ROLE OF TRANSIENT RECEPTOR POTENTIAL A1 (TRPA1) IN COLD-INDUCED CONTRACTION IN THE ISOLATED INTESTINAL STRIPS IN RABBITS

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ABESTRACT

Background: Transient receptor potential (TRP) A1, a member of TRP channel family, is activated by noxious cold. **Aim of the work:** The present study is designed to determine if TRPA1 contributed to cold-induced contractions in the isolated rabbit intestinal preparations and explore the potential mechanisms. **Method:** The small intestinal smooth muscle layers were surgically isolated from male rabbits and changes in isotonic tension of longitudinal muscle under various treatments were recorded as intestinal motilities. Cold stimuli were obtained by the reperfusion with Tyrode's solution at given temperature. **Results:** The contractions induced by cold stimuli (from 37 °C to 12 °C stepwise) were inversely proportional to the temperature with a maximum contraction at 17 °C in rabbit small intestinal preparations ($P<0.001$). Cold-induced intestinal contractions were specially inhibited by TRPA1 blocker, ruthenium red (30 μmol/ml bath fluid), ($P<0.001$). The cold-induced contractions of the small intestinal strips were almost inhibited by the pretreatments of the strips with TRPA1, Allyl isothiocyanate (AITC, 300 μmol/ml bath fluid) ($P<0.01$). Pretreatment of the strips with atropine 10^{-4}M/ml bath fluid did not affect the cold induced contractions in rabbit small intestinal strips ($P>0.05$). Lidocaine, Na^+ channel blocker, (10^{-2}) M/ml bath fluid reduced the cold induced contraction in the rabbit small intestinal strips ($P<0.05$). **Conclusions:** TRPA1 was involved in cold-induced contractions in the rabbit small intestinal smooth muscle. Neural mechanism might be involved in that response. Further studies should be done to clarify role of Ca^{2+} in cold induced contraction.

Keywords: TRPA1, smooth muscle contraction, cold

INTODUCTION

The transient receptor potential (TRP) A1 is a member of TRP channel family and characterized as a thermoreceptor activated by noxious cold (≤17 ºC), suggesting a nociceptive function [(1),(2),(3)]. TRPA1 activation induced by cold stimuli and chemical agonists was related to many physiological and pathological processes such as cold temperature sensing, gastrointestinal motility and cold hyperalgesia in inflammatory pain [(4),(5),6),(7)]. TRPA1 agonist, allylisothiocyanate (AITC), was found to induce dose-dependent contractions in the isolated mouse intestine, which were neurogenic [(8)].

Contractions of guinea pig ileum induced by AITC were mediated by increasing serotonin release from enterochromaffin cells located on the mucosal surface [(7)]. It has been well shown that cooling induces contractions of arteriole, gastrointestine, urinary bladder and trachea smooth muscle, which are myogenic [(9),(10),(11),(12)]. **Mustafa et al.** [(13)] reported that the stimuli of 20 ºC induced the contraction of smooth muscle in the rat gastric fundus via TRPM8 receptor and Rho-kinase activation, because TRPM8 antagonist capsazepine and Rho-kinase blocker-2763 inhibited the contractions. TRPM8 is activated by innocuous cooling (≤25 ºC) [(14)], which suggests TRPM8 may mediate the cold-induced contractions in the colon. In this study, the expression of TRPM8 in rat colon smooth muscle was examined to exclude its function on colonic contraction.

In the light of the previous data, the present study was designed to examine cold-induced contractions in the rabbit small intestinal strips and to determine the role of TRPA1 in this response. In addition, the underlying mechanisms were further explored.

MATERIALS AND METHODS

Animals: Healthy adult male rabbits weighing 1500-2000 grams were used in this work. The number of animals used was kept to the necessary for a meaningful interpretation of the data. The animals were housed under standard laboratory conditions at room temperature in clean cages, kept in a 12-12 h light/dark cycle and allowed free access to normal food and water until the day of experiment.

Chemical agents: AITC, ruthenium red were from Sigma-Aldrich Co. (CH-9471Buchs, Germany), atropine sulfate was from (Cid co., Egypt) Lidocaine HCl (El-Nasr pharm. Chemicals Co. Egypt).

