

A-type potassium channels are involved in resting membrane potential depolarization induced by Prostaglandin E2 in rat dorsal root ganglia neurons.

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Short Report

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Abstract

Prostaglandin E₂ (PGE₂) is an inflammatory mediator that directly sensitizes nociceptors. In the present study, we investigated the type of potassium channels involved in depolarization of the resting neuronal membrane potential induced by PGE₂ using the fluorescent probe DiBAC₄(3) in primary dorsal root ganglia cultures. It was observed that neurons isolated from ipsilateral L4 and L5 ganglia of rats treated with intraplantar PGE₂ injections are depolarized in relation to neurons obtained from contralateral ganglia. PGE₂ induced depolarization was not affected in the presence of tetraethylammonium (TEA) or glibenclamide, while it was reduced in the presence of 4-aminopyridine (4-AP). Furthermore, 4-AP did not further depolarize neurons previously depolarized by PGE₂ suggesting that PGE₂ acts on the same channels blocked by 4-AP. Therefore, resting membrane depolarization induced by PGE₂ during nociceptive sensitization appears to involve reduction of A-type potassium currents.

1. Introduction

Nociceptor sensitization is a hallmark of inflammatory pain and is produced by the action of inflammatory mediators on peripheral sensory neurons [1]. During sensitization, the threshold for activation of action potentials is reduced, facilitating impulse generation and subsequent conduction through pain pathways that result in pain perception [2]. Prostaglandin E₂ (PGE₂), an eicosanoid synthesized from arachidonic acid in a reaction catalyzed by cyclooxygenases (COX), is one of the most important mediators that can directly sensitize primary nociceptive neurons [3]. The molecular mechanism for PGE₂ induced nociceptor sensitization involves selective G-coupled EP receptors that activate adenylyl cyclases, resulting in cAMP accumulation, PKA and PKC activation [3, 4].

The final mechanism responsible for the enhanced neuronal excitability seems to depend on the modulation of sodium (Na⁺) and potassium (K⁺) channels [5, 6, 7]. The phosphorylation of specific subtypes of Na⁺ channels, the Nav1.8 and Nav1.9 have been implicated in nociceptor sensitization [6, 8] and are investigated as important targets for analgesic development [9]. The participation of K⁺ channels in nociceptive sensitization is, however, not well comprehended. These channels are key regulators of neuronal excitability, responsible for setting resting membrane potential and also for the repolarization phase of action potentials. K⁺ channels can be classified according to their electrophysiological properties into voltage-gated (Kv), calcium-activated (KCa), inward rectifier (Kir or IRK), and two-pore (K2P) potassium channels [10]. Voltage-gated K⁺ channels are subdivided, according to the characteristics of their currents, as fast-inactivating A-type currents or slowly-inactivating delayed-rectifier-type currents [11]. It was observed that prostaglandin E₂ administration at dorsal root ganglia neurons induces a reduction in the amplitude of voltage-gated K⁺ currents [5].

The identification of molecular mechanisms underlying sensitization by an inflammatory mediator, such as PGE₂, might provide therapeutic targets for pain treatment and possibly a better understanding of pain chronification processes. In the present study it was evaluated the depolarization of the resting

membrane potential induced by PGE₂ in primary cultures of rat peripheral nociceptive neurons and selective subtypes of K⁺ channels.

2. Methods

2.1. Animals

Females Wistar rats (100 g) at approximately 4 weeks old were used, manipulated, and euthanized according to the guidelines established by Guide for the Care and Use of Laboratory Animals [12] and was approved by the Committee for Ethical Use of Animals from Universidade Federal de Uberlândia (CEUA/ UFU N°078/17). The animals were maintained under standard conditions on light-dark 12/12h cycles and allowed a standard diet and water *ad libitum*. Female rats were used due to greater availability. Pilot experiments were performed with animals of both sexes and no difference was observed in the neuronal response to PGE₂.

2.2. Dorsal root ganglia primary cultures preparation

Rats were euthanized following isoflurane inhalation anesthesia. Lumbar and thoracic dorsal root ganglia (DRGs) were immediately dissected and transferred to Hank's Buffered Saline Solution supplemented with 10 mM Hepes. For enzymatic dissociation, ganglia were sequentially incubated with Type II collagenase (Sigma, 0.28 U/ml) for 75 minutes, followed by 0.25% trypsin (Sigma) for 12 minutes. The enzyme activity was quenched by performing three washes with DMEM containing 10% fetal bovine serum (FBS). Ganglia were then mechanically dissociated using a fire-polished glass Pasteur pipette. Dissociated cells were subsequently seeded onto glass-bottom dishes coated with Matrigel (BD). Cultures were maintained for 24 hours in a humidified 5% CO₂ atmosphere at 37°C.

