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**HYDROGEN SULFIDE AS AN ALLOSTERIC MODULATOR OF ATP SENSITIVE
POTASSIUM CHANNELS IN COLONIC INFLAMMATION**

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science
in Pharmacology at Virginia Commonwealth University.

by

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List of Abbreviations

ATP – Adenosine triphosphate

CBS – Cystathionine β synthase

CO – Carbon monoxide

CSE – Cystathionine γ lyase

DSS – Dextran sulfate sodium

DTT – Dithiothreitol

EGTA – Ethylene glycol tetraacetic acid

FIG – Figure

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GIT – Gastrointestinal tract

GLIB – Glibenclamide

H₂S – Hydrogen Sulfide

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IACUC – Institutional Animal Care and Use Committees

IBD – Inflammatory bowel disease

K_{ATP} – ATP sensitive potassium channel

LEVC – Levchromakalim

MPO – Myeloperoxidase

NaHS – Sodium hydrogen sulfide (H₂S Donor)

NEM – N-Ethylmaleimide

NO – Nitric Oxide

TEA – Tetraethylammonium

TNBS – 2,4,6-trinitrobenzene sulfonic acid

PA/PF – Current density

Abstract

HYDROGEN SULFIDE AS AN ALLOSTERIC MODULATOR OF ATP SENSITIVE POTASSIUM CHANNELS IN COLONIC INFLAMMATION

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The ATP sensitive potassium channel (K_{ATP}) in mouse colonic smooth muscle cell is a complex containing a pore forming subunit (Kir6.1) and a sulfonyl urea receptor subunit (SUR2B). These channels are responsible for maintaining the cellular excitability of the smooth muscle cell which in turn regulates the motility patterns in the colon. We used whole-cell voltage-clamp techniques to study the alterations in these channels in smooth muscle cells in experimental model of colitis (colonic inflammation). Colitis was induced in BALB/C mice following an intracolonic administration of trinitrobenzene sulfonic acid (TNBS). K_{ATP} currents were measured at V_h -60 mV in high K^+ external solution. The dose-response to levcromakalim (LEVC), a K_{ATP} channel opener, was significantly shifted to the left in the inflamed smooth muscle cells. Both the affinity and maximal currents induced by LEV were enhanced in inflammation. The EC_{50} in control was 6259 nM (n=10) and 422 nM (n=8) in inflamed colon while the maximal currents were 9.9 ± 0.71 pA/pF (60 μ M) in control and 39.7 ± 8.8 pA/pF (3 μ M) following inflammation. Similar to LEVC, K_{ATP} currents activated by sodium hydrogen sulfide (NaHS) (10-1000 μ M) were significantly greater in inflamed compared to controls. In control cells, pretreatment with 100

x

μM NaHS shifted the EC50 for LEV-induced currents from 2838 nM (n=6) to 154 nM (n=8). These data suggest that NaHS can act as an allosteric modulator for LEV-induced K_{ATP} currents. Decreased colonic motility may result from enhanced K_{ATP} activation by increased release of H_2S in colitis.

CHAPTER 1 INTRODUCTION

Ulcerative colitis is a form of inflammatory bowel disease that usually occurs in the colon. The main symptom seen in this disease is altered motility patterns in the colon, leading to a bloody diarrhea or constipation. These alterations are assumed to be mediated through the decreased contractile force generated by the smooth muscle cell^{21, 22, 23, 24}. Contractility of the smooth muscle cell is primarily dependent on K^+ and Ca^{+2} channels present on its membrane. Hence any alteration in the ion channel activity of colonic smooth muscle cell might alter the motility patterns in colon.

Several earlier reports suggested a decrease in the activity of voltage gated Ca^{+2} channels (L-type) of colonic smooth muscle cell in inflammation due to decreased expression or modification of the channel^{2,3,4}. Apart from these reports, previous findings from our lab have shown an enhanced activity of ATP sensitive potassium channels (K_{ATP}) of the colonic smooth muscle cell in an experimental model of colitis (DSS)¹. The underlying mechanism of this alteration was not clearly understood. As the name suggest, the activity of this channel is modulated by the intracellular concentration of ATP. Apart from this, recent reports also suggest the modulation of channel activity by different endogenous signaling molecules like H_2S ^{17, 18, 19}. Recent reports also suggest an increased activity of enzymes synthesizing H_2S in ulcerative colitis¹⁵. This showed a possible involvement of endogenous H_2S in the enhanced activity of K_{ATP} channels in ulcerative colitis.

In the present study, we used electrophysiological techniques (Patch Clamp) to examine the alterations seen in the K_{ATP} channel currents in TNBS model of colonic inflammation

(experimental model of colitis) through a dose-response study and demonstrate that, the affinity and efficacy of K_{ATP} channel opener, Levc induced currents were enhanced in inflammation. We examined the effect of exogenous H_2S on K_{ATP} channel in a similar setup and demonstrate that, H_2S activated the channel at high concentrations and at low concentrations increased the sensitivity of the channel to the opener Levc. We conclude that this modulation of K_{ATP} channel activity by H_2S might be a possible mechanism for the channel alteration in colonic inflammation.

CHAPTER 2 BACKGORUND

2.1 Ulcerative Colitis:

Ulcerative Colitis is a form of inflammatory bowel disease (IBD) seen in the colon. It is a disorder of modern society and its frequency has been increasing in developed countries since the mid-20th century. This relapsing and remitting disease is characterized with tissue inflammation and degradation. Inflammation is primarily restricted to the mucosal region of the wall of colon but in severe cases might go deep into the tissue. There is no known cause for this disease, but there is a presumed involvement of genetic component in its incidence. Evidence suggests that, defects in the immune system might lead to the incidence of the disease^{35, 36}. At any point of time, 50% of the patients are asymptomatic while 30% of them have mild symptoms and 20% of them have severe symptoms. Primary symptom seen in this disease is the altered motility patterns in colon leading to either a bloody diarrhea or constipation²¹⁻²⁴. A few extra intestinal symptoms are also seen in rare conditions.

This disease is quite similar to Crohns disease in symptoms but differs in several other aspects. While ulcerative colitis is restricted to colon and rectum, Crohns may spread to any part of the GIT. The etiology and pathophysiology involved in the incidence of these diseases are different from each other. The treatment involved might be similar in both the cases, as they both involve defects in immune system^{35, 36}.

2.2 ATP sensitive potassium channels (K_{ATP}):

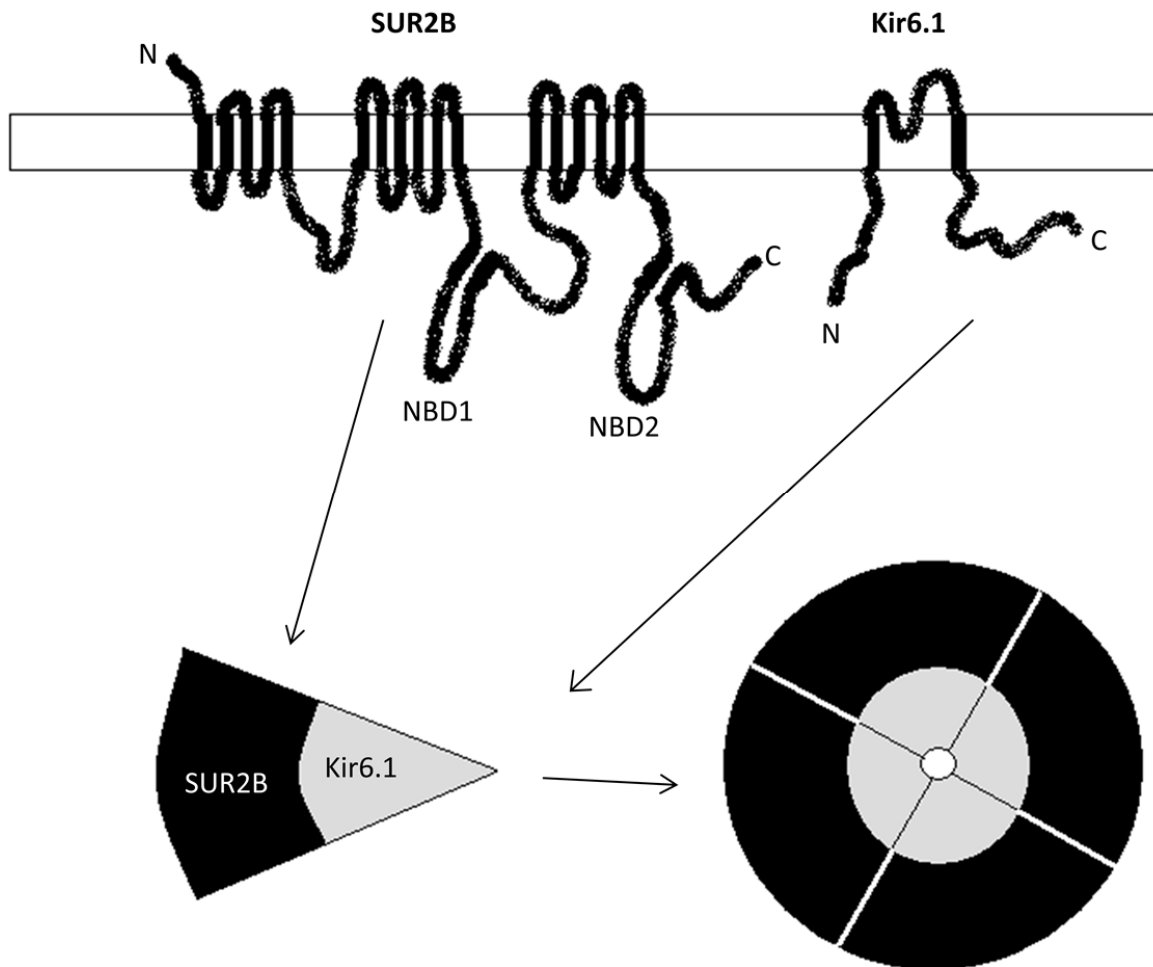
As the name suggests, these channels are sensitive to intracellular ATP and link the electrical activity of cell membrane to the cellular metabolism. K_{ATP} channels are hetero octomers comprised of two subunits, the sulfonylurea receptor subunit (SUR1 or SUR2A or SUR2B) and the inward rectifying pore forming K^+ channel subunit (Kir 6.1 or Kir6.2). Association of these two subunits forms a functional channel on the cell membrane. Sulfonylurea receptor subunit provides the binding site for channel opener and blocker on the extracellular side, whereas the K^+ channel subunit provides the binding site for ATP on the intracellular side³⁴.³⁸. Association of these subtypes of subunits is different in different parts of the body. This association of the sub-types of subunits determines the function of channel in the body. This combination of subunits also determines the sensitivity towards channel agonists and antagonists^{34, 12}.

K_{ATP} channels are known to play an important role in maintaining the contractility of the vascular smooth muscle cell through which it maintains the vascular tone and thus the blood pressure⁶⁻⁹.

K_{ATP} channels play a particularly important role in the pancreatic β cells, where it links the change in blood glucose to insulin secretion and modulates the uptake of glucose¹⁰⁻¹². This functional role led to the discovery of drugs targeting K_{ATP} channels (sulfonylureas) for the treatment of diseases like type-2 diabetes^{13, 14}.

K_{ATP} channel in the colonic smooth muscle cell is a complex containing a pore forming K^+ channel subunit (Kir6.1) and a sulfonyl urea receptor subunit (SUR2B)¹. These channels are inwardly rectifying. At resting membrane potential these channels regulate the contractility of

the smooth muscle cell. The activity of channel is thus crucial in maintaining the motility patterns of the colon⁵. The activity of this channel was found to be enhanced in an experimental model of colitis. This enhanced activity is assumed to be responsible for the reduced contractile force of the muscle in colonic inflammation¹.



Diagrammatic representation of the structure of ATP sensitive potassium channels. K_{ATP} channels in colonic smooth muscle cell are heterooctamers formed by the association of two subunits (SUR2B and Kir6.1). The central Kir6.1 subunit forms the channel pore through which the potassium ions move, whereas the SUR2B subunits are regulatory and associated to each Kir6.1 subunit³⁴.

