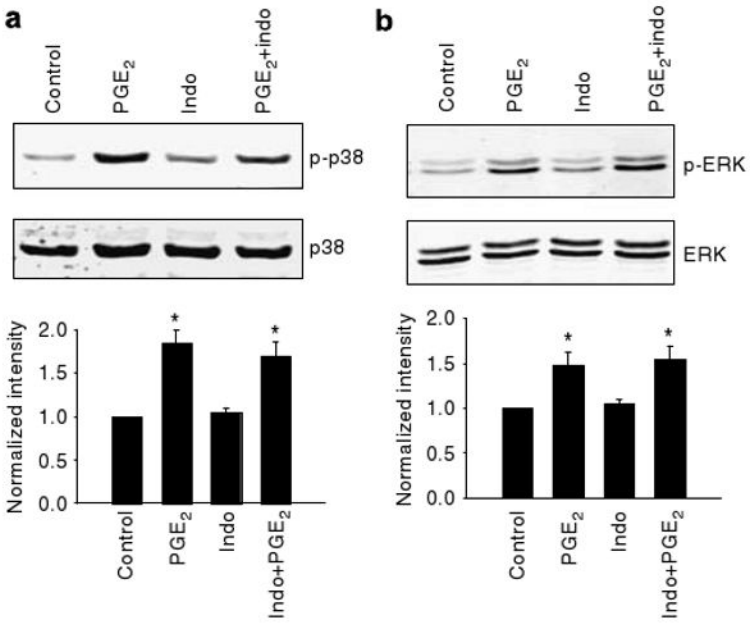

Figure 8. Effect of PGE_2 on the phosphorylation of p38 and ERK

Western blots show the effect of 10 μM PGE_2 on the phosphorylation of p38 (**a**) and ERK (**b**) in the presence or absence of 10 μM indomethacin. Data are summarized in a bar graph (bottom panel). The asterisk indicates a significant difference in comparison with the control.

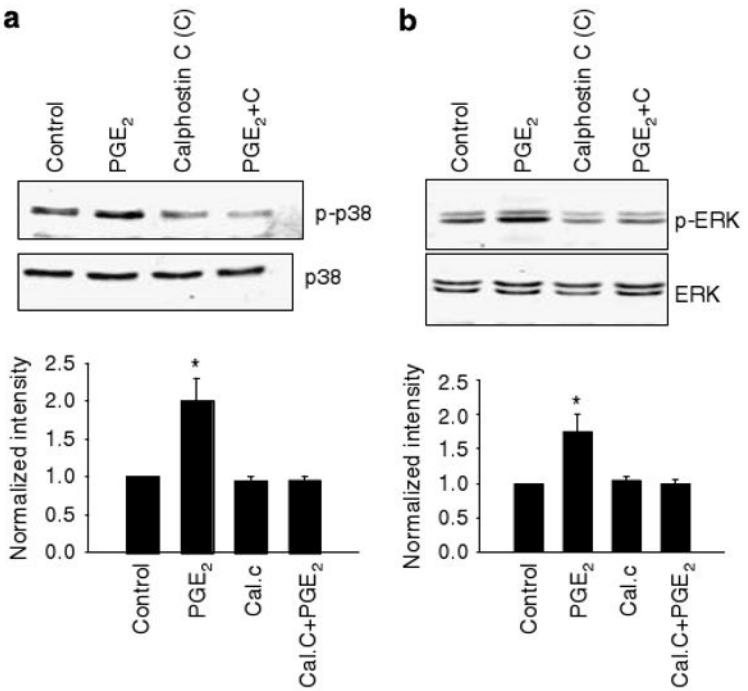

Figure 9. Effect of PGE_2 on the phosphorylation of p38 and ERK in the presence of PKC inhibitor

Western blots show the effect of PGE_2 on the phosphorylation of p38 (**a**) and ERK (**b**) in the presence or absence of 300 nM calphostin-C. Data are summarized in a bar graph (bottom panel). The asterisk indicates a significant difference in comparison with the control.

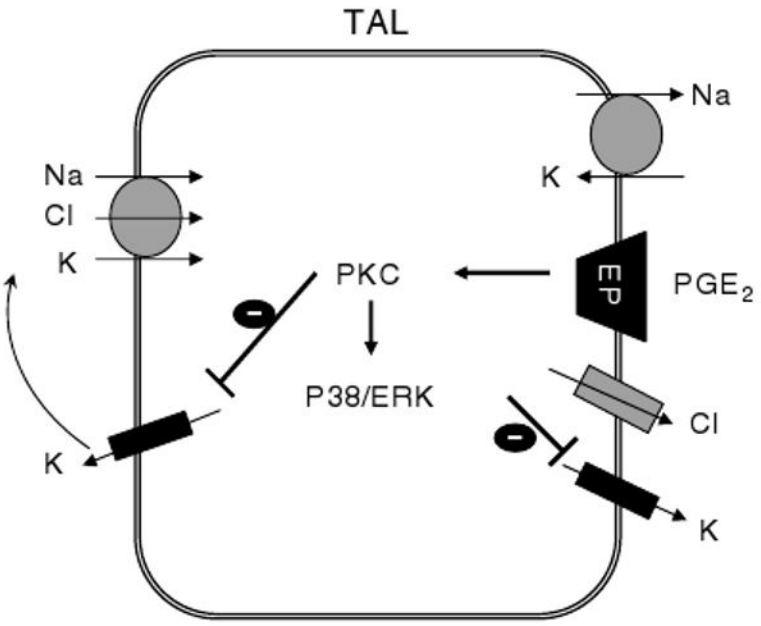

Figure 10. A cell model illustrating the mechanism by which PGE_2 inhibits the basolateral 50 pS K channels by a PKC–MAPK pathway in the TAL