CaMKII inhibition hyperpolarizes intestinal smooth muscle by closing a Cl- conductance

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Grant: NIH/NIDDK (DK062867)

Short Title: CaMKII activated chloride channels in nitrergic neurotransmission

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Conflicts of interest: None

Author Contributions: Study concept and design: RKG; acquisition of data: XDH; analysis and interpretation of data: XDH & RKG; drafting of the manuscript: XDH & RKG; critical revision of the manuscript

for important intellectual content: RKG; statistical analysis: XDH; obtained funding: RKG; study supervision: RKG.

ABSTRACT

Background: Ionic basis of nitrergic 'slow' inhibitory junction potential (sIJP) is not fully understood. Purpose of the present study was to determine the nature and the role of CaMKII -dependent ion conductance in nitrergic neurotransmission at the intestinal smooth muscle neuromuscular junction.

Methods: Studies were performed in guinea pig ileum. Modified Tomita Bath technique was used to induce passive hyperpolarizing electrotonic potentials (ETP) and membrane potential change due to sIJP or drug treatment in the same cell. Changes in membrane potential and ETP were recorded in the same smooth muscle cell, using sharp microelectrode. Nitrergic IJP was elicited by EFS in NANC conditions and chemical block of purinergic IJP. Modification of ETP during hyperpolarization reflected active conductance change in the smooth muscle

Results: Nitrergic IJP was associated with decreased membrane conductance. CAMKII inhibitor KN93 but not KN92, Cl^- channel blocker niflumic acid (NFA) and K_{ATP} channel opener cromakalim (CK) hyperpolarized membrane. However, KN93 and NFA were associated with decreased and cromakalim was associated with increased membrane conductance. After maximal NFA-induced

hyperpolarization, hyperpolarization associated with KN93 or sIJP was not seen, suggesting a saturation block of the Cl^- channel signaling.

Conclusions: These studies suggest that inhibition of CaMKII-dependent Cl^- conductance mediates nitrergic sIJP by causing maximal closure of the Cl^- conductance.

Keywords:

KN93, niflumic acid, cromakalim, Tomita bath, ETP

INTRODUCTION

Enteric motor nerves release two major inhibitory neurotransmitters: a purine and nitric oxide (NO). They produce two distinct inhibitory junction potentials (IJP), namely a prominent, large amplitude, purinergic, fast IJP (fIJP) and a subdued, low amplitude, nitrergic, slow IJP (sIJP) in the intestinal smooth muscles (4, 12, 25).

Signaling cascade of purinergic fIJP has been shown to involve the release of a purine and activation of P2Y1 receptor which stimulates Gq/11-phospholipase C to produce IP3. IP3 causes localized Ca^{2+} release from IP3 receptor-operated (IP3R) stores. Localized Ca^{2+} release activates small conductance K^+ (SK) channels (3, 35, 36).

Signaling cascade of nitrergic inhibitory junction potential associated hyperpolarization is more complicated. Nitrergic sIJP involves stimulation of guanylate cyclase (GC) by nitric oxide (NO·). However, some nitric oxide donors, in addition to causing guanylate cyclase (GC) stimulation, may also cause direct nitrosylation and activation of SK channels (10). NO.-GC stimulation leads to accumulation of cyclic guanylyl monophosphate (cGMP) and activation of protein kinase G (PKG) (10, 16). However, the ion channels involved in cGMP-PKG

mediated smooth muscle hyperpolarization has been controversial (18, 26, 43).

Potassium and chloride are two major conductances that exert opposing effects of smooth muscle membrane potential (39). Initial studies of ionic basis of the prominent IJP in the guinea pig (now recognized as purinergic fIJP) were performed by investigating the behavior of the IJP during prolonged conditioning hyperpolarization and ion-substitution, using the Tomita bath. These studies suggested that the IJP was mediated by opening of K^+ channels (1, 34). However, it also became known that in the guinea-pig intestinal smooth muscle, in addition to the prominent purinergic fIJPs, there was also a low-amplitude, sIJP (4, 12, 25). Studies of IJPs during induced passive hyperpolarization in the smooth muscle cells in the guinea-pig ileum suggested that while the fIJP was associated with increased K^+ conductance as originally proposed (34), the sIJP was associated with decreased Cl^- conductance (6, 7) and a non-cholinergic component of the excitatory junction potential was associated with increased Cl^- conductance (7). In the opossum esophageal circular muscle, there is no purinergic IJP. The sIJP in the opossum was also reported to be due to closure of a chloride conductance.(5) However, the view that the nitrergic sIJP could be due to closure of Cl^- conductance was not

readily accepted and some leaders in the field continued to believe that nitrergic IJP was also due an increase in K^+ conductance (see Daniel 1992) (8). The view that K^+ channel opening was responsible for the nitrergic IJP was fueled by the fact that NO donors were found to stimulate K^+ channels (19, 20). Over the last 30 year many reports regarding the involvement of K^+ currents in nitrergic IJP have appeared (19, 20, 40). On the other hand, many reports supporting the role of closure of Cl^- channels also appeared (6, 10, 41, 43). However, there is still no general agreement among the investigators, whether opening of K+ or closing of a Cl- conductance underlies the nitrergic sIJP.

It is generally thought that the ion channels that are involved in IJP are activated by Ca^{2+} (26, 42). In our preliminary studies we observed that CaMKII inhibitor KN93 but not a related analog KN92 hyperpolarized the smooth muscle membrane and suppressed the nitrergic IJP. However, CaMKII may be involved in the regulation of either K^+ or Cl^- channels, leaving unanswered the question whether K^+ or Cl^- channel mediated the sIJP.

Purpose of these studies was to investigate: 1) whether opening of K^+ channels or closure of Cl^- conductance was responsible for smooth

muscle hyperpolarization associated with sIJP or CaMKII; 2) the mechanism of block of sIJP by KN93.

MATERIALS AND METHODS

All experiments were approved by IACUC at VA Boston HealthCare System (VABHS) and were performed according to the Institutional guidelines.

Animals: Thirty five (male or female) guinea pigs weighing between 250 and 400 g were anesthetized by means of CO_2 narcosis and subsequently stunned and bled via the carotid artery.