Experimental protocol: Each rabbit used for experiments was scarified, a midline laparotomy was performed to expose the intestine and a portion of the small intestine approximately 15 cm was removed and divided into segments, each segment was about 1.5-2 cm length, and all segments were immediately immersed in Tyrode's solution composed of (in gm/L): (NaCl 8.00, KCl 0.20, NaH2PO4 0.13, MgSO4 0.26, $NaHCO_3$1.0, Glucose 1, $CaCl_2$ 0.18) [15]. Each segment was tied in both ends with fine silk sutures. The segments were mounted along its longitudinal axis in 50 ml organ bath (63100-Palmer, Washington) filled with Tyrode's solution**,** continuously aerated with a mixture of 95٪ O_2 and 5٪ CO_2 and maintained at 37ºC. One end is connected to the aerator and the other end was connected to an ink- writing lever (Remote release fixit-811-50100-0), which record the amplitude of isotonic contraction (in mm) on a rotating kymograph (011-10550-5 Bioscience-Palmer, Washington). The load on the lever was 2 gm [16].

In these experiments, cold stimuli were obtained by reperfusion with Tyrode's solution at an invariable temperature. For each cold stimulus, the reperfusion was maintained for 5 min. It took 1-2 min to reach the desired temperature. The tissue was allowed to equilibrate for 30 min before the next stimulus was supplied. The contraction induced by the temperature of 17 ºC, which was the maximal one observed in preliminary experiments, was used as control.

After the control contraction was determined, the bath temperature returned to 37 ºC and equilibrated for 30 min, allowing the muscular tone to return to baseline. Then the preparations were incubated for 20 min with inhibitors before the reperfusion with Tyrode's solution containing inhibitors at 17 ºC for 5 min.

To investigate the desensitization character of TRPA1 channel, the preparations were incubated with TRPA1 agonists (AITC) for 5 min and washed more than three times, then subjected to 30 min of equilibrium and the following cold stimuli.

At the end of each experiment, the muscle was blotted and weighed. Each response was expressed as the amplitude of contraction in (mm) per gram of intestinal smooth muscle strips (mm·/ gm).

Statistical analysis: The data were expressed as mean ± SD for quantitative variables and statistically analyzed according to the methods described by **Pickey, (2003) [17].** The statistical analysis is done by using SPSS program (version 17) (SPSS Inc. Chicago, IL, USA). Multiple comparisons against a single control group were made by one-way analysis of variance **(ANOVA).** The statistical significance of differences between two groups was assessed using **Student "t" test** for comparison of means of two independent groups. Test was considered significant at P values < 0.05.

RESULTS

1- Cold-induced contractions:

As shown in **tracing 1, table 1 and figure 1**: The tonic contractions of longitudinal smooth muscle in the isolated rabbit intestinal strips were induced by lowering the bath temperature from 37 ºC to 32, 25, 17 and 12 ºC stepwise. The contractions increased as the temperature decreased (n=6, P<0.001). The maximum contractions [(40.8 ±2.7) mm/gm] were achieved at the temperature of 17 ºC. During the period of cold stimulation, the tone was enhanced and maintained at a constant level, but the spontaneous rhythmic contractions were decreased or abolished. When the temperature returned to 37 ºC, the tone rapidly returned to the basal level and spontaneous rhythmic contractions reappeared.

2- TRPA1 antagonist ruthenium red inhibited cold-induced contractions:

To clarify whether TRPA1 contributed to cold induced colonic contraction, the effects of 30 µmol/ml bath fluid ruthenium red; the antagonist of TRPA1, on cold-induced contractions were investigated. The results showed that ruthenium red decreased the contractions of rabbit small intestinal strips from (33.7±2.7) mm/gm to (23±1.4) mm/gm (n=6, P<0.001) **(tracing2, table 2 and figure 2).**

3- Pharmacology feature of TRPA1 desensitization:

To test the possible desensitization for the contractile responses evoked by cold stimuli, the preparations were repeatedly exposed to the cooling of 17 ºC with a 30 min interval. The results showed that cold stimuli did not cause desensitization of their own contractile effects (n=6, P>0.05) **(tracing 3a, table 3a and figure 3a)**. However, it was shown that cold-induced contractile responses were significantly decreased by the pretreatment of the strips with 300 µmol/ml bath fluid AITC (n=6, P<0.01) **(tracing 3b, table 3b and figure 3b)**.