2.3 Hydrogen Sulfide (H₂S):

Hydrogen sulfide is a colorless toxic gas well known for its harmful effects. Research done in the past few years established that H₂S is synthesized in different parts of the mammalian body. H₂S is synthesized from the amino acid L-Cysteine by the enzymes Cystathionine β synthase (CBS) and Cystathionine γ lyase (CSE)^{16, 17}. Apart from these enzymes it is also known to be produced by the gut bacteria. H₂S has been placed along with NO and CO in the list of gasotransmitters^{16, 46}. Gasotransmitters are endogenously produced gaseous molecules known for their involvement in modulating physiological functions through different signaling processes.

H₂S signals by targeting the thiol groups of free cysteines residues on the target protein (s-sulfhydration)¹⁷. Unlike nitrosylation of cysteine residues by NO, sulfhydration by H₂S is a stimulating signal most of the time¹⁶. There are many targets in the mammalian body, which were shown to be sulfhydrated by exogenous H₂S. Some of the important targets include ion channels like K_{ATP} channels and enzymes like GAPDH whose activity was increased upon sulfhydration with H₂S^{17, 18, 20, 48}. Endogenously produced H₂S was shown to be involved in the modulation of different physiological functions. Recent reports suggest H₂S as an endothelium derived hyperpolarizing factor responsible for the vasodilatation of blood vessels through its action on K_{ATP} channels of vascular smooth muscle cell¹⁸. It is also shown to act on different enzymes like GAPDH in liver and increase their activity¹⁶.

H₂S is also assumed to play an important role in several diseases, primarily inflammation. Recent reports from Wallace et al.¹⁵ demonstrated an increased activity of the enzymes CBS and CSE in colonic inflammation. There were also other reports suggesting an increase in the sulphate reducing bacteria in patients with colonic inflammation³⁷. These bacteria are known to produce H₂S in the gut. These findings clearly showed a possibility of increased H₂S levels in

inflammation. However, it is still argued if the role played by H₂S is pro-inflammatory or anti-inflammatory.

The goal of this study is to identify if the increased H₂S concentration is modulating the enhanced activity of K_{ATP} channel in colonic inflammation. To test this hypothesis we studied the correlation between the actions of H₂S on K_{ATP} channels with the enhanced channel activity seen in colonic inflammation.

CHAPTER 3 MATERIALS AND METHODS

3.1 Materials:

TNBS (Tri-Nitro Benzene Sulfonic acid), Glib and Trypsin (from Bovine Pancrease) were purchased from Sigma. Levc was purchased from Tocris bioscience. Hydrogen sulfide was purchased from Cayman chemicals. Collagenase was purchased from Worthington (Lakewood, NJ). Serum Bovine Albumin was purchased from American Bio-Analytical. Sodium chloride (NaCl), magnesium chloride (MgCl₂), calcium chloride (CaCl₂), glucose, ATP disodium salt, HEPES, EGTA and tetraethylammonium chloride (TEA) were purchased from Sigma. MPO activity assay kit was purchased from Invitrogen.

3.2 Animals:

Adult male BALB/C mice that weighed 25-30g were housed in animal care quarters under a 12:12 hr light-dark cycle with food and water. All the experiments were done according to protocols approved by the Virginia Commonwealth University IACUC.

3.3 Methods:

3.3.1 Induction of Inflammation:

Inflammation was induced in the colon of the BALB/C mice through intracolonic administration of TNBS (0.1 ml). Weights of the mice were monitored on a daily basis and MPO assay was performed on the colon tissue at different time points after the administration of TNBS to determine the severity of inflammation. TNBS solution was prepared by diluting the stock with 50% ethyl alcohol in 1:1 ratio. Vehicle controls were prepared by diluting 1X PBS solution with 50% ethyl alcohol in 1:1 ratio.

3.3.2 MPO Assay:

Colon samples were collected from control and inflamed mice. Cell lysate was prepared from these samples and centrifuged. Supernatant collected from centrifuged sample was used for the assay. Chlorination activity assay was performed to determine the MPO activity of the sample. Assay was performed as directed by the protocol provided along with the MPO assay kit.

3.3.3 Cell-isolation:

Smooth muscle cells were isolated from the colon of male BALB/C mice (25-30gms) as described previously¹. Mice were euthanized and the colon was isolated. The colon was then cut open across the myenteric border and the mucosa is scrapped off to isolate the muscle layer. This whole process was carried out in a low calcium tyrode solution. The muscle layer was then cut into small pieces and transferred into tyrode solution containing 1.5 mg collagenase, 1mg trypsin and 5mg serum bovine albumin in 5ml for 10-12 minutes at 37°C. Then the tissue was subjected to gradual trituration with a flame polished glass bore. The partially digested tissue was then transferred into the enzyme free solution and subjected to further trituration and monitored under microscope to check for the dispersed cells. The dispersed cells were stored on ice and used within 6 hrs. All the electrophysiological recordings were done at room temperature (22-25°C).

3.3.4 Electrical Recordings:

Standard whole-cell configuration was used for all recordings. The Patch-clamp amplifier used was HEKA EPC 10. The micropipettes were prepared on a Flaming-Brown horizontal puller (P-87; Sutter Instruments) and fire polished. Resistance of the pipettes used was 5-10MΩ. The currents were recorded in a gap-free protocol and an I-V protocol. In gap-free protocol, the cell was held at a voltage of -60mV and currents were measured continuously for a period of 15 mins

where as in I-V protocol, the cell was held at a voltage of -60mV and the currents were measured from the -120mV to 0 mV in series of pulses.

3.3.5 Solutions:

Different solutions were used for recordings in the whole-cell configuration. The solutions used are listed in the table 1. The low calcium tyrode solution was equilibrated with 95%O₂ – 5%CO₂. pH of all bathing solutions was adjusted to 7.4 using 3N KOH. The K_{ATP} currents were recorded in a High K⁺ (140 K⁺ External) external bath solution which specifically isolated and amplified the K_{ATP} currents by blocking other possible potassium channels.

Table 1. Solutions used for cell isolation and electrophysiological recordings (values are in millimolar).

Low Ca⁺² Tyrode	Whole Cell Recording		
	Internal	5 K+ External	140 K+ External
137 NaCl	100 K+	135 NaCl	140 KCl
2.7 KCl	asparate	5.4 KCl	10 HEPES
0.008 CaCl ₂	30 KCl	0.33 NaH ₂ PO ₄	1 MgCl ₂
0.88 MgCl ₂	5 HEPES	5 HEPES	0.1 CaCl ₂
0.36 NaH ₂ PO ₄	1 MgCl ₂	1 MgCl ₂	1 TEA
12 NaHCO ₃	10 EGTA	2 CaCl ₂	
5.5 Glucose	0.1 ATP	5.5 Glucose	

3.3.6 Data-Analysis:

Sigma-Plot 11.0 was used for the analysis of the data and to plot the graphs. EC50 values were calculated using 4 parameter logistic nonlinear regression model in sigma plot. Significance levels were determined using unpaired t-tests. P≤0.05 was considered significant.

CHAPTER 4 RESULTS

Mice treated with TNBS displayed a significant loss in weight on day 1 and 2 after the treatment. MPO assay performed with the colon tissues also displayed a significant increase in the MPO activity on day 1 and 2 after treatment with TNBS. This increase in the MPO activity also showed significant differences when compared with mice treated with control vehicle (Fig 1).

4.1 Enhancement of the K_{ATP} Channel opener induced currents in inflammation:

In order to study the alterations in K_{ATP} channel activity in inflammation, currents were recorded from freshly dispersed smooth muscle cells of distal colon using the whole cell configuration of the voltage clamp technique. To identify the K_{ATP} channel currents, cells were bathed in a high K^+ (140 mM) external bath solution, held at V_h -60 mV and dialyzed with low ATP (0.1 mM) in the pipette solution as previously described¹. Perfusion from a low (5.4 mM) to high K^+ solution resulted in inward currents. The basal currents recorded in the high K^+ solution were 0.9 ± 0.12 pA/pF (n=14) in controls, and 5.5 ± 1.4 pA/pF (n=10) in colonic smooth muscle cells from TNBS treated mice (day 2 after treatment with TNBS), henceforth referred to as inflamed cells (Fig 3A). The high K^+ -induced currents were abolished by Glib suggestive of increased basal activity of K_{ATP} in inflamed cells. The average capacitance was 58.93 ± 2.05 pF (n=39) in control and 45.40 ± 2.28 pF (n=20) in inflamed cells. The K_{ATP} channel opener, Levc, further enhanced inward currents at -60 mV. The channel opener- induced currents measured after subtraction of baseline currents in high K^+ showed a remarkable increase from 9.9 ± 0.71 pA/pF (n=12) in

control cells to 39.7 ± 8.8 pA/pF (n=10) in cells from inflamed colon demonstrating an enhancement of almost 7 fold in inflammation (Fig: 3B).

Test depolarizations from -120 mV to 0mV in 10 mV increments (Vh -60 mV) resulted in time-independent and weakly voltage dependent currents. Fig 4 and 5 shows current-voltage relationships for Levc-induced currents in control and inflamed cells in the presence of various concentrations of Levc. Compared to control cells, inflamed cells induced significantly larger currents at each potential and were more sensitive to the channel opener. A dose-response curve for Levc-induced currents was plotted at each voltage. Fig 6 shows the dose-response at -60 mV for control (open circles) and inflamed (closed circles). There was both a leftward shift in the dose-response and an enhancement of the maximal current in inflamed cells. The significant shift in potency was evident when current amplitudes at each concentration were plotted as a fraction of the maxima (Fig 6B). The EC_{50} values calculated for Levc shifted from 6259 nM (95% C.L: 4909 – 7625 nM) (n=10) in control cells to 422 nM (95% C.L: 273 – 522 nM) (n=8) in cells from the inflamed colon showing a tenfold difference. This suggested that inflammation results in both an increase in affinity and efficacy for the K_{ATP} channel opener. To further examine whether there was a voltage-dependency to the affinity for Levc, Dose-response curves were plotted and the EC_{50} values were plotted for each potential (Fig 7A). The EC_{50} values were not different at any of the different potentials with inflamed cells being more sensitive to Levc (Fig 7B).