Drugs and chemicals: Drugs and chemicals used in this study are: atropine, guanethidine, nifedipine, cromakalim (CK), apamin, substance P, niflumic acid (NFA) and N^W-nitro L-arginine (L-NNA) and were obtained from Sigma Chemical Co (St Louis, MO). KN93, KN92, calmidazolium and W7 were obtained from Research Biochemicals Incorporated, Natick, MA. Nifedipine was dissolved in 95% ethanol at 10 mM/L and stored in a light protected container. L-NNA was dissolved in 0.01 N HCl and diluted in Kreb's solution before use. NFA, KN93, KN92 and calmidazolium were dissolved in 10% DMSO and shielded from light. The Krebs solution consisted of

(in mM): 11.5 glucose, 21.9 bicarbonate, 1.2 phosphate, 138.5 sodium, 2.5 calcium, 1.2 magnesium, 4.6 K+, and 125 Cl-. The pH of the Krebs solution after 30 min of bubbling with 95% O_2-5% CO_2 ranged between 7.34 and 7.39.

Tomita bath: In order to determine whether the nerve mediated junction potential was passively conducted from a neighboring cell or was actively generated in the same cell (generated potential), we used a modification of technique described by Abe and Tomita (1) (Fig 1). Other recording parameters being the same, an increase in conductance in the recording cell would be expected to be associated with a decrease in the amplitude of the ETP and a decrease in conductance would be associated with increase in the amplitude of the ETP. This technique can similarly be used to investigate whether drug-induced hyperpolarization are conducted from a neighboring cell or generated in the same smooth muscle cell. In these studies, long-term intracellular recording from the same smooth muscle cells were performed during the entire study, in order to record changes in ETP with treatments in relation to the pretreatment control period.

Intracellular Recording: Intracellular recordings of membrane potential and ETP were obtained from the same smooth muscle cell

using microelectrodes made from glass of 1.2 mm external diameter (Frederick Haer, Brunswick, ME) and filled with 1M K^+ methyl sulfate and 3M KCl. The resistance of the microelectrodes was between 30 and 80 MΩ as described earlier (6, 7). All membrane potential values were determined by the difference between the stable potential recorded within the cell compared with the balanced zero potential upon withdrawal, as described earlier (6, 7).

Generation of ETP: Direct current, hyperpolarizing potentials of 0.5 sec in duration were generated by passing current between the two stimulating plates in this bath and was monitored by a constant-current monitor unit (Grass Instruments CCUI) positioned in series between the plates and the stimulator. The direct current potentials were conducted to other coupled smooth cells in the strip and produced passive electrotonic potential (ETP) in the coupled cells. The ETP were recorded using an intracellular microelectrode positioned within 2 mm of the stimulating plate next to the compartment B. The effect of nitrergic IJP and various treatments on membrane potential and amplitude of the applied ETP was recorded. Details of handling of current leakage and other technical issues have been described elsewhere (6, 7).

Generation of the fIJP and sIJP

Two Ag-AgCl electrodes (0.26 mm diameter) positioned above and below the intestinal preparation perpendicular to its longitudinal axis and 5 mm away from the recording microelectrode were used to deliver transmural nerve stimulation. These electrodes were insulated up to 2 mm from their tips and connected to a stimulator (Grass S-88) in series with a stimulus isolation unit (Grass SIU5) and a constant-current unit (Grass CCUl). Optimal stimulus parameters (70V, 1 millisecond duration square pulses at 10Hz for 0.5-5 sec) were used (12).

Atropine (1 μM) and guanethidine (5 μM) were added to create nonadrenergic, noncholinergic (NANC) conditions. Nifedipine (0.1 μM) was added to the perfusing solution to suppress L-type calcium channels and reduce contraction of the muscle. To further reduce the substance P mediated muscle excitability, substance P tachyphylaxis was used to as described earlier (6). Apamin (0.3 μM) was added to the perfusing solution to block the fIJP or L-NA (100 μM) to mask the sIJP (6, 7).

Effect of CaM and CaMKII inhibitors on fIJP and sIJP

Intracellular recordings of pharmacologically isolated fIJP and sIJP junction potentials were also performed without the use of Tomita Bath as described earlier (4, 12). Effects of CaM inhibitors, calmidazolium and W7, as well as CaMKII inhibitor, KN93, were examined on the fIJP and the sIJP.

Application of chemicals

Chemicals KN93, KN92, NFA and CK were dissolved in DMSO and diluted in Kreb's solution to achieved desired concentration and perfused in the bath at a rate of 3 ml/min. Smooth muscle cell in which impalement was maintained for the entire duration of study lasting ~45 minutes were included for analysis.

Statistics

Statistical comparisons were made using standard Student paired and unpaired t statistics and covariance analysis. All data are expressed as means ± SD. One-way ANOVA with $p < 0.05$ was accepted as statistically significant.

RESULTS

ETP during sIJP

Using Tomita bath setup, passive hyperpolarizing ETP of ~7mV and ~ 0.5 sec duration were applied every 1-4 sec, before and after, and once during the sIJP. An example of ETP during sIJP in a smooth muscle is shown in Fig 2. Note that in this example, amplitude of the ETP was 6.6 mV during the control period and increased to 13.5 mV during the IJP associated hyperpolarization and returning to baseline level of 6.7mV. In this example the ETP increased by 104 % during the nitrergic IJP as compared to period of baseline. In 3 studies in 3 separate animals, the mean ± SD value of MP was - 41±0.6 mV, after apamin to suppress the fIJP, prior to the sIJP and -50±0.6 during the sIJP. Spontaneous small amplitude MP fluctuations described earlier were not consistently recorded due to technical reasons (41). Amplitude of the sIJP was -8.3±0.6 mV and the amplitude of the ETP increased from 7±0.3mV to 14.3±0.6mV during the sIJP ($p<0.0001$). Since increased ETP indicates reduced membrane conductance, these observations suggest that the nitrergic sIJP is associated with decrease in membrane conductance or closure of ion channels.