4- Effects of atropine and Lidocaine on cold-induced contractions

Pretreatment of the strips with atropine 10^{-4}M/ml bath fluid did not affect the cold induced contractions in rabbit small intestinal strips (P>0.05, n=6) **(tracing 4a, table 4a and figure 4a)**, so the contractions were not related to activation of muscarinic receptors. On the other hand, lidocaine, Na^+ channel blocker, (10^{-2}) M/ml bath fluid reduced the cold induced contraction in the rabbit small intestinal strips (n=6, P<0.05) **(tracing 4b, table 4b and figure 4b)**, which indicated that neuronal pathway was involved in cold induced contraction partly in the rabbit small intestine.

1) Cold-induced contractions in the isolated rabbit small intestinal strips

Tracing (1):

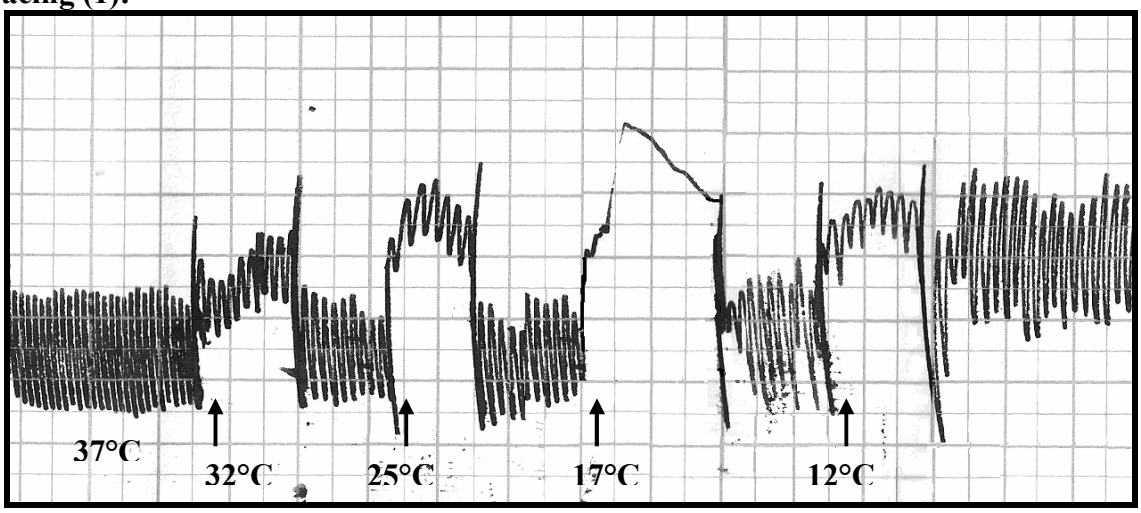
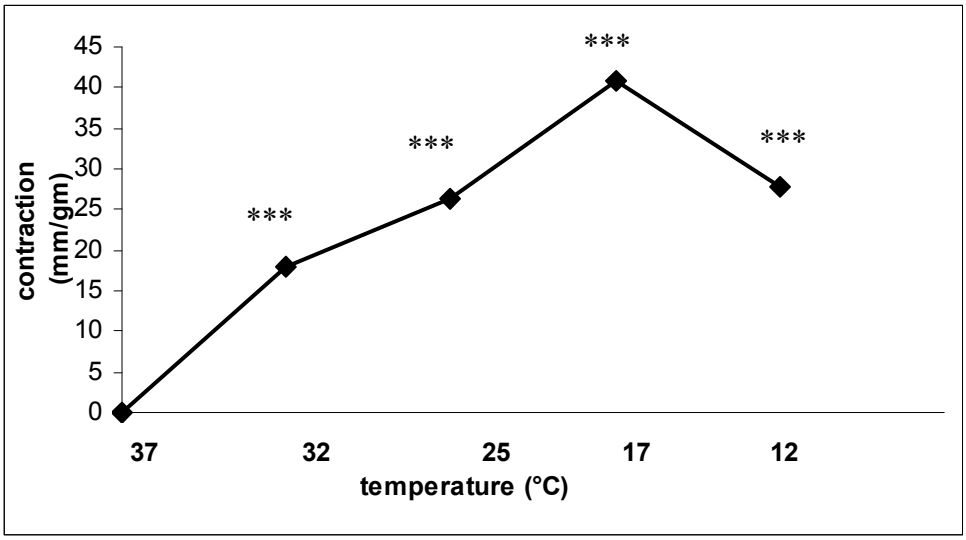