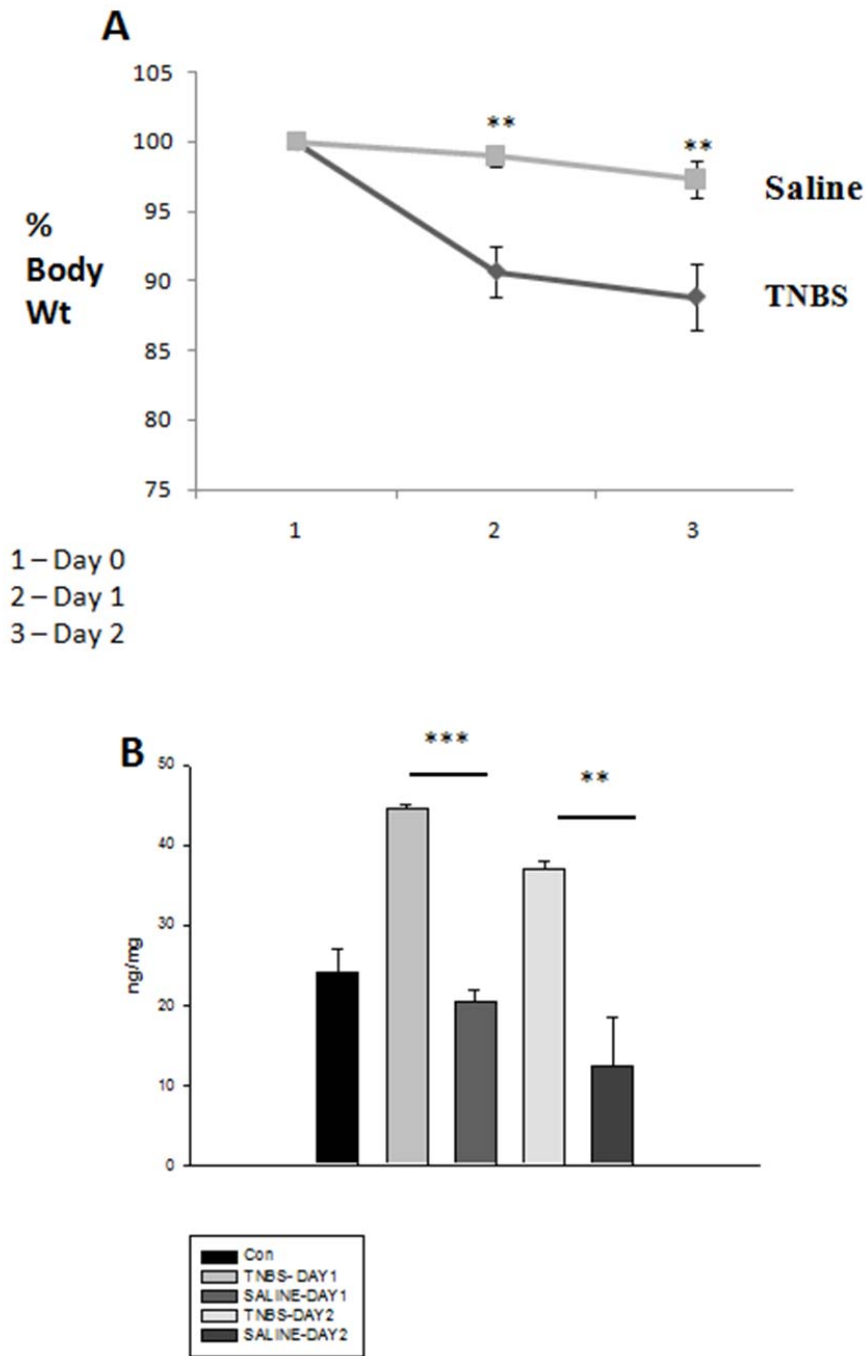


Figure 1: Disease activity index. A: % decrease in the weight of animals after treatment with TNBS (n=13) and Saline (n=5). B: MPO activity in mice in control and after treatment with TNBS (n=4) and Saline (n=5). ***P<0.001, **P<0.01

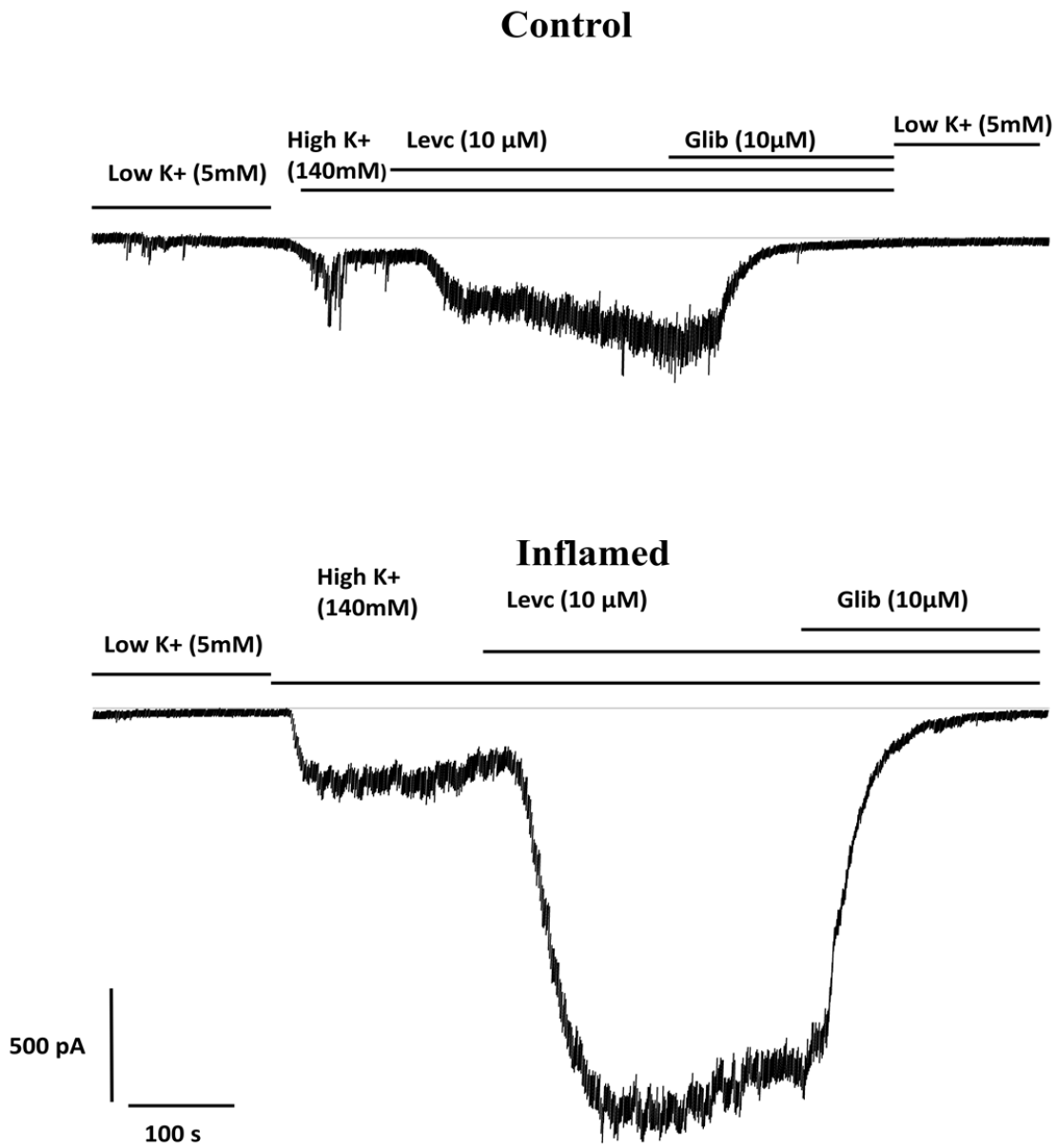


Figure 2: Levc induced currents in control and inflamed cells. A. Raw traces showing the inward current induced by the channel opener levc in a whole-cell recording of gap-free protocol at -60mV in the control and inflamed cells.

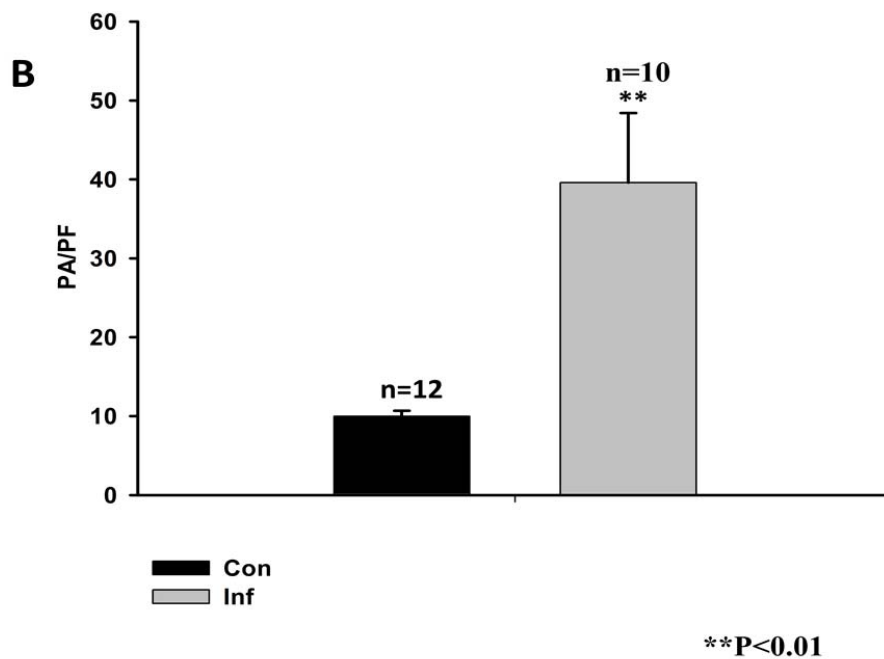
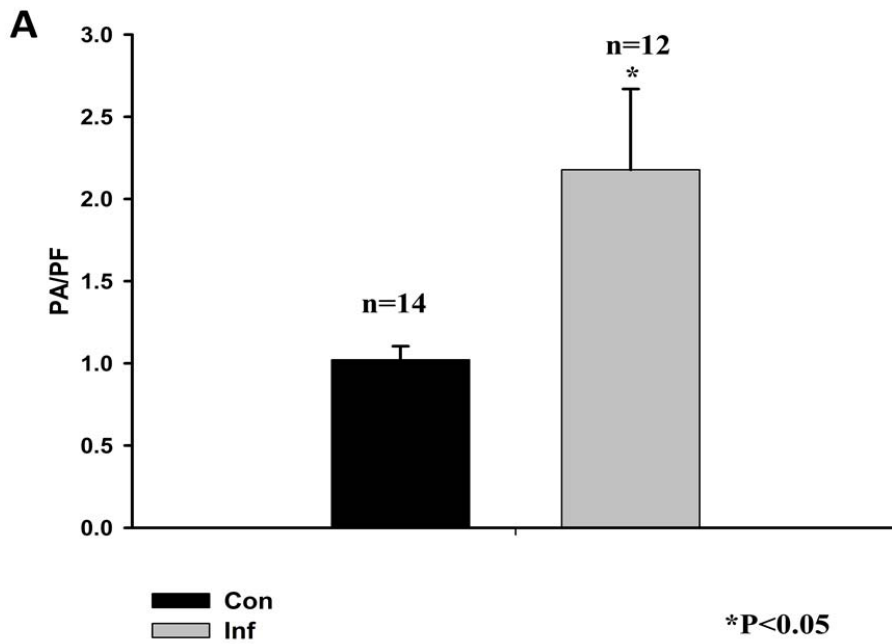


Figure 3: Amplitude of K_{ATP} currents in control and inflamed cells A: Normalized amplitude of basal K_{ATP} currents in control and inflamed cells. (con: 0.9 ± 0.12 pA/pF, Inf: 5.5 ± 1.4 pA/pF) B: normalized amplitude of levc ($10 \mu\text{M}$) induced currents in control and inflamed cells. (con: 9.9 ± 0.71 pA/pF, Inf: 39.6 ± 8.8 pA/pF)