2.3 Drugs

Prostaglandin E₂ - PGE₂ (Sigma), dibutyl-cAMP (Sigma), tetraethylammonium - TEA (Sigma), glibenclamide (Sigma). 4-aminopyridine - 4-AP (Sigma), capsaicin (Sigma), DiBAC₄(3) (Invitrogen). All reagents and drugs were diluted in a Hanks/Hepes solution and applied at the primary cultures using 10X the desired final concentration during image acquisition. Capsaicin was diluted from a stock solution of 10 mM in dimethyl sulfoxide (Vetec).

2.4. Confocal microscopy

Cultures were washed with Hanks/Hepes buffered solution and incubated for 30 minutes with 5 μM of DiBAC₄(3) in Hanks/Hepes at room temperature in the dark. The fluorescent probe remained at the culture dishes during experiments as the molecule movement across the plasmatic membrane according to its electrochemical gradient determines fluorescence variations. is a slow-response, lipophilic, anionic fluorescent dye used to monitor changes in cellular membrane potential. Because of its negative charge, the dye is excluded from resting cells with a negative interior potential; upon membrane depolarization, the dye partitions across the plasma membrane into the cytosol and binds

intracellular proteins and membranes, resulting in an increase in fluorescence intensity. Conversely, hyperpolarization drives the dye out of the cell, leading to a decrease in fluorescent signal. Fluorescent images were acquired using a confocal microscope (LSM 510 meta, Zeiss). Images were registered for 10 minutes at 3-second intervals at basal conditions followed by immediate administration of vehicle or drugs at 10X the final concentration. After each test, capsaicin (1 μ M) was applied and fluorescence images were registered for 2 minutes. Only neurons that responded to capsaicin with membrane depolarization were selected for analysis. Capsaicin sensitivity allowed the selection of nociceptive neurons that were alive and functional in the primary cultures.

Each experiment was repeated in 3 different plates and images were analyzed using Image J software (developed by Wayne Rasband, NIH, USA). Regions of interest containing individual neurons were selected and fluorescence intensities were quantified at the image series. For determination of fluorescence variation data were calculated as $(\delta F/F_0)$, in which $\delta F = F - F_0$ and F_0 is the fluorescence intensity of the first image in the time series, acquired before any drug administration. Because DiBAC₄ (3) response kinetics are inherently slow, it is not prone to rapid baseline fluctuations, allowing the initial frame to be reliably used as F_0 .

2.5. Effect of *in vivo* intraplantar injection of PGE₂ on the resting potential of cultured sensory neurons.

PGE₂ (100 ng in 50 μ L of sterile saline) was injected into the right hind paw of three animals. After 120 minutes, the animals were euthanized and the ganglia from the L4 and L5 regions of the right and left sides were removed. Dorsal root ganglia cultures were performed as previously described and divided into four culture plates of the right ganglia (ipsilateral to the treatment) and four culture plates of cells from the left ganglia (contralateral). Plates were left at the incubator for 60 minutes for cell adhesion and then treated with the resting potential indicator DiBAC₄(3) (5 μ M, 30 min). The fluorescence emitted by the cells was acquired through images obtained by confocal microscopy as previously described.

2.6. Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 8.2 (GraphPad Software Inc., San Diego, CA). The comparison of data between two groups was analyzed by Mann-Whitney test or T Test and three or more groups by Kruskal-Wallis test followed by Dunn's multiple comparisons test. Nonparametric tests were used in most of the analyses of the results, since the data did not present a normal distribution. The selection of neurons to be considered for the analyses was based on their response to capsaicin, which was administered at the end of all tests. Thus, we included mainly viable nociceptive neurons in the analyses, but not all of them responded to PGE₂. The responses, therefore, were closer to a bimodal distribution. The differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. PGE₂ induces depolarization of nociceptive neurons in primary cultures of dorsal root ganglia

Administration of PGE₂ (1, 10, 100 nM) depolarized nociceptive neurons in a concentration-dependent manner. The higher concentration tested (100 nM) induced an increase of 72% in fluorescence (n = 30, median = 0.4645, p < 0.0001) while 10 nM induced a smaller depolarization (n = 37, median = 0.1772, p = 0.02) and 1 nM (n = 20, median = 0.0379, p > 0.09) did not provoke detectable alterations after 10 minutes comparing to vehicle administration (n = 150, median = 0.0076) (Fig. 1).