Table (1):

	Amplitude of contraction of isolated rabbit small intestinal strips (mm/gm)			
	32°C	**25°C**	**17°C**	**12°C**
Mean	+18	+26.3	+40.8	+27.7
±SD	2.4	2.2	2.7	2.4
F	89.51***			
p	<0.001			

Figure (1):



***P<0.001 vs 37 ºC.

2) TRPA1 antagonist ruthenium red inhibited cold-induced contractions:

Tracing (2):

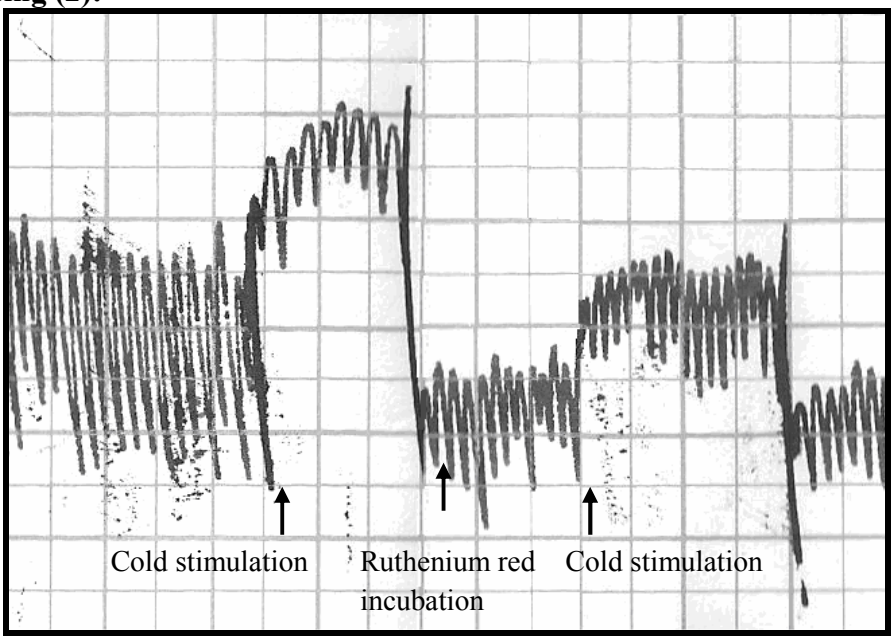
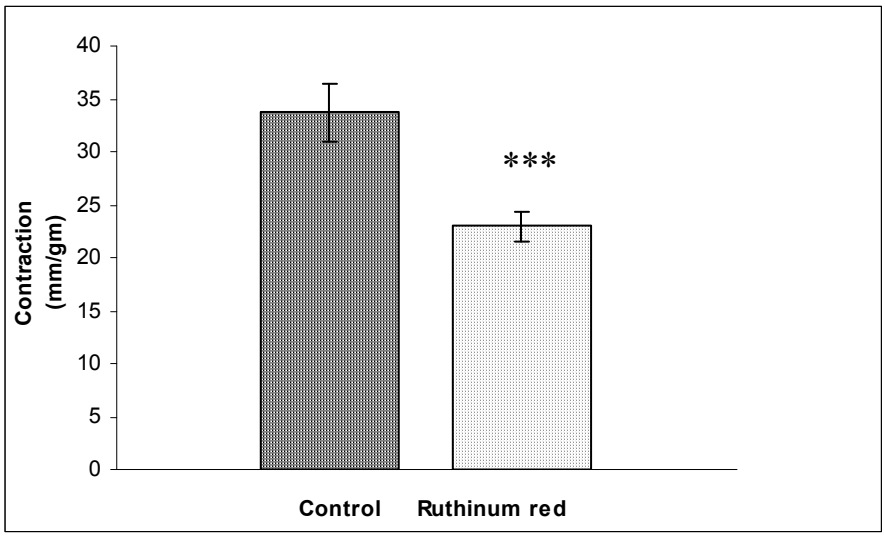