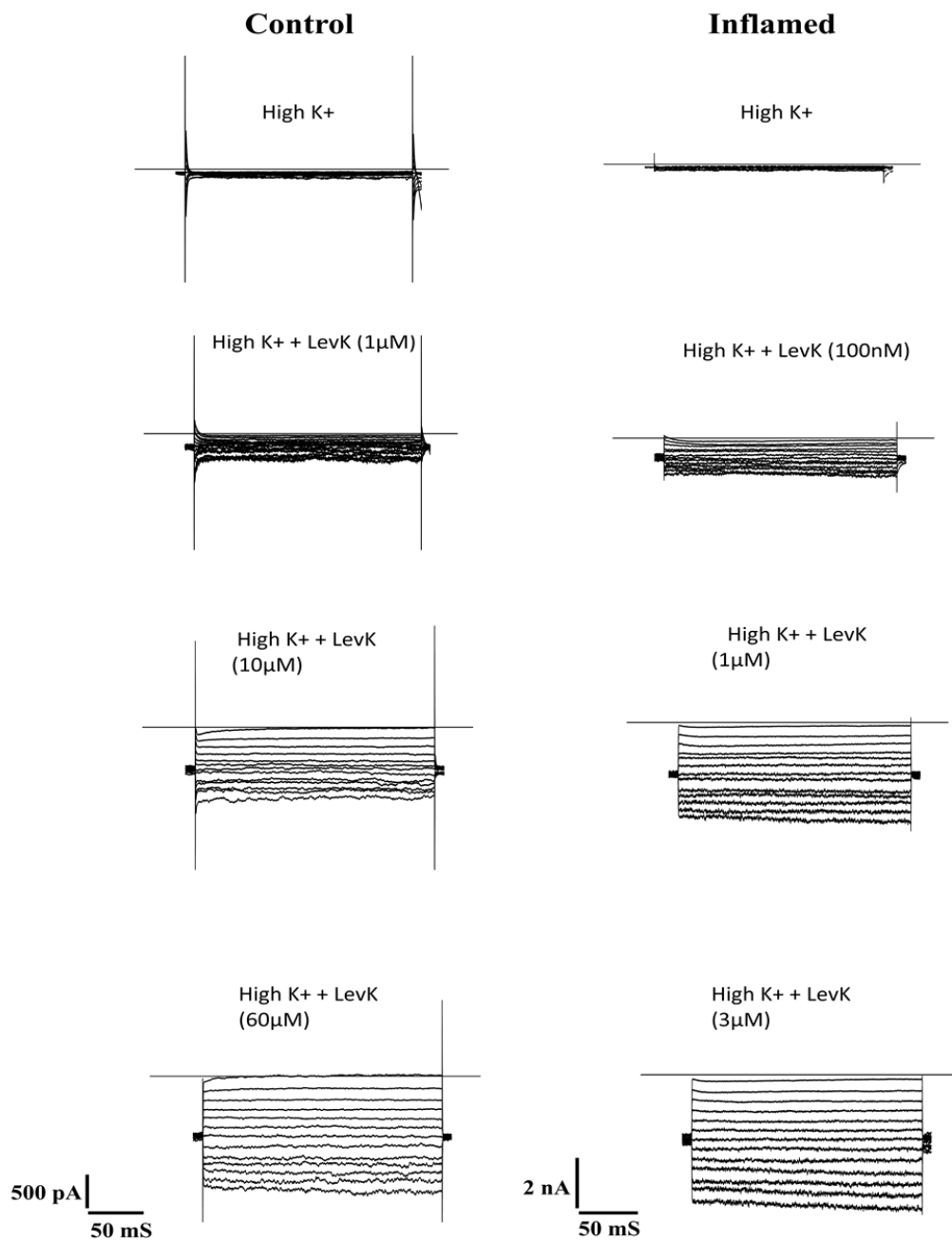


Figure 4: Voltage and dose dependence of levk induced currents. Currents were recorded in a series of step voltages applied from -120 to 0mV in 10mV increments from a holding potential of -60mV in high K⁺ solution. A,B: current traces in high K⁺ and in different concentrations of levk in control and inflamed cells. Note the difference in scale bars.

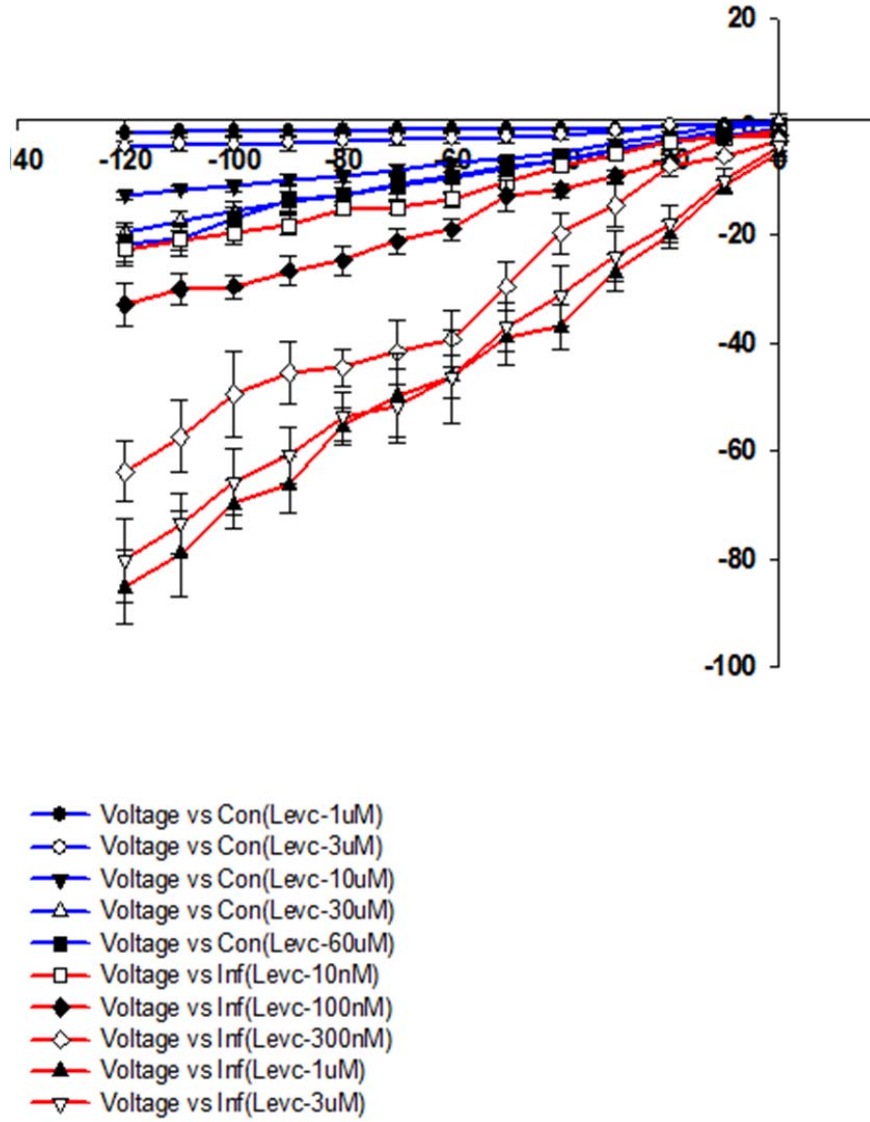


Figure 5: Currents-voltage relationship. Current-voltage relationship with different concentrations of levc in control and inflamed cells.

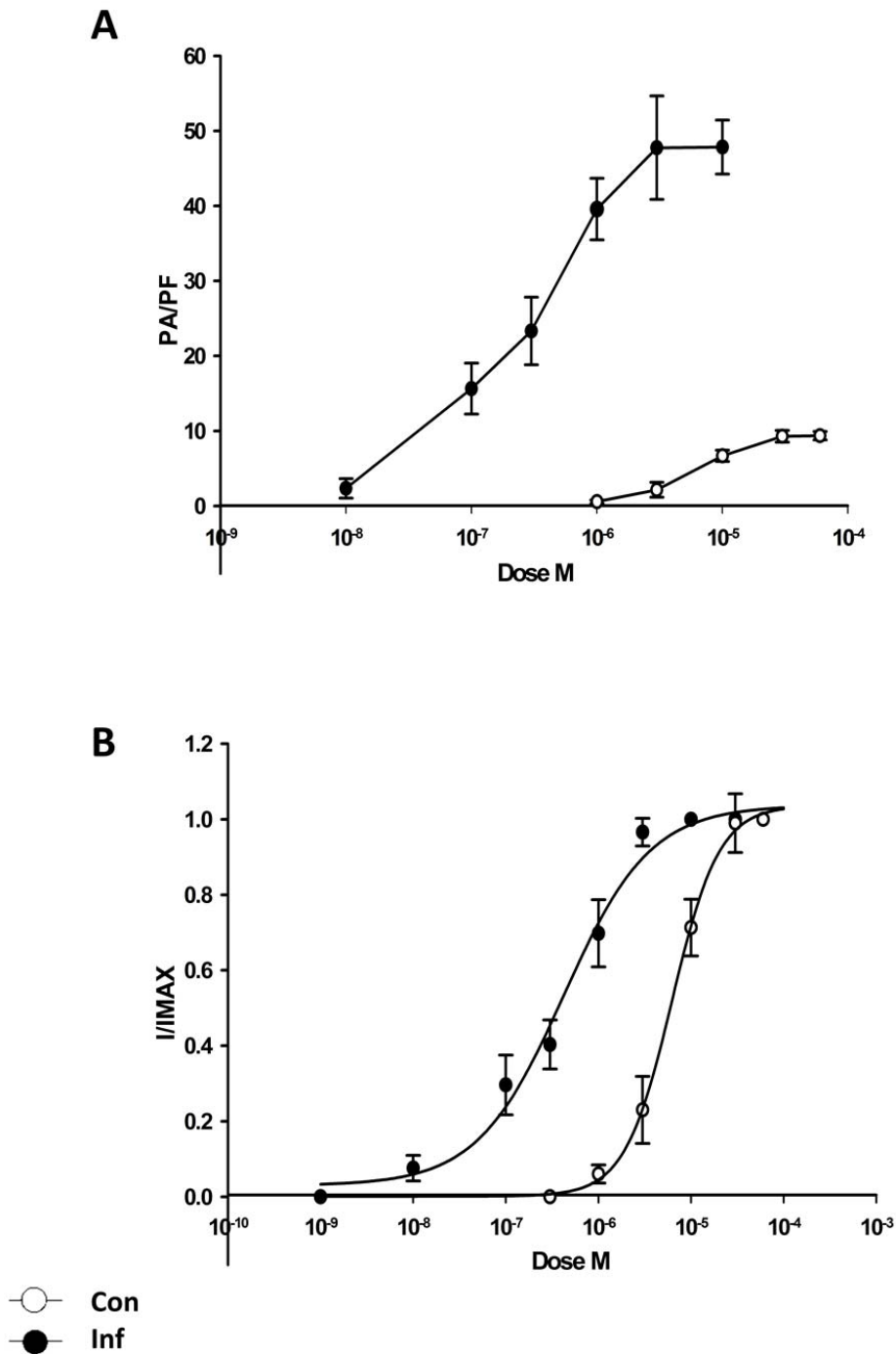


Figure 6: Dose-response relation of levc. A: The normalized amplitude of current induced at each dose of levc measured at a holding voltage of -60mv in the smooth muscle cells from control and inflamed colon. B: The % of current induced at each dose of levc as a function of the maximum current induced at a holding voltage of -60mv in the smooth muscle cells of the control and inflamed colon. Con (n=10) Inf (n=8).