To verify the possible involvement of membrane depolarization in PGE₂ induced hyperalgesia, rats were treated with intraplantar PGE₂ injections (100 ng in 50 µL of sterile saline) and euthanized after 120 min. Dorsal root ganglia cultures were prepared from L4/L5 levels ganglia. Figure 2 show the fluorescence, in arbitrary units, emitted by incubation with DiBAC₄(3) of neurons obtained from ipsilateral and contralateral ganglia. Nociceptive neurons obtained from ganglia ipsilateral to the PGE₂ injected paw show increased fluorescence when compared to neurons from contralateral ganglia, indicating that *in vivo* PGE₂ injection induced resting membrane depolarization in nociceptive neurons.

3.2. The cell permeable cAMP analogue db-cAMP induces depolarization of cultured nociceptive neurons.

PGE₂ induced hyperalgesia depends on the activation of the adenylyl cyclase/cAMP/PKA pathway [4]. To test if PGE₂ induced depolarization is related to hyperalgesia and depends on the same intracellular mechanisms, we tested the effects of the cAMP analog, dibutyryl-cAMP (db-cAMP) [13]. The administration of db-cAMP (100 µM) resulted in a depolarization of the nociceptive neurons (n = 35, median = 0.3589, p < 0.0001) that was not different from that induced by PGE₂ (100 nM) when comparing to vehicle administration (Fig. 3).

3.3. Evaluation of K⁺ channels participation in the PGE₂ induced depolarization of nociceptive neurons

As resting membrane potential is mainly maintained and modulated by K⁺ channels it was tested the effects of selective K⁺ channels blockers in the fluorescence intensities of nociceptive neurons in culture. The effects of the non-selective delayed rectifier K⁺ channels blockers, tetraethylammonium (TEA), the A-type potassium channel blocker, 4-aminopyridine (4-AP) and the ATP dependent potassium channel (K_{atp}) blocker, glibenclamide, were tested in the resting membrane potential of primary sensory neurons in culture (Fig. 4A). The concentrations of 4-AP, TEA and glibenclamide used in these experiments were selected based on other studies by [14, 15]. Administration of 4-AP resulted in a resting membrane potential depolarization of nociceptive neurons (n = 12, median = 0.4200) that was

stronger than that induced by glibenclamide ($n = 8$, median = 0.0245, $p = 0.001$) or TEA ($n = 11$, median = 0.1780, $p = 0.01$) administration.

When PGE_2 was administered 15 minutes after the K^+ channel blockers (Fig. 4B) it was observed that PGE_2 was not able to further depolarize neurons that were depolarized by 4-AP ($n = 11$, median = 0.061, $p = 0.03$). PGE_2 induced depolarization was not affected in the presence of TEA ($n = 7$, median = 0.2640) or glibenclamide ($n = 5$, median = 0.4770). Also, 4-AP administration was tested after 15 minutes of PGE_2 administration (Fig. 4C). In the presence of PGE_2 (100 nM), 4-AP (10 mM) was able to depolarize only neurons that were not affected by PGE_2 ($n = 5$, median = 0.4900), but did not further depolarize neurons that were previously depolarized by PGE_2 administration ($n = 6$, median = 0.00, $p = 0.0079$).

4. Discussion

The molecular mechanism involved in depolarization of the resting neuronal membrane potential induced by PGE_2 was investigated using fluorescence microscopy. Previous studies have already used fluorescence to assess variations of neuronal resting potential [15, 16, 17]. Using DiBAC₄ (3) fluorescence potentiometry, this study demonstrates that PGE_2 induces resting membrane depolarization in nociceptive DRG neurons via downstream cAMP signaling and functional suppression of 4-AP-sensitive, A-type potassium conductances.

PGE_2 is an inflammatory mediator able to sensitize nociceptors and the molecular mechanism involved is associated with the activation of the cAMP /PKA pathway [4]. In electrophysiological studies of neurons isolated from the DGR, it was demonstrated that the activation of PKA by PGE_2 leads to inhibition of K^+ currents and also an increase in Na^+ currents due to phosphorylation of tetrodotoxin-resistant Na^+ channels, two important mechanisms for neuronal excitability [5, 6, 7]. PGE_2 via EP receptors also enhances the nociceptive actions of other mediators of inflammation, such as bradykinin [18, 19], and potentiates TRPV1 activity and pain response evoked by capsaicin [20, 21].