Table (2):

	Control	Cold stimulation after ruthenium red incubation
Mean	+33.7	+23
±SD	2.7	1.4
T	8.49***	
P	<0.001	

Figure (2):



***P<0.001.

3) Pharmacology feature of TRPA1 desensitization:

Tracing (3a):

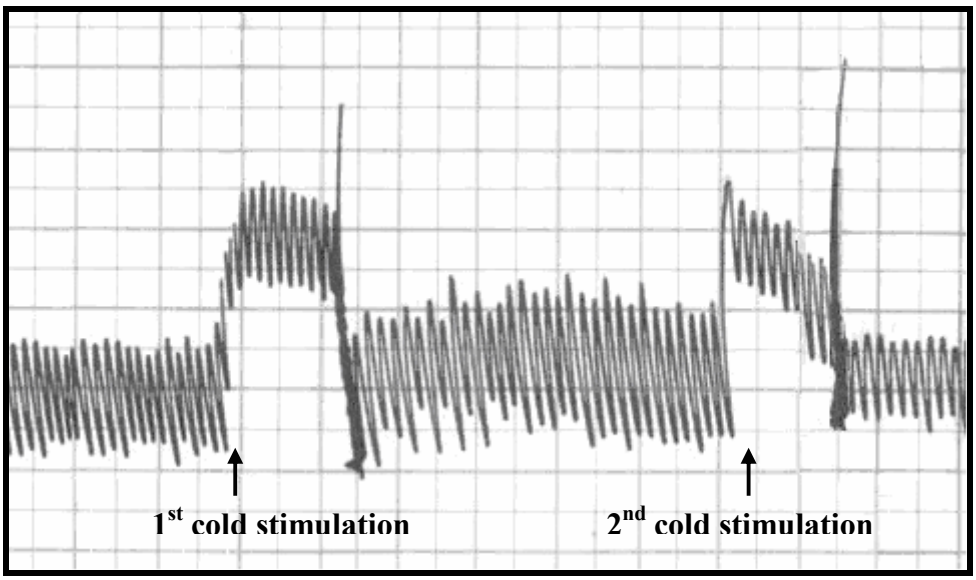
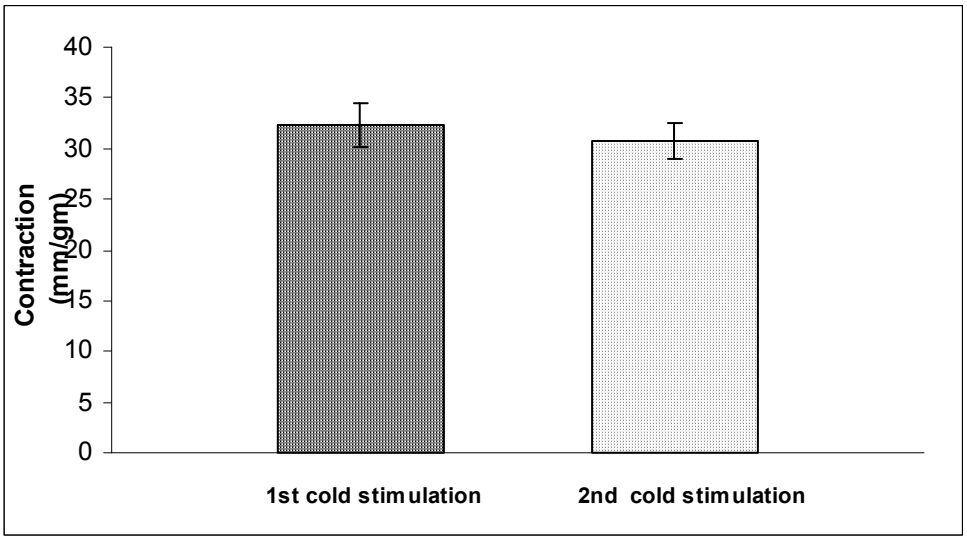


Table (3a):

	1st cold stimulation	2nd cold stimulation
Mean	+32.3	+30.8
±SD	2.16	1.83
T	1.296	
P	>0.05	

Figure (3a):



Tracing (3b):