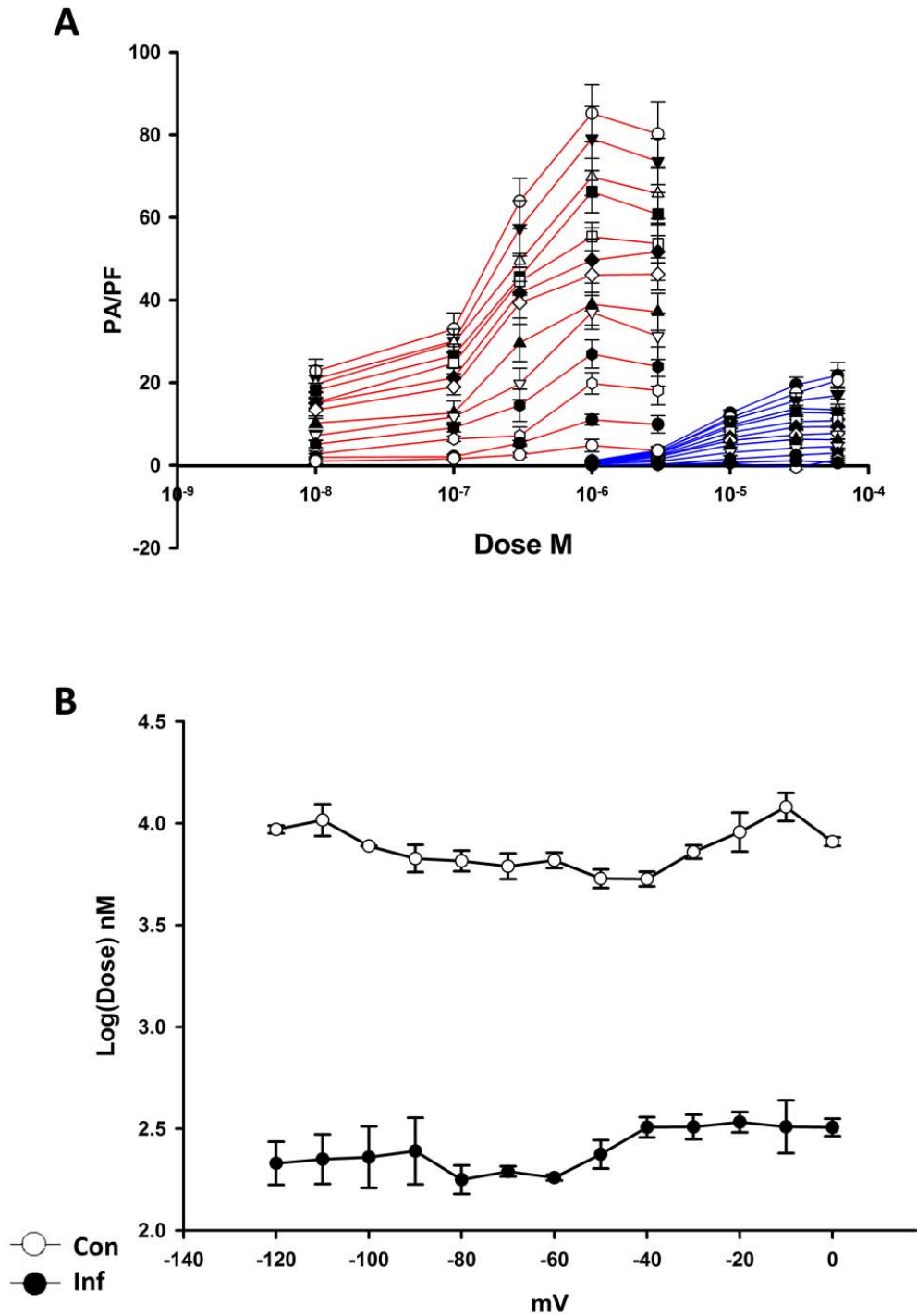


Figure 7: Voltage dependence of calculated EC50. A: The normalized amplitude of current induced at each dose of levc measured at voltages ranging from 0mV to -120mV (Blue lines – Control, Red lines – Inflamed). B: Voltage dependence of calculated EC50 values in control and inflamed cells.

4.2 Effect of K_{ATP} channel blocker in inflammation:

We next tested whether the K_{ATP} channel blocker, Glib demonstrated any difference in the potency towards inhibition of Levc-induced currents during inflammation. A cumulative dose-response for glibemclamide induced inhibition of the K_{ATP} currents were conducted in the presence of 10 μ M Levc. (Fig: 8). While there were significantly larger Levc-induced currents in inflamed cells, the dose-response relationship showed no difference in the potency of Glib to inhibit K_{ATP} currents in control or inflamed cells. The IC_{50} values were 183 nM(95% C.L: 154 – 217nM) (n=6) in control and 144 nM(95% C.L: 128 – 162nM) (n=5) in the cells from inflamed colon (Fig 9).

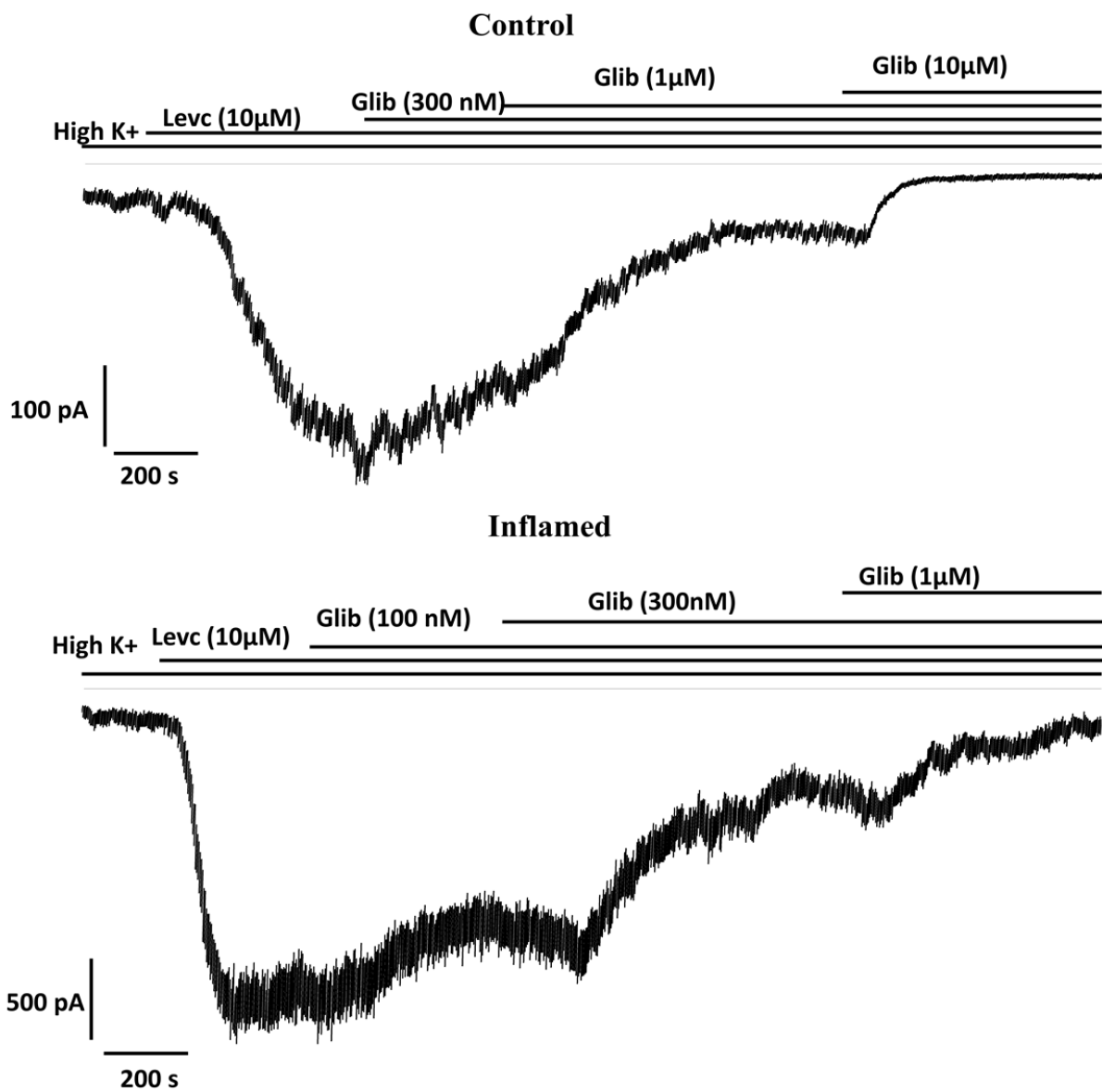


Figure 8: Effect of Glib on K_{ATP} channels in inflammation. Raw traces showing the inhibition of the levc induced currents by different concentrations of glib in a whole-cell recording through the gap-free protocol at -60mV in control and inflamed cells.

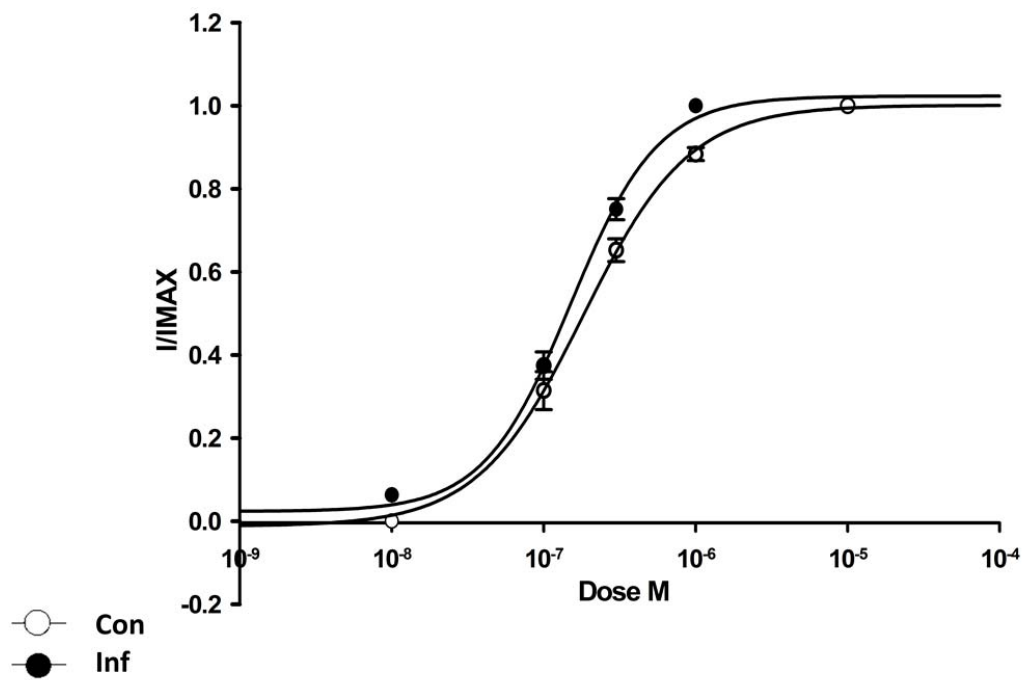


Figure 9: Dose-Response of Glib in control and inflamed cells. Dose-response curve plotted with the % of current inhibited at different concentrations of glib as a function of maximum current inhibited. Con (n=6) and Inf (n=5).

4.3 Effect of hydrogen sulfide on K_{ATP} channels of colonic smooth muscle cell:

We next examined the effect of hydrogen sulfide, an endogenous signaling molecule whose levels have been shown to be increased in colonic inflammation¹⁵. Exogenous sodium hydrogen sulfide (NaHS) (1 mM) when added to the external bath solution induced inward currents at -60mV in a gap-free protocol. The currents were abolished by Glib (10 μ M). Similar to the effects of Levc, the inward currents activated by 1M NaHS were significantly larger in inflamed cells 8.6 ± 1.4 pA/pF (n=6) than control 2.47 ± 0.1 pA/pF (n=7)

We also tested the dose-dependence of NaHS in control and inflamed cells in a similar setup. There was a significant shift in the dose-response curve to the left in inflamed cells with an increase in the maximal currents. When plotted as the fraction of maximal currents, the EC_{50} values shifted from 461 μ M (95% C.L: 376 – 564 μ M) (n=6) in control cells to 199 μ M (95% C.L: 140 - 283 μ M) (n=6) in inflamed cells (Fig: 11B).