ETP during hyperpolarization due to various drug treatments

During drug treatments, hyperpolarizing ETP of durations between 0.5 sec and 1.5 sec with an interval 1.5 to 3 sec were continuously applied during the control period and period of drug infusion. Membrane potential and ETP were simultaneously recorded in the same smooth muscle cell for over 45 minutes.

KN 93 (20μM) perfusion caused membrane hyperpolarization. Hyperpolarization started within 30 sec, reached a peak in around 2 minutes and persisted for about 45 minutes. The steady state hyperpolarization was 11.2±1mV (from -55±2 mV to -66±2 mV) (Fig 2a; Table1). KN93 associated hyperpolarization of smooth muscles was associated with increase in the amplitude of ETPs which increased by 20.3±5.9%. On the other hand, the inactive analog KN92 did not affect membrane potential or the ETP (Fig2B; Table1). These observations show that KN93 is associated with decreased membrane conductance and mimics the action of nitrergic IJP.

A putative Cl^- channel blocker, NFA (200 μM) perfusion hyperpolarized the smooth muscle membrane by 10.8±1.3 mV (from -58±1.2 mV to -69±1.5 mV, n= 3-8 observations in 3 animals) associated with increase in the amplitude of ETPs by 25±2.2% (Fig 2C; Table 1), suggesting that NFA hyperpolarization is associated with

closure of Cl^- conductance and mimics the action of KN93 or nitrergic IJP.

CK is a well-known K_{ATP} channel opener. CK (10μM) infusion strongly hyperpolarized the smooth muscle by 15±1 mV (from -59±4 mV to -73±4 mV). However, the hyperpolarization was associated with decrease in the amplitude of ETP by 41±6% (Fig 2D; Table 1), suggesting that, as expected, CK hyperpolarization was associated with increase in K^+ conductance.

Effect of NFA and cromakalim on responses to KN93

We also examined the effect of KN93 on membrane potential and ETP after treatment with NFA or CK. The membrane potential after NFA treatment was -69±4.4 mV. During NFA hyperpolarization, KN93 hyperpolarization was obliterated (being only 0.2±0.2 mV, from -69±4.4mV to -69.2±4.2 mV) and there was little change (+3.80±2%) in the amplitude of ETP (Fig 3a; Table1). These observations show that NFA antagonizes the action of KN93. However, the hyperpolarizing action of KN93 was not affected by CK. CK perfusion hyperpolarized the smooth muscle membrane to -70±5 mV. During CK-induced hyperpolarization, KN93 caused further hyperpolarization of 8.3±0.3 mV from -70±5 mV to -78±5 mV and an increase in the

amplitude of ETPs by 46±6% (Fig 3b; Table1). These observations show that KN 93, by closing Cl^- conductance, can cause additional hyperpolarization on top of CK induced hyperpolarization associated with closure of membrane conductance. These observations are consistent with the fact that KN93 and CK cause membrane hyperpolarization via effects of different ion conductances.

Effect of CaMKII inhibitors on fIJP and sIJP

In a separate study, effect of CaMKII inhibitors was investigated on the fIJP and the sIJP. The fIJP was elicited after suppression of nitrergic IJP with L-NNA (100 μM). In the control studies, the membrane potential after L-NNA treatment was -52±2.2 mV and the amplitude of fIJP was 28±2.5 mV. CaM inhibitors, calmidazolium and W7 as well as CaMKII inhibitor KN93 hyperpolarized the membrane from -52±2.2 mV to -68±2.7, -65±5.9 and -63±3.4 mV, respectively. However, these treatments did not affect the amplitude of fIJP (Fig 4; Table2). During fIJP, the membrane potential reached value up to -91mV.

Nitrergic sIJP was isolated by blocking the purinergic IJP with apamin. Apamin (0.3 μM) treatment depolarized smooth muscle from -52±2.2 mV to -40±1.6 mV. EFS elicited nitrergic sIJP of 6.6±0.9 mV.

KN93 hyperpolarized the membrane potential to -50±2.4 mV and suppressed the sIJP to -0.2±0.2 mV ($p<0.01$) (Fig 4; Table 2). In contrast, KN92 was without effect on membrane potential or the sIJP. CaM inhibitors, calmidazolium and W7, which also indirectly inhibit CaMKII also hyperpolarized the smooth muscle and suppressed the sIJP (Fig 4; Table 2).

DISCUSSION

In our preliminary studies, we observed that the CaMKII inhibitor KN93, that inhibits all of its isoforms (32) , caused suppression of the nitrergic IJP without affecting the purinergic IJP. The nitrergic IJP is due to production and release of NO from the nitrergic nerve terminals (31). In the prejunctional nerve terminals, NO production from nNOSα is known to be regulated by CaMKII that acts to phosphorylate nNOSα and suppress its action and CaMKII inhibitors should counteract this inhibition and may enhance NO production and the nitrergic IJP (22). Therefore the inhibitory action of KN93 on the nitrergic IJP appeared paradoxical and was puzzling.

The present studies show that: 1) the nitrergic IJP is associated with decrease in membrane conductance; 2) CaMKII inhibitor KN93, and

CaCC blocker, NFA, mimicked the action of nitrergic IJP and caused smooth muscle membrane hyperpolarization associated with a decrease in membrane conductance; 3) Hyperpolarization associated with NFA but not K^+ channel opener CK blocked the action of KN93 by a 'maximal' block of CaCC; 4) the nitrergic IJP, but not the purinergic IJP, was blocked by CaM or CaMKII inhibitors and by NFA. These results suggest that nitrergic IJP, KN93 and NFA act by closing CaMKII activated CaCC in postjunctional smooth muscle cells.

Tomita bath technique has been used to investigate the junction potentials during prolonged ETP. Such studies had originally suggested that the fIJP was due to opening of K^+ channels and the sIJP may be due to closure of Cl^- conductance (5-7, 34). In this study, we directly investigated membrane conductance during the nitrergic IJP. Because of the brief time period, membrane conductance in the fIJP could not be investigated. However, amplitude of the ETP was significantly increased during the sIJP, indicating that the sIJP due to decrease in membrane conductance. .