Data presented in the current study showed that PGE_2 induced depolarization in a concentration-dependent manner in nociceptive neuron cultures (Fig. 1) and the depolarization induced by PGE_2 was mimicked by a permeable cAMP analog, db-Camp (Fig. 3). Because cAMP signaling is a recognized mediator of PGE_2 -induced inflammatory pain, and intraplantar db-cAMP directly triggers mechanical hyperalgesia, this resting membrane depolarization likely represents a cellular mechanism driving peripheral sensitization *in vivo*. To further investigate the participation of membrane depolarization in PGE_2 induced hyperalgesia, rats were treated with intraplantar PGE_2 injections and neurons isolated from L4/L5 ipsilateral ganglia were compared with neurons from contralateral ganglia (Fig. 2). Neurons from ganglia that contained neurons that were sensitized by PGE_2 injections had membrane potential that were depolarized when compared with neurons from the contralateral ganglia. We used capsaicin as a positive control to select nociceptive neurons that were functional in culture. Capsaicin activates TRPV1 receptors expressed mainly in peripheral terminals of C-fiber nociceptive neurons [22]. Thus, using

fluorescence microscopy it was possible to show nociceptive sensitization by PGE₂ and the involvement of cAMP in this mechanism, reproducing the mechanism previously described by electrophysiological and *in vivo* studies (5).

K⁺ channels are a large family of membrane proteins and the main regulators of resting membrane potential, repolarization and the frequency of action potentials in neurons and other electrically excitable cells; their activation reduces excitability, whereas channel inhibition increases excitability [23]. Studies have already described the importance of K⁺ channels in neuronal sensitization by PGE₂ [7, 24]. Evans et al. (1999) [7] demonstrated in an electrophysiological study of sensory neurons isolated from rat embryos that an analog of cAMP (cpt-cAMP) or PGE₂ caused a suppression of K⁺ currents, an effect dependent on activation of PKA. In this study, the authors conclude that PGE₂ is acting on delayed rectifiers of K⁺ channels blocked by tetraethylammonium (TEA). Jiang et al. (2003) [24] tested the effect of PGE₂ on sensory neurons of knockout mice for Kv1.1 channel, a type of delayed rectifier K⁺ channel, and observed that PGE₂ continued to inhibit K⁺ currents in such neurons. To better understand the role of delayed-rectifiers K⁺ channels we use the blocker TEA, a non-selective delayed rectifier K⁺ channel blocker. In addition to TEA, the other blockers used were 4-AP, which nonspecifically blocks A-type K⁺ channels, and the inhibitor of Katp channels glibenclamide.

The results showed that only administration of 4-AP was able to significantly depolarize neurons in culture (Fig. 4A). In addition, the effect of PGE₂ was not verified when the cells were previously depolarized by 4-AP (Fig. 4B). Furthermore, 4-AP did not further depolarize neurons previously depolarized by PGE₂ (Fig. 4C). These results indicate that PGE₂ acts on the same channels blocked by 4-AP, the A-type K⁺ channels. As PGE₂ remains capable of depolarizing neurons in the presence of the blockers TEA and glibenclamide, delayed rectifier channels and Katp channels seem not to be involved in the observed PGE₂ induced depolarization. These results do not exclude a PGE₂ effect in such K⁺ channels. As the method presented in this study is able to evaluate only resting membrane potential, PGE₂ might likely modulate other K⁺ channels that are involved in other events, for example, in the repolarization phase of the action potential. The participation of A-type K⁺ currents in pain were previously described in a rat model of chronic pancreatitis [25], corroborating with a possible role for these channels in nociceptive sensitization. A variety of A-type K⁺ channels were detected in nociceptive neurons at the DRG, including Kv1.4, Kv3.4, Kv4.1, and Kv4.3 [26]. Also, alterations in A-type channels expression, function and modulation in DRG neurons have already been demonstrated in pain models, including sciatic nerve ligation [27], diabetic neuropathic pain [28], and bone cancer [29]. Our findings support a model where inflammatory prostanoids directly target these conductances to elevate baseline membrane potential, shifting primary afferents closer to the activation threshold for voltage-gated sodium channels and amplifying peripheral excitability.

5. Conclusion

PGE₂ induces sustained resting membrane depolarization in primary nociceptive sensory neurons through the activation of cAMP-dependent downstream signaling and subsequent inhibition of 4-AP-sensitive A-type potassium channels. Pharmacological strategies aimed at preventing the downregulation of these specific conductances may represent a viable approach for mitigating inflammatory peripheral sensitization.