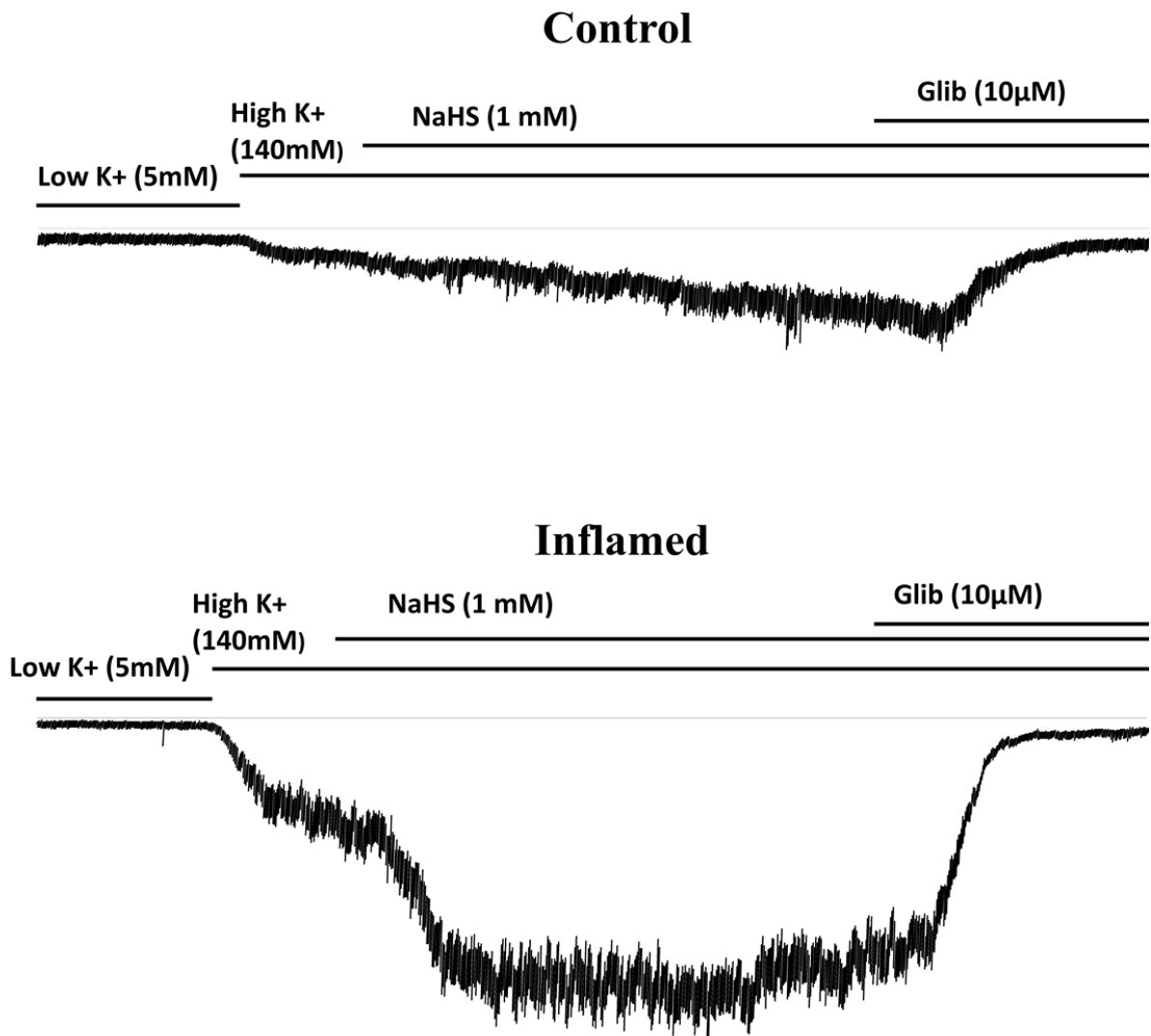


Figure 10: Effect of H₂S on K_{ATP} channels of colonic smooth muscle cell. Raw traces showing the NaHS (H₂S donor) induced currents in a whole cell recording at -60mV voltage through a gap-free protocol in control and inflamed cells.

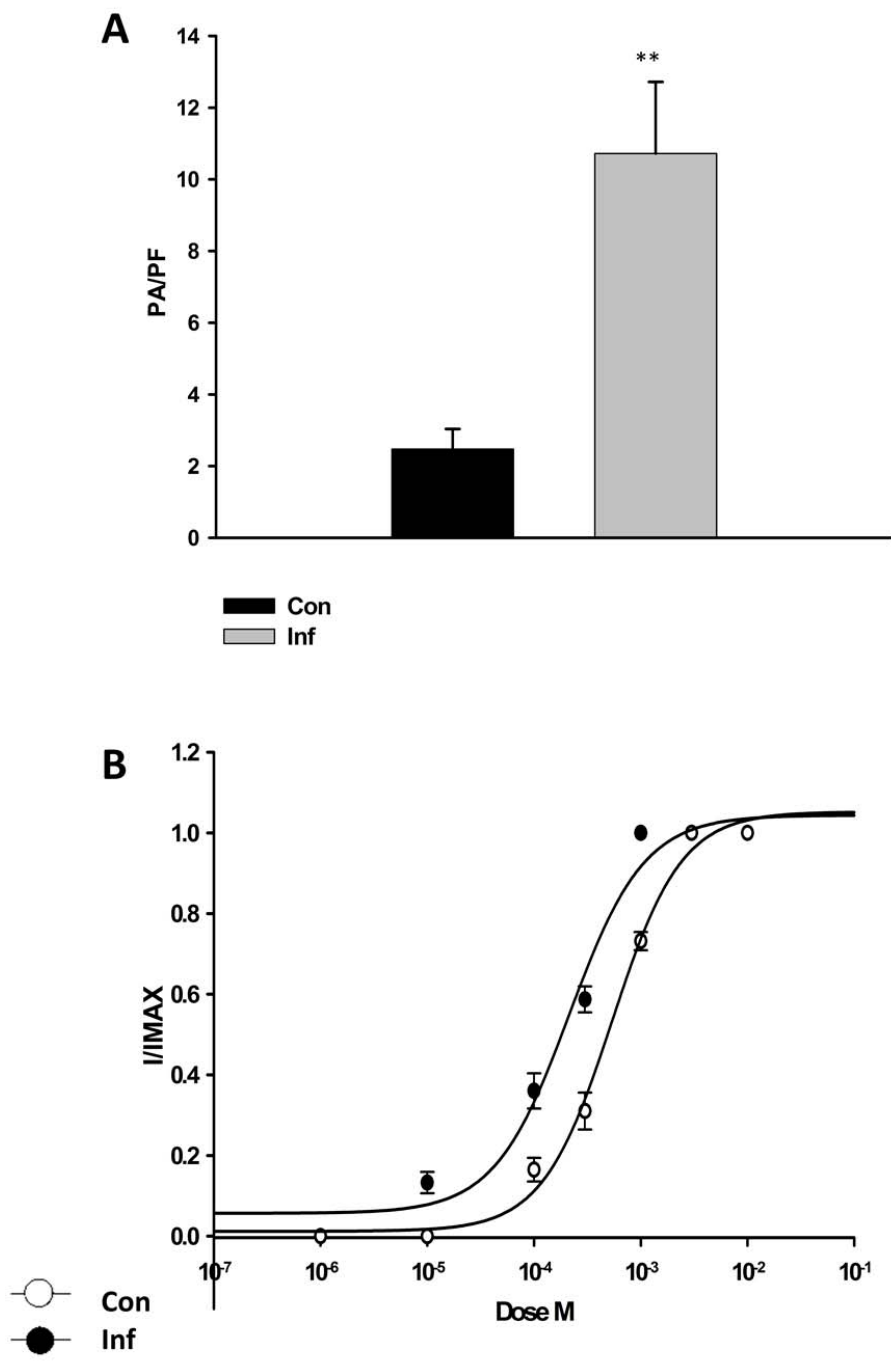


Figure 11: Amplitude of H₂S induced currents in control and inflamed cells A: Normalized amplitude of inward currents induced by NaHS in control and inflamed cells. (Con: 2.47±0.5 pA/pF (n=6), Inf: 10.72±1.9 pA/pF (n=7)) B. The % of current induced at each dose of NaHS as a function of the maximum current induced at a holding voltage of -60mv in the smooth muscle cells of the control and inflamed colon. ****P<0.01**

4.4 Effect of H₂S on K_{ATP} opener induced currents:

In order to examine if NaHS acts as an allosteric modulator of Levco-induced K_{ATP} currents, a low dose of the hydrogen sulfide (100μM) was bath applied prior to conducting Levco dose-response. In presence of 100 μM, the currents activated were 0.47±0.04 pA/pF. In the presence of this concentration of the H₂S the channel opener showed an increased affinity and induced currents at lower doses. The curve plotted shifted to the left and the EC₅₀ values calculated shifted from 2838nM (95% C.L: 954 – 4625 nM) (n=6), In 100μM NaHS:154.9nM (95%C.L: 94 – 251nM) (n=8) in the presence of 100μM NaHS (Fig 13) , demonstrating an increase in affinity of the drug similar to what was seen in the case of inflammation. At this concentration of NaHS, there was no increase in the maximal amplitude of current induced by Levcomakalim (Fig 13A).

This study was repeated in the presence of a higher concentration of NaHS (1mM) where a maximal concentration of the channel opener (10μM) was used to induce K_{ATP} currents. The inward currents induced by the opener showed an increase from 10.5±1.6 pA/pF (n=6) in the presence of 100μM H₂S to 22±5.4 pA/pF (n=5) in the presence of 1mM of H₂S demonstrating an increase in the efficacy of the drug in the presence of higher concentration of hydrogen sulfide (Fig 14, 15).

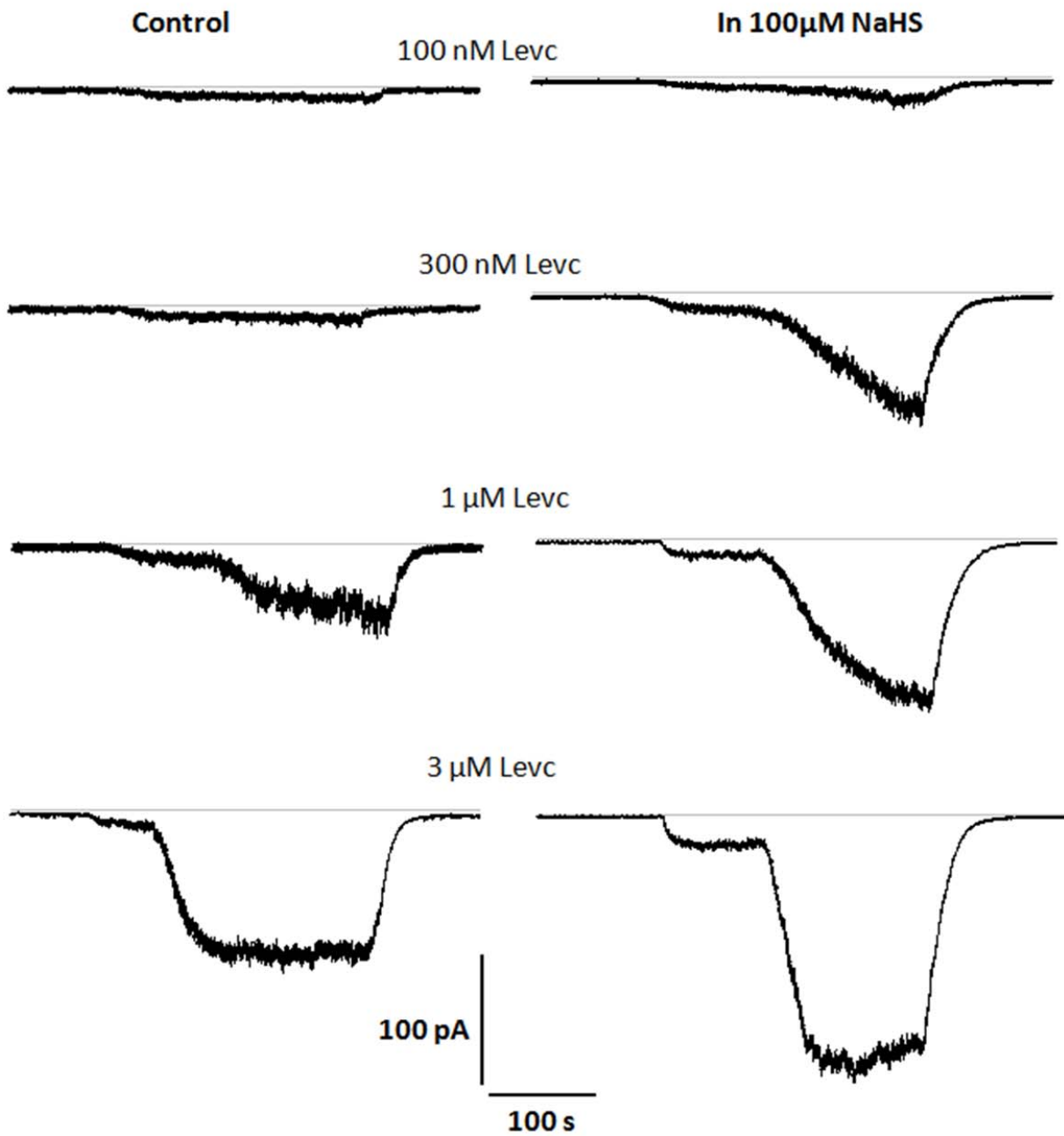


Figure 12: Effect of H₂S on levc induced currents. Current traces in the presence of different concentrations of levc in control and in the presence of 100µM NaHS.