CaMKII inhibitor KN93 is selective inhibitor of CaMKII and its structural analog KN92 is inactive on CaMKII (30, 33). KN93 also

have other effects: it suppresses L-type Ca^{2+} channels (9). Moreover, KN93 and its analog KN92 both have been reported to block K^+ channel opening (27). However, these nonselective effects do not explain the hyperpolarizing action of KN93 and suggest that KN93-produced hyperpolarization of the smooth muscle was due to modulation of a CaMKII dependent signaling. In smooth muscle cells, the Cl^- levels are higher than those predicted by Cl^- equilibrium potential. Therefore, resting Cl^- conductance is responsible for depolarized state of smooth muscle. Closure of Cl^- conductance by putative Cl^- channel blockers has been shown to cause membrane hyperpolarization of intestinal smooth muscles (6, 7). Many Ca^{2+}-activated CaCC have been described in smooth muscles (11, 17), including those in the gut (2, 37, 43). Moreover, calcium activated Cl^- channels have been described in the ICC that may possible mediate sIJP (15, 44) . However, only the CaMKII regulated CaCC may explain the action of CaMKII inhibitor. CaMKII by its property of auto-phosphorylation can convert transient Ca^{2+} releases into sustained CaMKII mediated activation of the Cl^- channels that maintain resting Cl- conductance.

CaMKII regulation of CaCC is somewhat complicated (Hartzell2005; Leblanc2005). In arterial and tracheal smooth muscles, CaMKII

inhibits $I_{Cl\text{-}Ca}^{2+}$ that is stimulated by CaMKII inhibitors which cause membrane depolarization (24). These CaCC would not explain KN93 induced hyperpolarization observed in this study. However, CaMKII stimulated CaCC have been recognized in many cell types including smooth muscle (13). Molecular identity of CaMKII activated CaCC have recently been identified as a member of the ClC-3 subfamily. ClC-3 was originally described as intracellular channel that is present endosomes and synaptic vesicles, but is now known to be widely expressed also on the cell surface. Recent studies have shown that ClC-3 exists in at least 3 splice variants (28). They have different tissue distribution and regulated by different signaling molecules. Only one of these isoforms (containing 818 amino acids) is directly regulated by CaMKII (14, 28). Our studies provide strong functional support for suppression of CaMKII activated Cl^- channel in nitrergic IJP. We speculate that functional current described here may be due to 818 amino acid containing CaMKII binding, CLC3A (28). Further studies are needed to test this hypothesis.

NFA is a non-steroidal anti-inflammatory agent with multiple other effects including complex effects on Cl^- channels. However, it has been extensively used as a relatively selective blocker of Cl^- channels (38). Because KN93 and NFA blocked the sIJP, we investigated the

actions of KN93 and NFA and their interactions on the smooth muscle membrane potential and conductance in order to understand the mechanism of their inhibitory action. The effect of CaMKII inhibitor, KN93, was similar to that of NFA- a putative CaCC channel blocker. NFA also hyperpolarized smooth muscle membrane by ~10 mV and decreased membrane conductance. Thus, CaMKII inhibitor closely mimicked the action of NFA, suggesting that they both may act to suppress a resting Cl^- conductance. Moreover, hyperpolarizing response of nitrergic IJP was associated with decrease in membrane conductance and mimicked the actions of KN93 or NFA, suggesting that nitrergic IJP, KN93 and NFA acted on a common ion channel to produce hyperpolarization of the smooth muscles.

This study shows that CaCC blocker, NFA, may suppress the action of KN93 by saturation block of the Cl^- channels. Thus, after maximal closure of Cl^- channels by NFA, KN93 causes no additional closure of CaCC. Therefore, any hyperpolarization by KN93 is blocked. Block of nitrergic IJP by KN93 or NFA can be explained in a similar way.

Recently, CaMKII regulated potassium channels have been described which may account for the effect of KN93 on the sIJP (26, 29). It has been shown that in murine colon myocytes, CaMKII increases the

open probability of some isoforms of SK channels such as SK2 channels (23, 35). Therefore, it is possible that PKG stimulated CaMKII may also lead to enhanced opening of SK channels and associated hyperpolarization. If so, KN93 and apamin would be expected to depolarize the membrane and suppress both the purinergic and the nitrergic IJPs. However, we found that while apamin depolarized, KN93 hyperpolarized the membrane; and whereas apamin blocked the fIJP without affecting the sIJP, KN93 did not affect the fIJP but blocked the sIJP. These observations do not support the possibility that nitrergic IJP may involve activation of CaMKII dependent SK channels.

Recently, K^+ channels of Kv4 family and Kv1.4 have also been shown to be regulated by CaMKII. Kv4.3 is molecular marker of the A current in smooth muscles (21). CaMKII has been shown to slow inactivation and accelerate rate of recovery from inactivation of Kv4.3. Basal CaMKII activity is thought to allow these K^+ channels to moderate depolarization at resting potentials and KN93 may shift this moderation towards more depolarized states (21). This effect would not explain the hyperpolarizing action of KN93 on the smooth muscles.

One of the prerequisite of hyperpolarization due to opening of channel, such as K^+ channel, is that it is associated with increased ion conductance. The present study shows that a well-known K_{ATP} channel opener, CK, hyperpolarized smooth muscle membrane associated with an increase in membrane. However, KN93 associated hyperpolarization was not associated with increased membrane conductance. These observations argue against the possibility that the effect of KN93 or the nitrergic IJP is due to opening of any outward conductance.

In conclusion, these studies show that purinergic fIJP and nitrergic sIJP are due to distinct K^+ and Cl^- conductance changes, respectively. They also provide strong functional evidence to support the view that closure of resting CaMKII activated CaCC mediates the nitrergic IJP in the intestinal smooth muscle. These studies also show that the suppression of the nitrergic IJP by CaMKII inhibition and niflumic acid is due to 'maximal' block of the Cl^- channels. Further studies are needed to establish molecular identity of the functionally defined CaMKII activated Cl^- channels. These findings may enhance our understanding of pathophysiology and rational treatment of neuromuscular disorders of the gut.