Abbreviations

• 4-AP	
4-Aminopyridine	
• COX	
Cyclooxygenase	
• db-cAMP	
Dibutyl Cyclic Adenosine Monophosphate	
• dF/F ₀	
Fluorescence Variation over Initial Fluorescence	
• DiBAC4(3)	
Bis-(1,3-Dibarbituric Acid)-Trimethine Oxonol	
• DRG / DRGs	
Dorsal Root Ganglion / Dorsal Root Ganglia	
• EP	
Prostaglandin E Receptor	
• K2P	
Two-Pore Domain Potassium Channel	
• KCa	
Calcium-Activated Potassium Channel	
• Kir / IRK	
Inward Rectifier Potassium Channel	
• K _{ATP}	
ATP-sensitive Potassium Channel	
• PGE ₂	
Prostaglandin E2	
• PKA	
Protein Kinase A	
• PKC	
Protein Kinase C	
• TEA	
Tetraethylammonium	
• TRPV1	
Transient Receptor Potential Vanilloid 1	

Declarations

Conflict of Interest Statement:

The authors declare no conflict of interest.

Author Contribution

DOS and CMCL conceived and designed the study. CMCL developed the methodology. DOS and TCL performed the experiments and investigation. Formal data analysis was carried out by DOS, TCL, and CMCL. CMCL supervised the project and was responsible for project administration. DOS, TCL, and CMCL wrote the original draft, and all authors contributed to reviewing and editing the final manuscript.

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Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