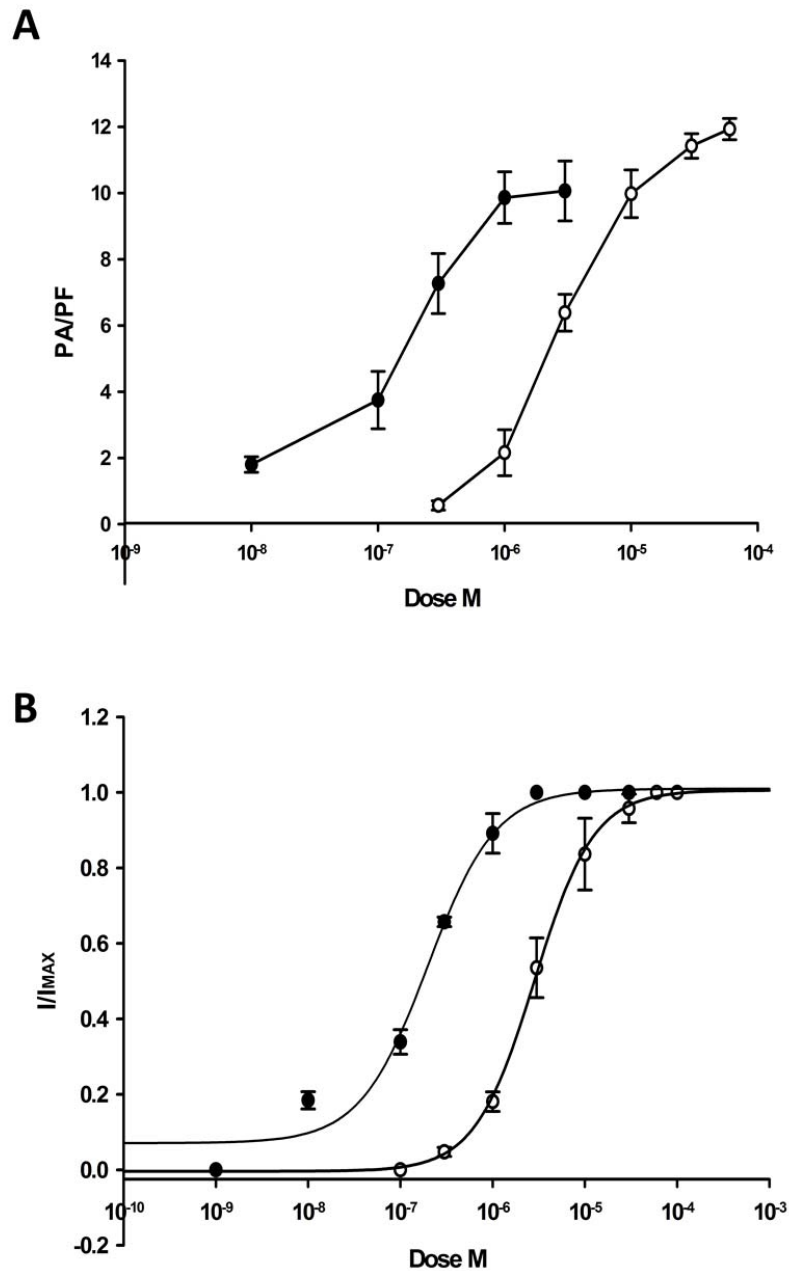


Figure 13: Levc Dose-Response in the presence of H₂S A: The amplitude of current induced at each dose of levcromakalim measured at a holding voltage of -60mv in the smooth muscle cell under control conditions (n=6) and in the presence of 100µM NaHS (n=8). B: The % of current induced at each dose of levcromakalim as a function of the maximum current induced at a holding voltage of -60mv in the smooth muscle cell under control conditions and in the presence of 100µM NaHS.

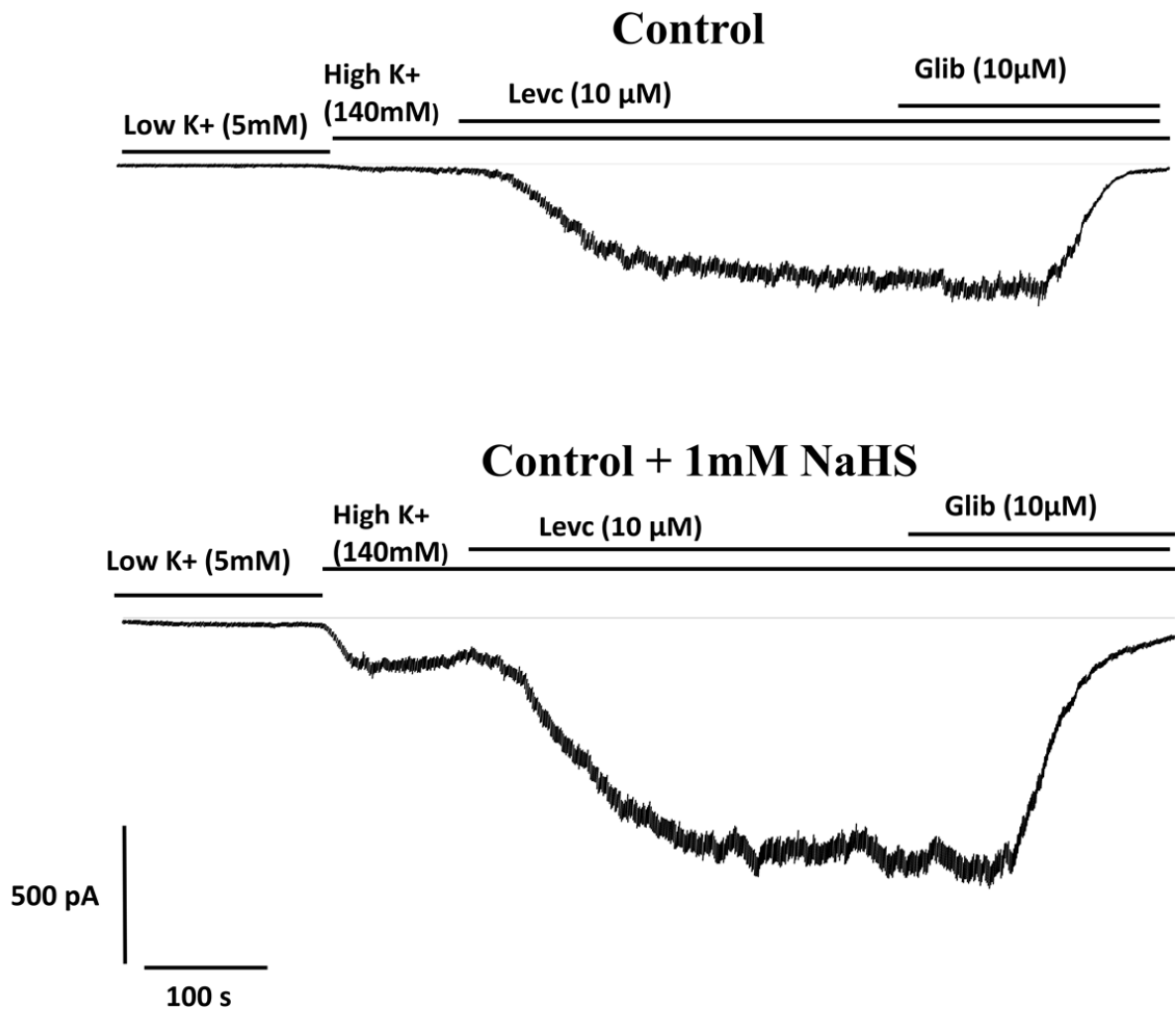


Figure 14: Response of Levc in 1mM NaHS. Current traces showing the response to Levc in control and in the presence of 1mM NaHS.

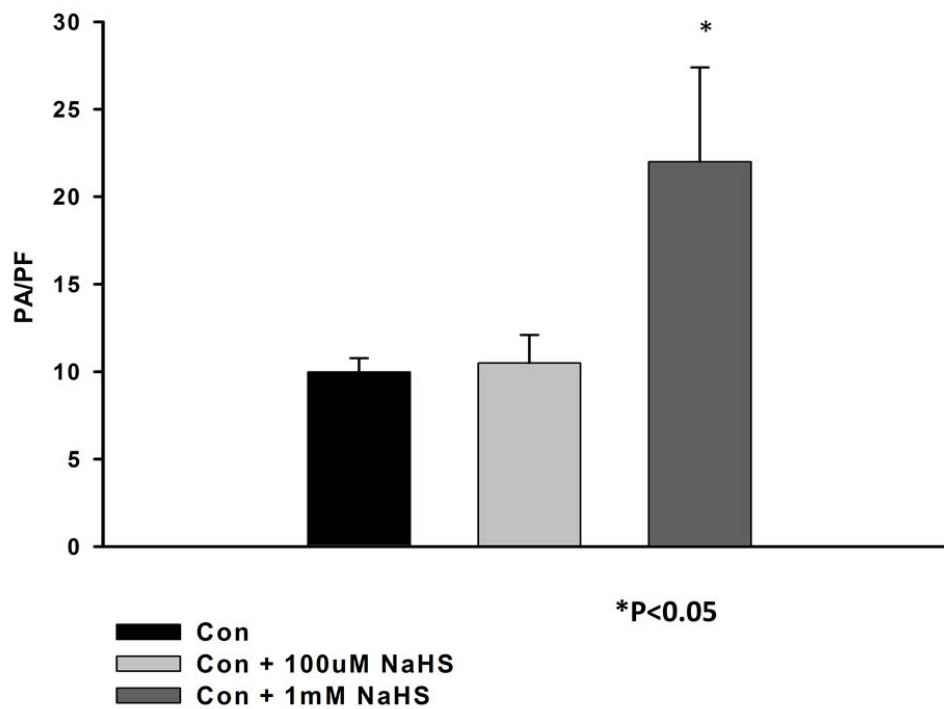


Figure 15: Lev-induced currents in different concentrations of NaHS. Amplitude of current induced by 10 μ M lev in the control cells and in the presence of different doses of NaHS (H₂S donor). Amplitude of Lev-induced currents is enhanced in the presence of high NaHS demonstrating an increase in efficacy of lev. (Con: 9.9 \pm 0.71 pA/pF (n=6), Con+100 μ M NaHS: 10.5 \pm 1.6 pA/pF (n=8), Con+1mM NaHS: 22 \pm 5.4 pA/pF (n=5)).

4.5 Effect of NEM on opener and H₂S induced current:

In order to examine the involvement of cysteine residues in the action of H₂S, the effect of NEM (an alkylating agent of free cysteine residues) was tested on NaHS and Levc induced currents. In the presence of 2mM NEM, the responses produced by NaHS and Levc showed a huge decrease indicating a strong involvement of cysteine residues on their action (Fig 16) (NaHS induced currents reduced from 2.47 ± 0.56 pA/pF in control to $.0397 \pm 0.001$ (n=4) in the presence of NEM. Levc induced currents reduced from 9.9 ± 0.71 in control to 0.45 ± 0.3 in the presence of NEM (n=4)) (Fig 17).