ACKNOWLEDGMENTS

We thank Arun Chaudhury, MD for his critical comments and suggestions.

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TABLES

Table 1 Effects of perfusion of KN93, NFA and CK on MP and ETP and interaction of KN93 with NFA and CK

	Membrane Potential (MP, mV)			Electrotonic Potential (ETP, mV)		
Treatment	control	treatment	Change	control	treatment	Change (%)
KN93	-55±2	-66±2*	-11.2±2.1	6.2±2	7.1±2.1	Up 20.3±5.9 *
KN92	-56±2.3	-56±2.3	0±0.	5.7±0.7	5.8±0.6	0±0
NFA	-58±1.2	-69±1.5*	- 10.8±1.3	9±1.3	11.4±1.8*	Up 25±2.2*
CK	-59±4	-73±4*	- 15±1	6.3±1.2	3.7±0.3*	Down 41±6*
KN93 after NFA	-69±4.4	-69.2±4.3	-0.2±0.2	14.5±1.7	15±1.4	Up 3.8±2
KN93 after CK	-70±5	-78±5*	- 8.3±0.6	3.7±0.3	5.3±0.3*	Up 46±6*

Values represent mean±SD of 3-8 observations in 3 animals

*significantly different from control value ($p<0.05$)

Table 2. Effect of CaM and CaMKII inhibitors and NFA and CK on purinergic and nitrergic IJP

Treatment	MP After LNA	Purinergic IJP		MP After apamin	Nitrergic IJP (mV)	
		Before treatment	After Treatment		Before treatment	After treatment
control	-52±2.2	28±2.5	---	-40±1.6	6.6±0.9	--
Calmidazolium	-68±2.7*	27±0.8	27±0.4	-52±1.9*	6.6±0.3	0.5±0.4*
W7	-65±5.9*	27.8±0.9	27±0.5	-53±2.4*	7.1±0.3	0.2±0.2*
KN93	-63±3.4*	28±2.2	28±0.4	-50±2.4*	6.7±0.1	0.2±0.2*
KN92	-52±2.6	27.5±1.8	27.5±0.5	-41±1.6	6.7±0.1	6.7±0.3

Values represent 4-7 observations in 3-6 animals

*significantly different from control ($p<0.001$)

FIGURE LEGENDS

Fig 1 Modified Tomita Bath. Details of the bath have been described in detail elsewhere (1, 6,7, 34). Briefly, it consists of two chambers, called chamber A and chamber B, that are isolated from each other except a small hole through which a muscle strip passes from one chamber to the other. Chamber A is designed to apply a direct current that causes a passive change in the membrane potential (electrotonic potential) of smooth muscles in the part of the muscle strip that in this chamber. The electrotonic potential applied to the smooth muscles in chamber A is conducted to smooth muscles in the part of the muscle strip that in chamber B where it can be recorded. This technique was investigated to study cable properties of the smooth muscles (1). The chamber B was modified to add electrodes to provide transmural electrical stimulation and induce inhibitory junction potential that can be recorded from the same cell from which the ETP are recorded and effect of the IJP on ETP investigated. Drugs and chemical can also be perfused in chamber B to study the interaction of drug induced changes, IJP and ETP in the same smooth muscle cell.

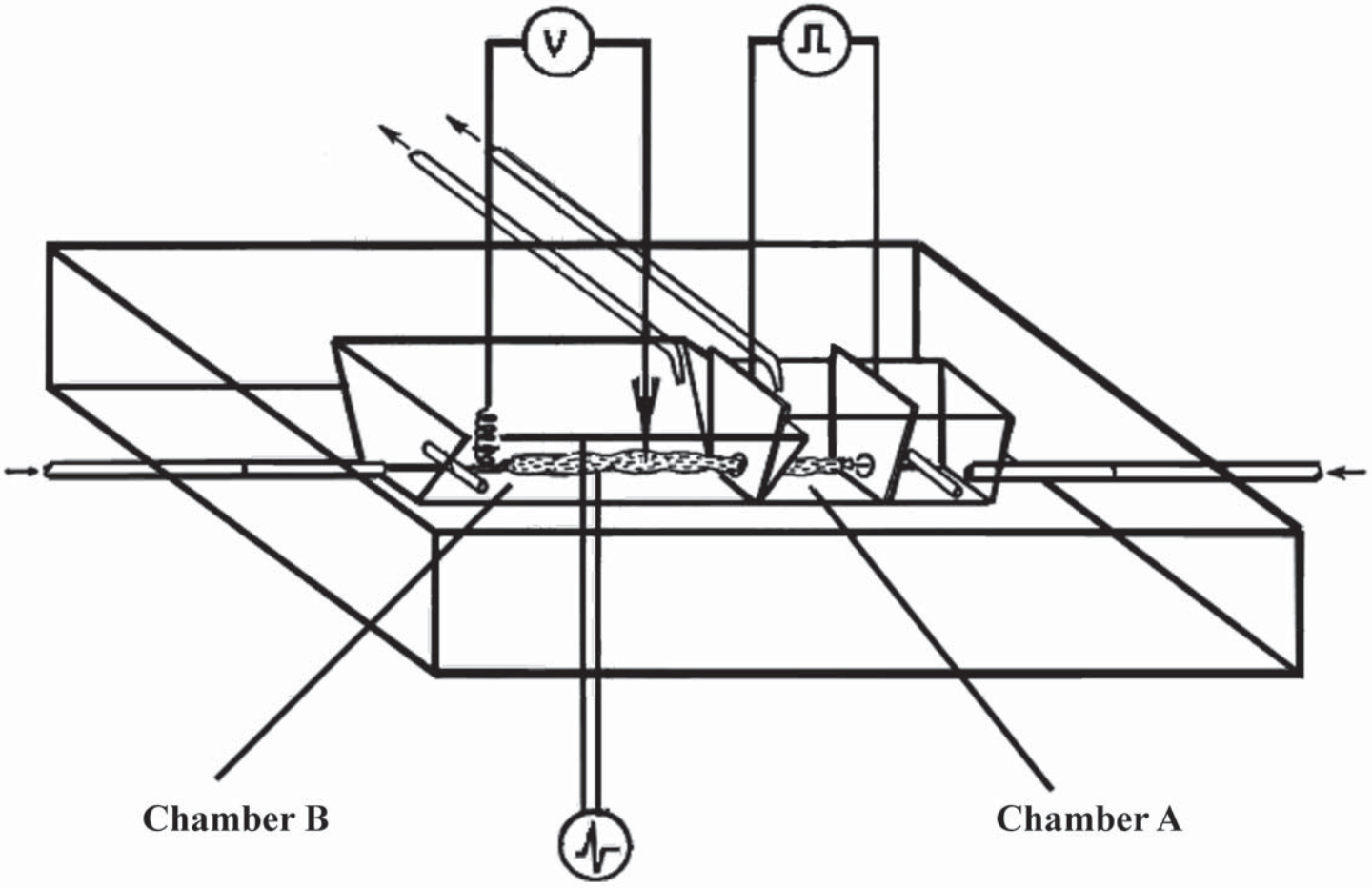
Fig 2 Effect of hyperpolarization associated with sIJP on amplitudes of hyperpolarizing ETPs in circular muscle of guinea pig ileum. The sIJP of ~8 mV, which was followed by a small depolarization before the