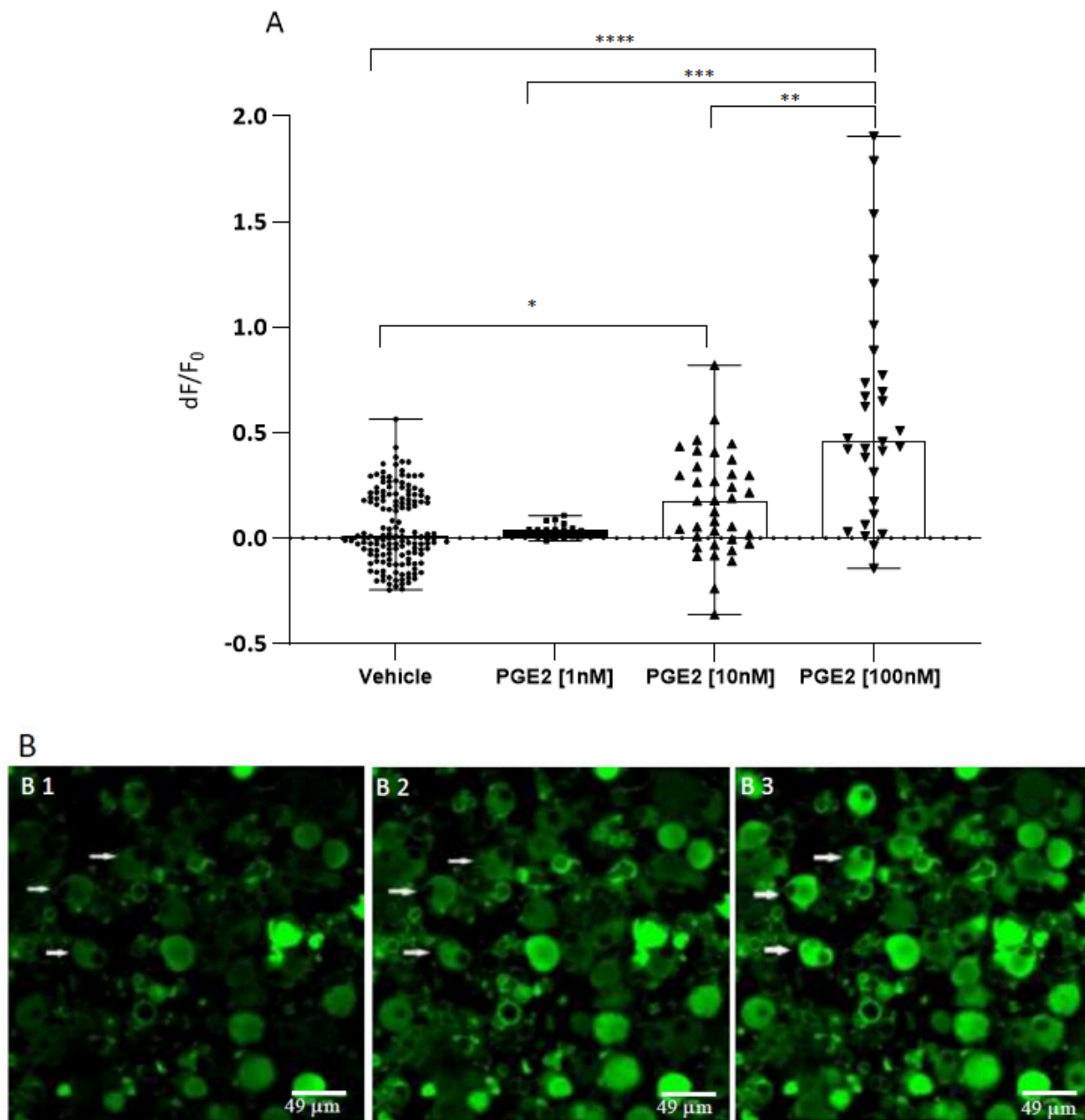


Figure 1

A: PGE2 concentration-dependent effect on resting membrane potential of cultured nociceptive neurons. It is shown the variation of fluorescence intensities after 10 minutes of PGE2 (1, 10 or 100 nM) or vehicle (control) administration. Data is shown as median with a range of 20 to 150 neurons. ****p < 0.0001, ***p < 0.001, **p < 0.01 e *p < 0.05 (Kruskal-Wallis followed by Dunn's multiple comparisons test). B: Confocal fluorescent images of primary cultures of dorsal root ganglia in the presence of the resting membrane potential probe DiBAC4(3). B1: Basal fluorescence. B2: At 10 minutes after PGE2 100nM. B3: At 2 minutes after capsaicin administration. Arrows indicate some of the neurons that responded to capsaicin and PGE2. Images were obtained using 40x magnification.

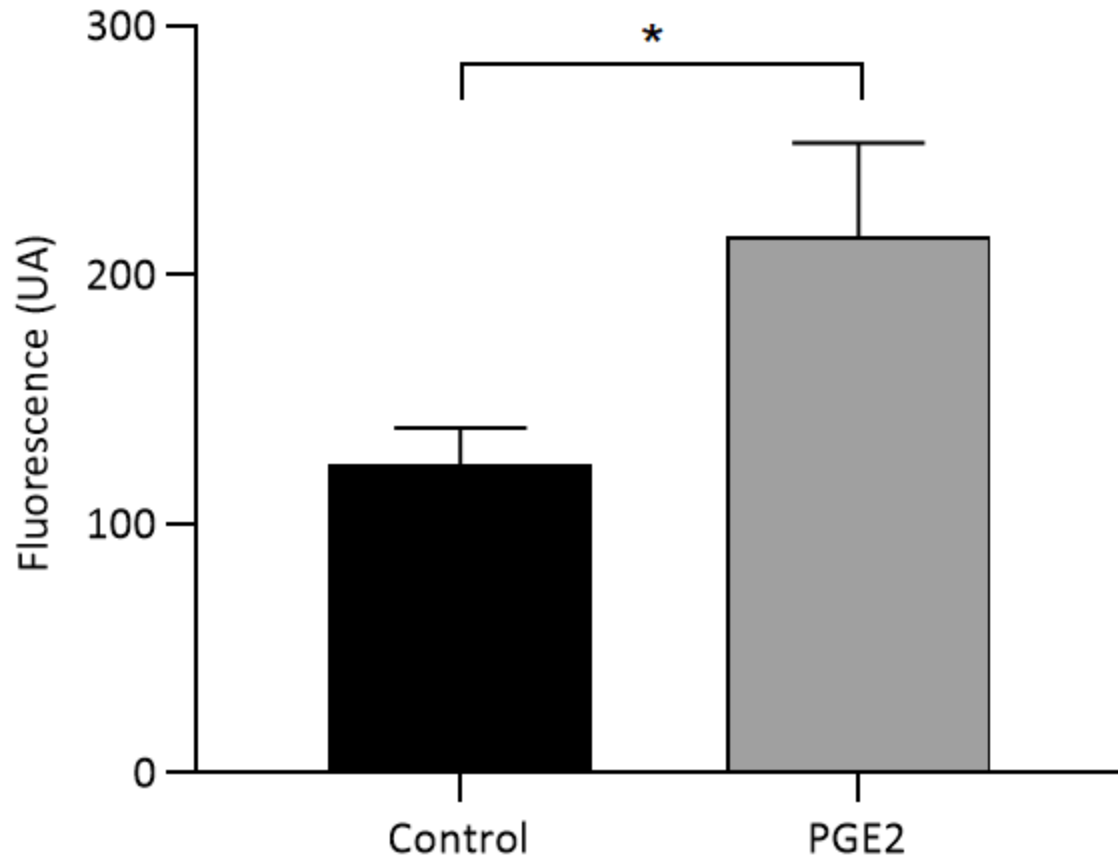


Figure 2

Effect of intraplantar (right paw) administration of PGE₂ on resting potential of cultured nociceptive neurons. Ipsilateral and contralateral neurons of dorsal root ganglia (L4 e L5) were collected after 2 hours of PGE₂ treatment and the fluorescence emitted by incubation with DiBAC₄(3) was registered in capsaicin sensitive neurons. Data shown as mean $\pm$ S.E.M. of 20 to 23 neurons. * $p < 0.03$ (unpaired t test).