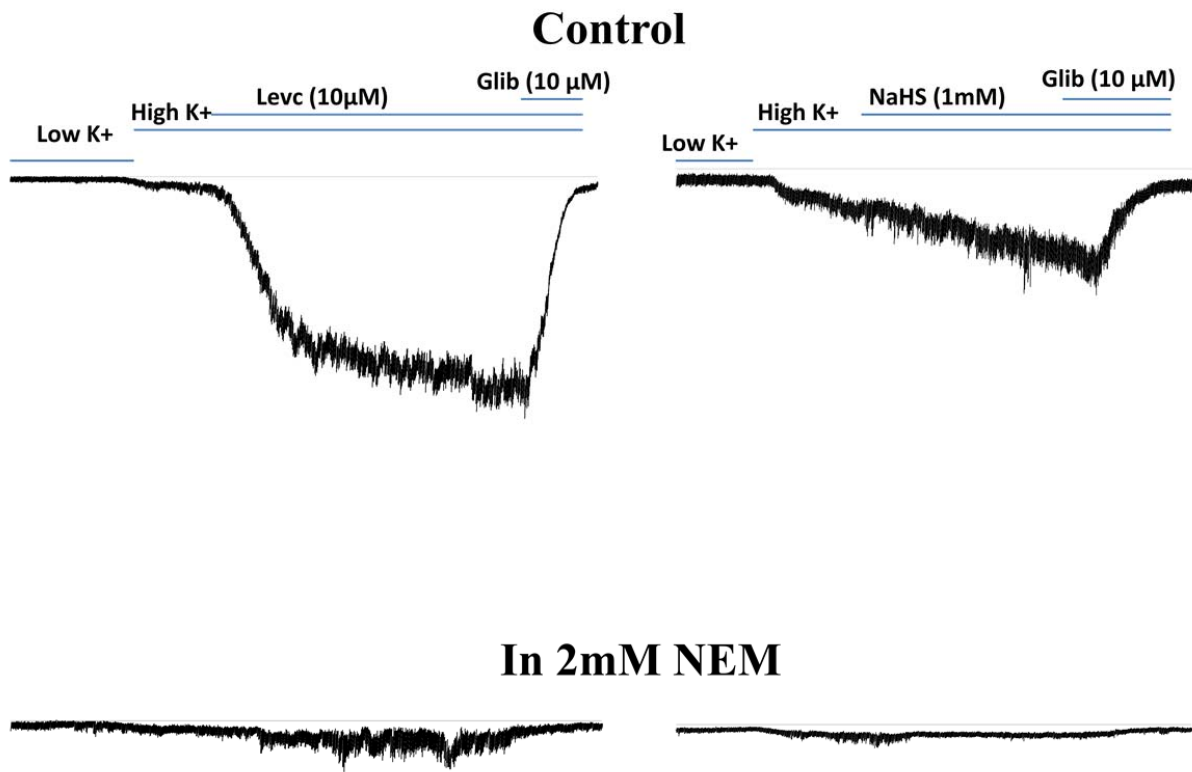


Figure 16: Effect of NEM on Levc and H₂S induced currents. Current traces showing the response to Levc and NaHS in control and in the presence of 2mM NEM.

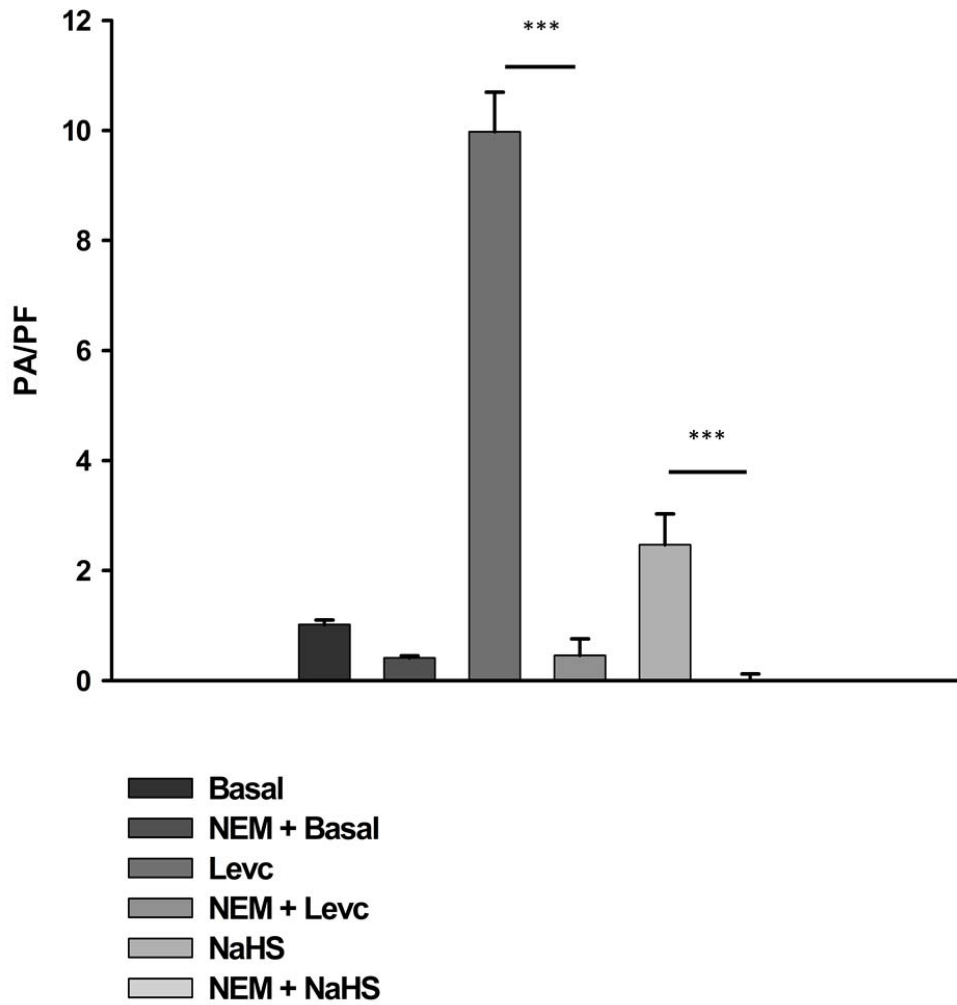


Figure 17: Amplitude of current inhibited by NEM. Bar-Graph showing the quantified differences in the amplitude of drug induced currents blocked by NEM. *** $P < 0.001$

CHAPTER 5 DISCUSSION

H₂S was previously shown to play an important role in inflammation^{15, 19} and is also known to act on K_{ATP} channels^{17, 18, 20}. Therefore, we examined the correlation between the effect of H₂S and the enhanced activity seen in K_{ATP} channel of colonic smooth muscle cell in inflammation.

Ulcerative colitis is a form of inflammatory bowel disease (IBD) that usually occurs in the colon. There are frequent alterations seen in the motility patterns of colon in people with this disease^{21, 22, 23, 24}. Previous studies have demonstrated changes in the ion channel activity of colonic smooth muscle cell which might be mediating these altered motility patterns. Previously, Jin et al.¹ demonstrated an enhanced Levc induced whole cell and single channel activity of K_{ATP} channel in the colonic smooth muscle cell of DSS treated mice. To further examine this role of K_{ATP} channels in inflammation, we conducted a dose response study with the channel opener, Levc on the colonic smooth muscle cell of TNBS treated mice. Through this study we demonstrated an increase in basal activity (Fig: 3A) of the K_{ATP} channel along with a change in efficacy and potency of the channel opener Levc in colonic inflammation (Fig: 6). This study displayed that, colonic inflammation is associated with increased sensitivity of K_{ATP} channels of colonic smooth muscle cell towards the channel opener Levc. In a similar setup, a dose-response study was conducted with a K_{ATP} channel blocker Glib, whose effect didn't show any significant enhancement in potency in the colonic smooth muscle cell of TNBS treated mice (Fig: 9). This suggests that, the colonic inflammation has induced an increase in sensitivity of K_{ATP} channel towards the channel opener but not the channel blocker.

H₂S is a gaseous signaling molecule produced in different parts of the body. Previous reports from Wallace et al.¹⁵ demonstrated an increased capacity of the colonic tissue (Increased activity of enzymes CBS and CSE) to synthesize H₂S in TNBS treated rats. These enzymes are known to be expressed in the smooth muscle cells, mucosal and myenteric neurons etc of the colon^{15, 39}. Wallace study demonstrates a possibility of increased H₂S concentrations in colon in inflammation which can expose the K_{ATP} channels to more amount of H₂S. Wallace also showed that, upon inhibiting the synthesis of H₂S or blocking the K_{ATP} channel increased the mortality rate in animals with colonic inflammation. This demonstrates that, H₂S and K_{ATP} might play a protective role in colonic inflammation. K_{ATP} channel is a well established target of H₂S, which was previously shown to activate the K_{ATP} channels in the different parts of the body^{20, 25, 26, 27, 29}. In our study we demonstrated that, H₂S induced activity of K_{ATP} channel in colonic smooth muscle cell is similar to that of a channel opener, Levc. Dose-response study conducted with H₂S on smooth muscle cell from control and inflamed colon tissues also displayed a similar behavior as seen with Levc (Fig 11).

Apart from that, our drug combination studies with H₂S and Levc demonstrated an increase in potency of Levc in the presence of exogenous H₂S (Fig: 6)... This increase in the potency was observed in the presence of lower concentrations of H₂S (Fig 13). In the presence of higher concentrations of H₂S, there was also an increase in the efficacy of the channel opener (Fig 15)... This behavior of Levc was similar to what was seen in the case of colonic inflammation. Our study along with Wallace previous reports, demonstrate a possibility of H₂S mediated enhancement of K_{ATP} channel activity in colonic inflammation.

H₂S usually targets the thiol groups of free cysteine residues on the target protein and forms a persulfide (s-sulfhydration). It was well established in the literature by Snyder's group and others

that, H₂S signals through the s-sulfhydration of the free cysteine residues on the target protein^{16, 17, 28}. This signaling mechanism was assumed to modulate different physiological functions^{16, 17, 29}. The mechanism involved or target sites involved in the H₂S mediated effects on the K_{ATP} channels are not well understood. Mustafa et al.¹⁸ recently demonstrated that, Kir 6.1 subunit of the K_{ATP} channel was sulfhydrated upon exposure to H₂S through a biotin switch assay. Work done by Minho Kang in our lab used a similar approach to study the sulfhydration of the K_{ATP} channel in colonic smooth muscle after exposure to the H₂S. We found that SUR2B subunit of the K_{ATP} channel gets sulfhydrated upon exposure to H₂S. This sulfhydrated subunit might be mediating the H₂S induced allosterism of the K_{ATP} channel.

H₂S induced effect can be reversed by reducing agents like DTT, which specifically break the disulfide bonds between the cysteine residues or can be inhibited upon pre-treatment with NEM, which alkylate the free cysteine residues and prevent s-sulfhydration. S-Sulfhydration of the SUR2B seen through the biotin switch assays was reversed upon treatment with DTT and was inhibited upon pretreatment with NEM (Kang et al. unpublished data). In contrast to the earlier results, H₂S induced activity of the channel was not reversed after exposure to DTT (data not shown) but was completely inhibited upon pretreatment with NEM, an alkylating agent of free cysteine residues (Fig 16, 17). Not only H₂S induced currents but also Levc induced currents were inhibited upon pretreatment with NEM. Thus the functional data along with the biochemical data demonstrated that, sulfhydration of cysteine residues of SUR2B is responsible for the activation produced by H₂S.

There is increasing evidence showing the contributions of H₂S as a modulator of inflammation^{15, 40, 41, 47}. Although there were several earlier reports suggesting its contribution to the pathogenesis of ulcerative colitis and colon cancer⁴²⁻⁴⁵, there are also equal numbers of reports

suggesting its protective role. Moreover, Wallace previous study has shown the protective role of both H₂S and K_{ATP} channel in colonic inflammation and results from the present study demonstrate the allosteric modulation of K_{ATP} channel by H₂S but it is still to be determined if the protective role of H₂S is mediated through the K_{ATP} channels.

CHAPTER 6 CONCLUSIONS AND FUTURE WORK

6.1 Conclusion:

In summary, the present study provides electrophysiological evidence showing an enhanced sensitivity of the K_{ATP} channel towards channel opener in colonic inflammation and in the presence of H_2S . This correlation in the activity of K_{ATP} channel in inflammation and in the presence of H_2S , along with previous reports display a possible role of H_2S mediated enhancement of K_{ATP} channel activity in colonic inflammation. This study along with other biochemical evidence (Kang et al., unpublished data) in our lab demonstrated a possible mechanism involved in the action of H_2S , which might be by an allosteric modulation through the s-sulfhydration of SUR2B subunit of the channel.

6.2 Future Work:

The work done so far has shown the alterations seen in K_{ATP} channels in colonic inflammation and its allosteric modulation by H_2S . Future studies can be done to identify the cysteine residues involved in the s-sulfhydration of the channel by H_2S using a site directed mutagenesis. Behavioral studies can be done to identify on the role played by K_{ATP} channels and H_2S in colonic inflammation.

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