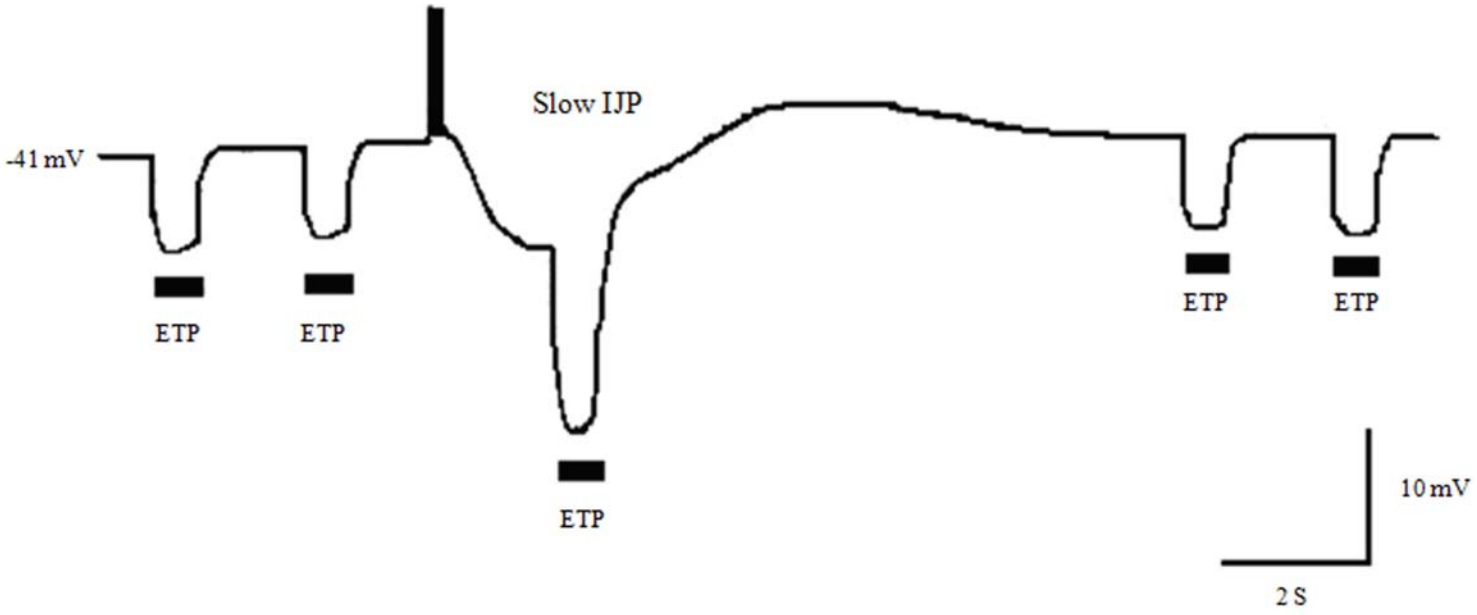
membrane potential returned to the base line. Note a clear increase in the amplitude of ETP during the IJP, suggesting a marked decrease in membrane conductance during the IJP. These studies were performed using the modified Tomita bath. ETP were applied in the chamber A. sIJP was produced by electrical field stimulation of the muscle in the recording chamber, under NANC conditions and addition of apamin to block the fIJP.

Fig 3 Examples of the effects of prolonged perfusion of KN93, KN92, NFA and CK on the smooth muscle membrane potential and ETP on the same cell Panel A shows that perfusion of CaMKII inhibitor, KN93, hyperpolarized the smooth muscle and increased the amplitude of the ETP. The increase in the amplitude of the ETP is consistent with decrease in membrane conductance. Panel B shows that perfusion of KN92, an analog of KN93 that is inactive against CaMKII, did not affect membrane potential or the ETP. Panel C shows that perfusion of a known Cl^- channel blocker, NFA, hyperpolarized the smooth muscle increase in the amplitude of the ETP that is consistent with decrease in membrane conductance. Panel D shows that perfusion of a known K_{ATP} channel opener, cromakalim, hyperpolarized the smooth muscle and decreased in the amplitude of the ETP that is consistent with increase in membrane conductance.