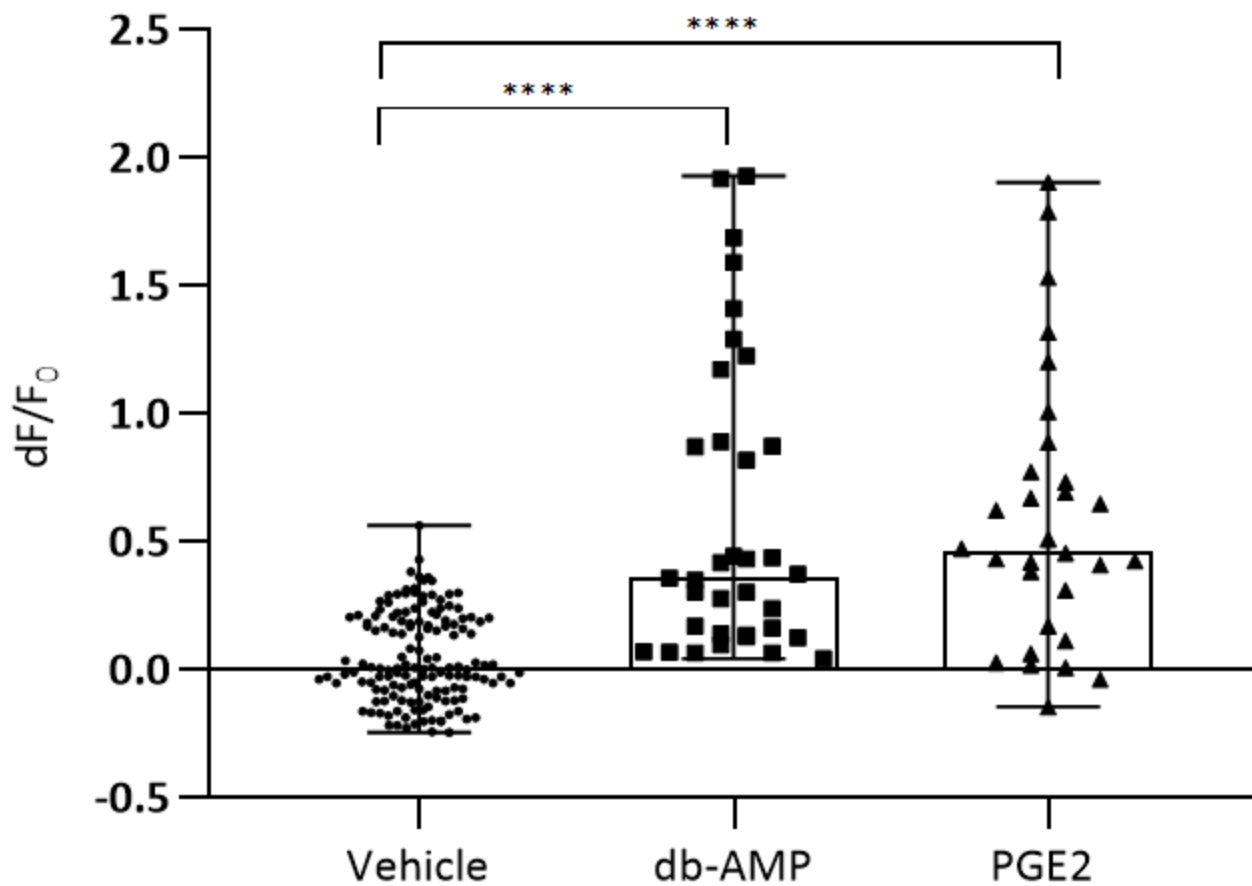


Figure 3

Effects of the cAMP analog db-cAMP on the resting membrane potential of primary nociceptive neurons. Fluorescence alteration induced after 10 minutes of db-cAMP (100 mM) or PGE2 (100 nM) administration. Data shown as median with range of 23 to 150 neurons. **** $p < 0.0001$ (Kruskal-Wallis followed by Dunn's multiple comparisons test).

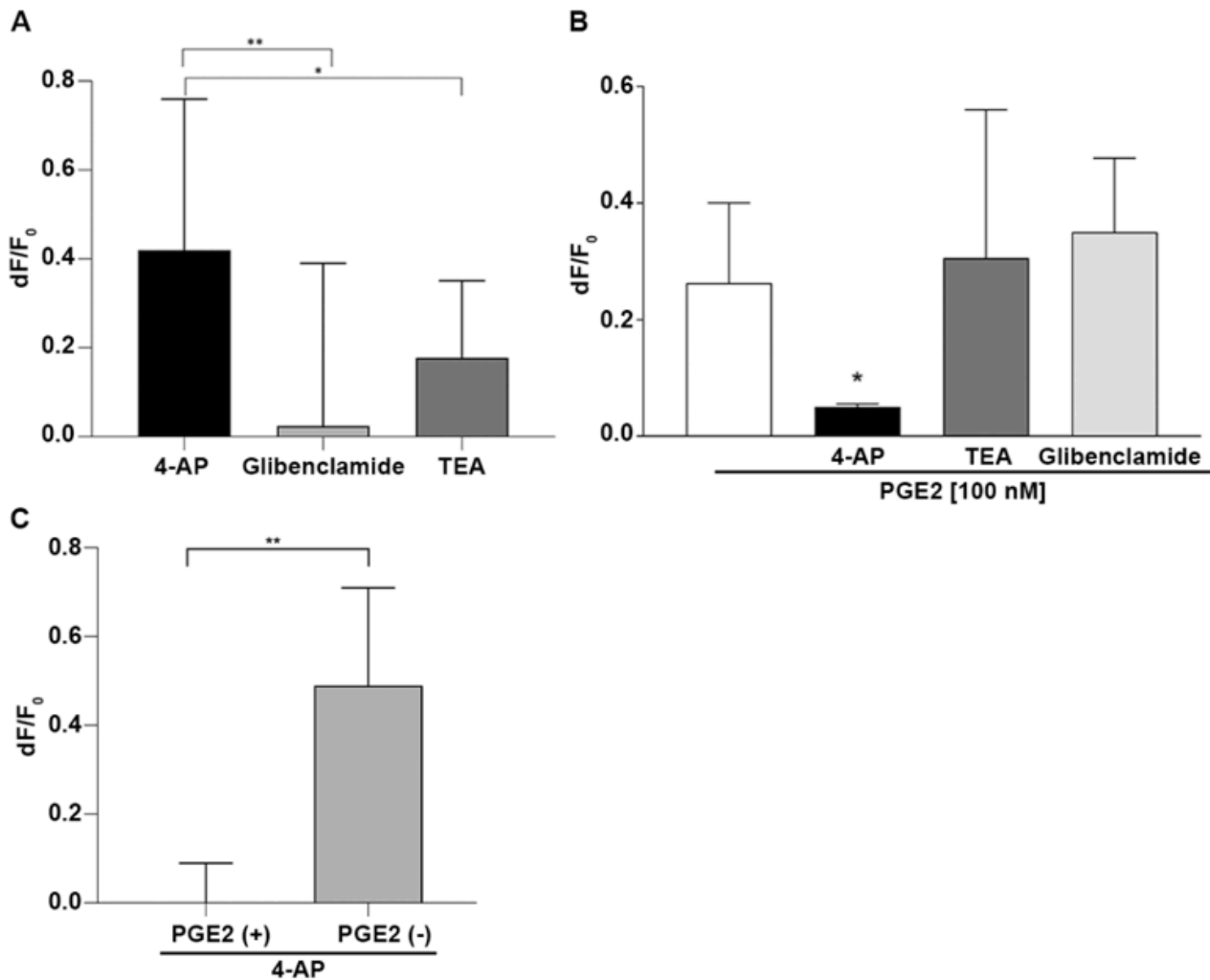


Figure 4

Effects of selective K⁺ channel blockers on the resting membrane potential of primary nociceptive neurons. A: Fluorescence variation induced by administration of 4-AP (10mM), glibenclamide (5 μ M) or TEA (10 mM). Data are shown as median with range of 5 to 12 neurons. ** $p < 0.01$ e * $p < 0,05$ (Kruskal-Wallis followed by Dunn's multiple comparisons test). B: Effect of PGE2 (100nM) administration in the presence of 4-AP (10 mM), glibenclamide (5 μ M) or TEA (10 mM). Potassium channel blockers were administered 15 minutes before PGE2 administration. Data are shown as median with range of 5 to 12 neurons. * $p < 0,05$ (Kruskal-Wallis followed by Dunn's multiple comparisons test). C: PGE2 (100 nM) was administered 15 minutes before 4-AP (10 mM) and the neurons were categorized according to PGE2 response. The effect of 4-AP is shown in neurons that responded to PGE2 (+) (n=6) and in neurons that were not affected by PGE2 administration (-) (n=5). Data is shown as median with range ** $p < 0.01$ (Mann-Whitney test).