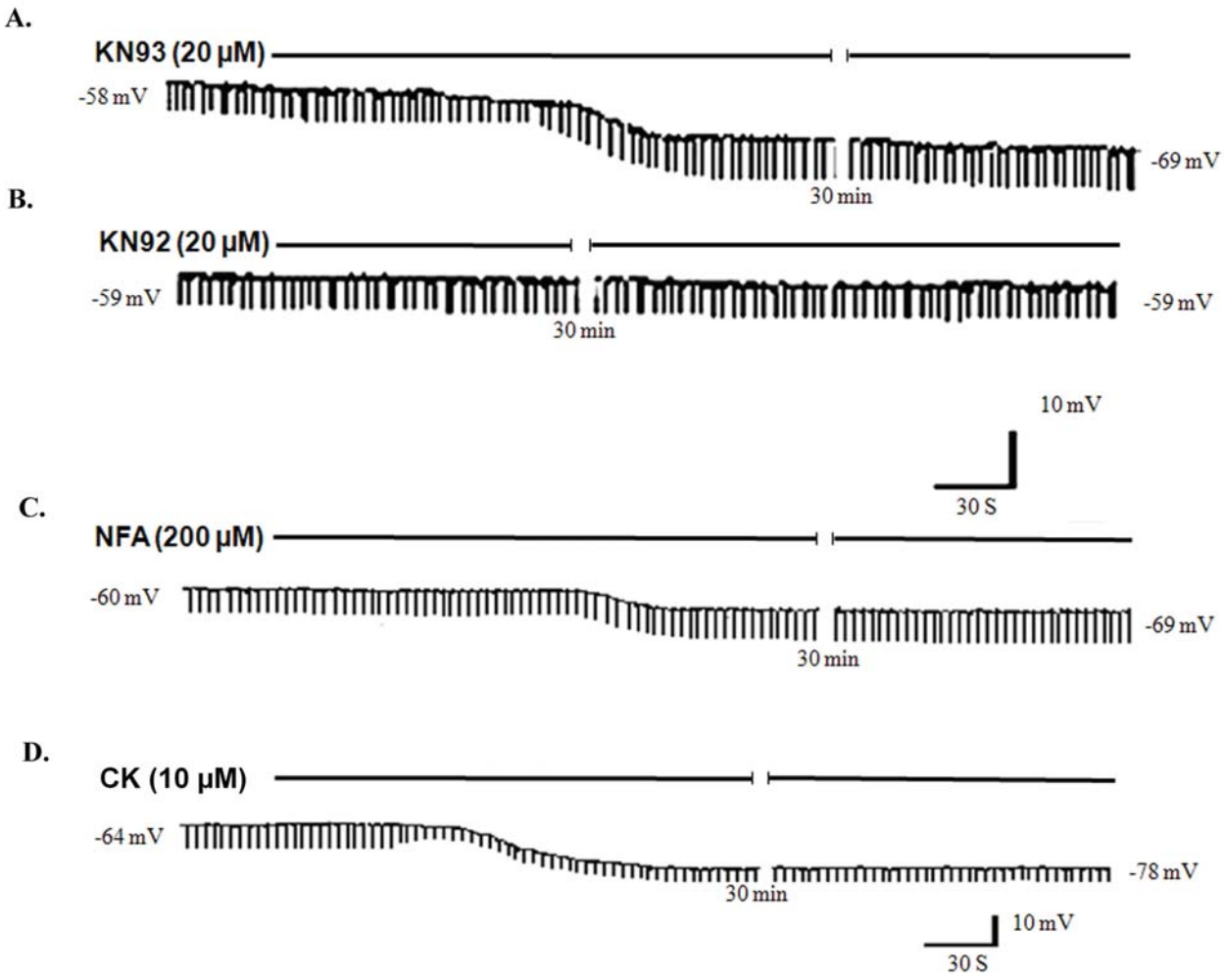
Fig 4 Examples of effect of perfusion of NFA and CK on the action of KN93 on the smooth muscle membrane potential and ETP on the same cell. Panel A shows that during perfusion of NFA, smooth muscle had MP of -69 mV and ETP of 11 mV. Addition of KN93 did not change either the MP or the ETP, suggesting that NFA blocked the action of KN93 by saturation block of Cl^- channels. Panel B shows that after perfusion of cromakalim, the smooth muscle was hyperpolarized (membrane potential: -78 mV) and had small amplitude ETP of 3.7 mV. Addition of KN93 further hyperpolarized the membrane by 8 mV to -86 mV, and increased the amplitude of the ETP, suggesting that CK hyperpolarization does not block the action of KN93 which can still act to close Cl^- channels.

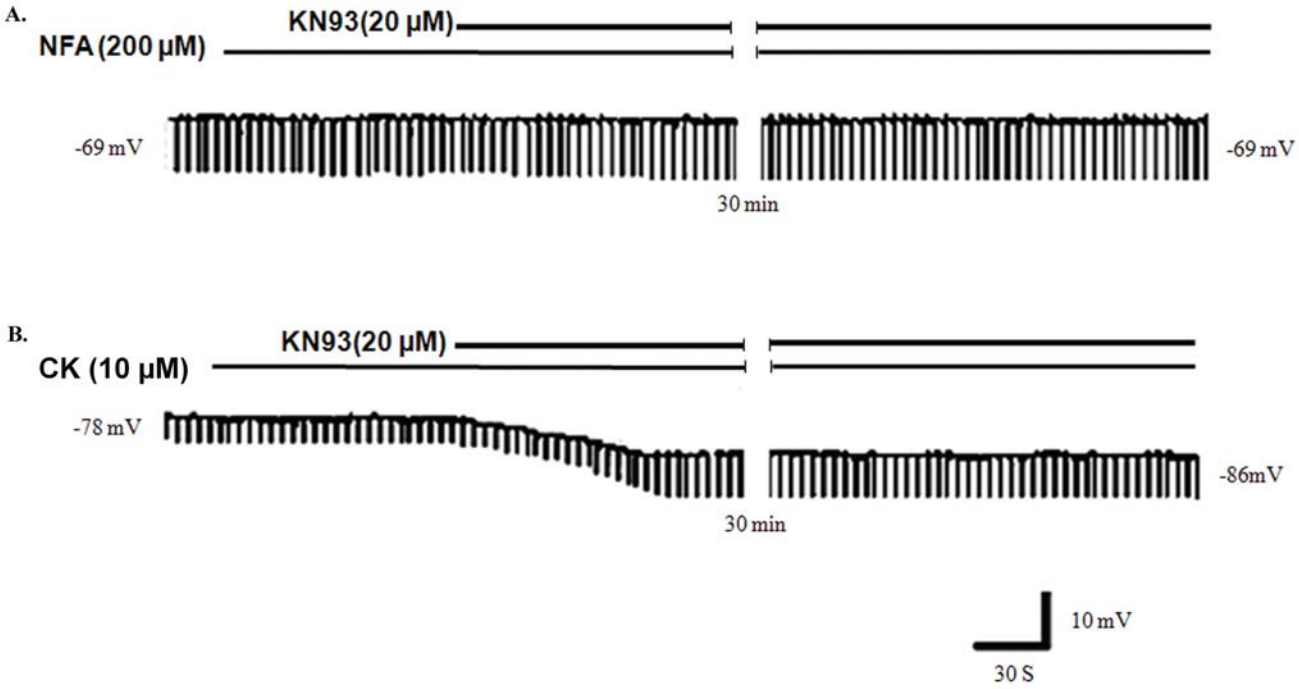
Fig 5 Examples of effects of various inhibitors of CaMKII on the fIJP and the sIJP. IJPs were elicited by EFS. The fIJP was isolated by blocking the sIJP by L-NNA and sIJP was isolated by blocking the fIJP by apamin. Note that the fIJP has a large amplitude and is not suppressed by CaM inhibitors calmidazolium or W7 that indirectly inhibit CaMKII and KN93 that directly inhibit CaMKII. However, calmidazolium, W7 and KN93 strongly suppressed the sIJP.

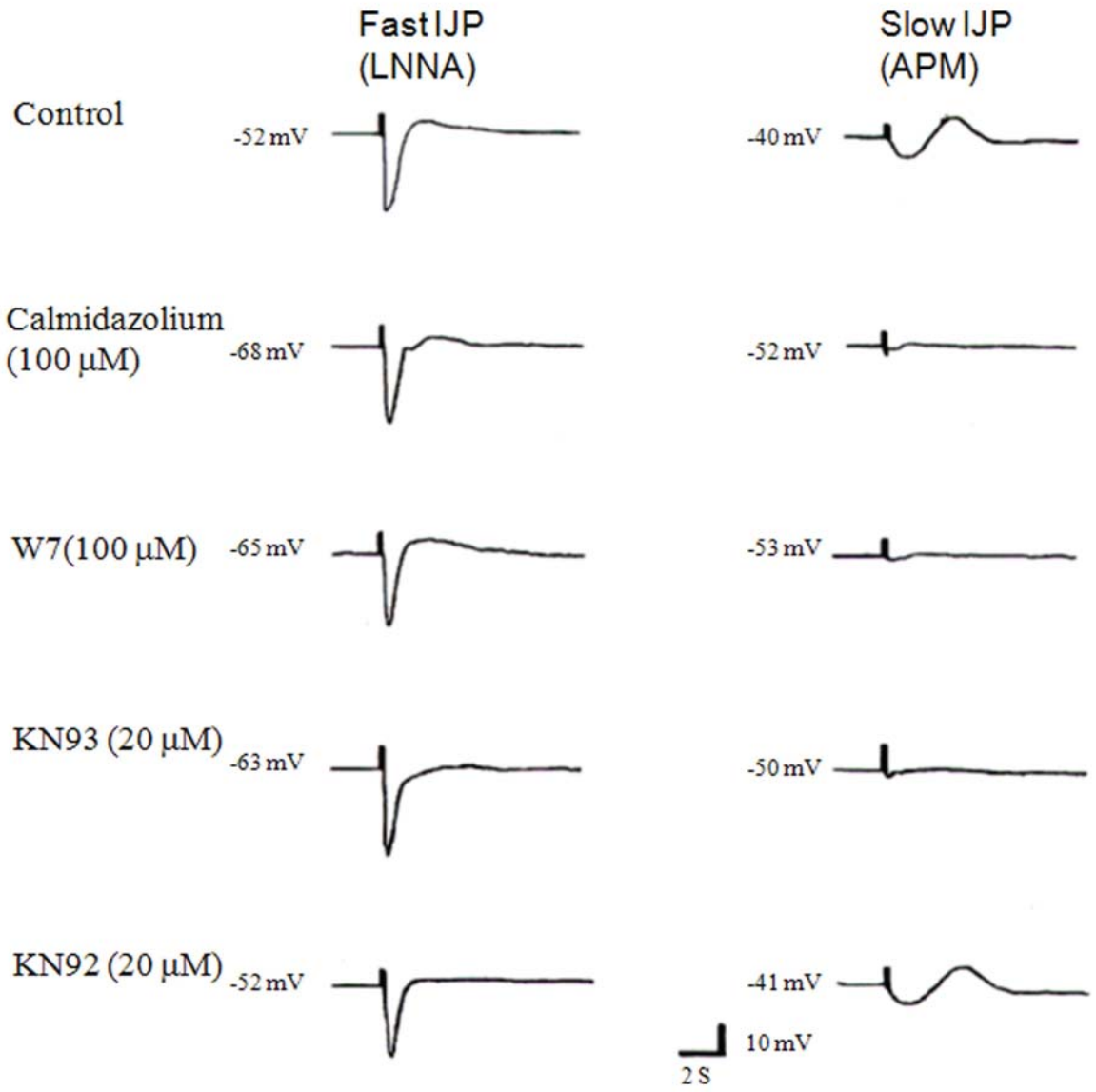

V
Chamber B
Chamber A



ETP: electrotonic potential


A.
KN93 (20 μM)
-58 mV
-69 mV
30 min
B.
KN92 (20 μM)
-59 mV
-59 mV
30 min
10 mV
30 S
C.
NFA (200 μM)
-60 mV
-69 mV
30 min
D.
CK (10 μM)
-64 mV
-78 mV
30 min
10 mV
30 S


A.
KN93(20 μM)
NFA (200 μM)
-69 mV
-69 mV
30 min
B.
KN93(20 μM)
CK (10 μM)
-78 mV
-86mV
30 min
10 mV
30 S


Fast IJP
(LNNA)
Slow IJP
(APM)
Control
-52 mV
-40 mV
Calmidazolium
(100 μM)
-68 mV
-52 mV
W7(100 μM)
-65 mV
-53 mV
KN93 (20 μM)
-63 mV
-50 mV
KN92 (20 μM)
-52 mV
-41 mV
10 mV
2 S