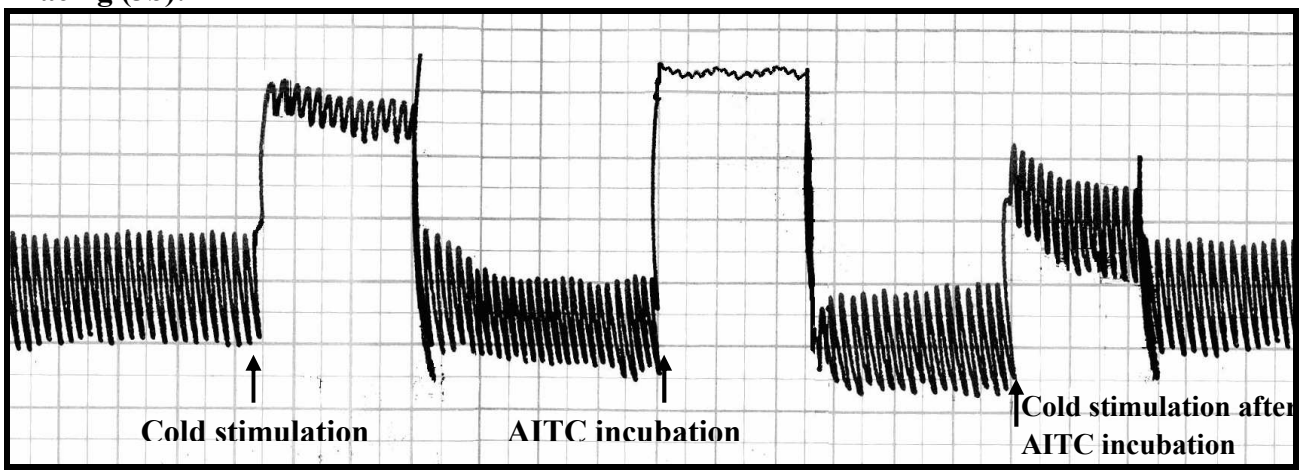
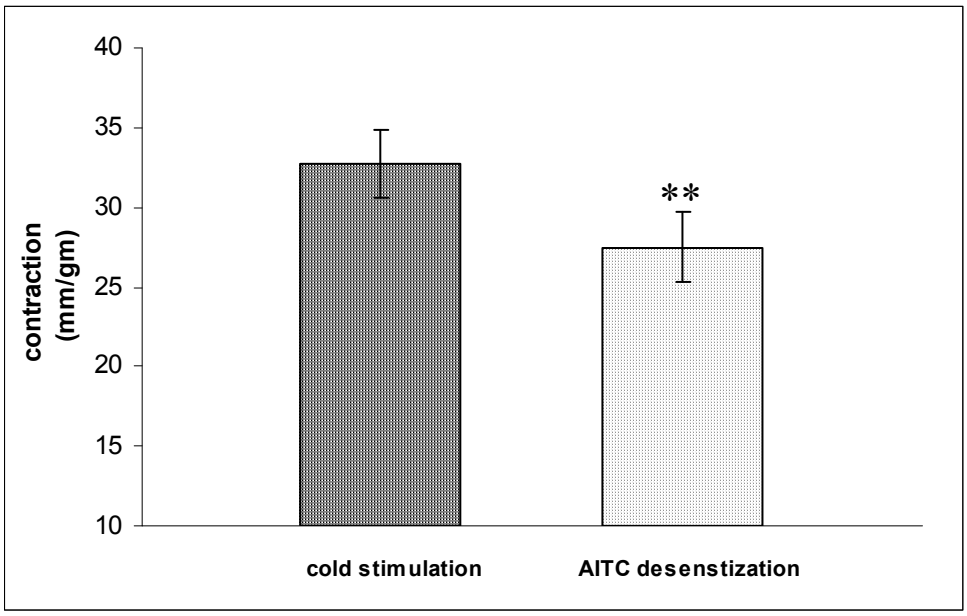


Table (3b):

	Control	AITC desesnstization
Mean	+32.7	+27.5
±SD	2.16	2.17
T	4.135**	
P	<0.01	

Figure (3b):



**P<0.01.

4- Effects of Atropine and Lidocaine on cold-induced contractions

Tracing (4a):

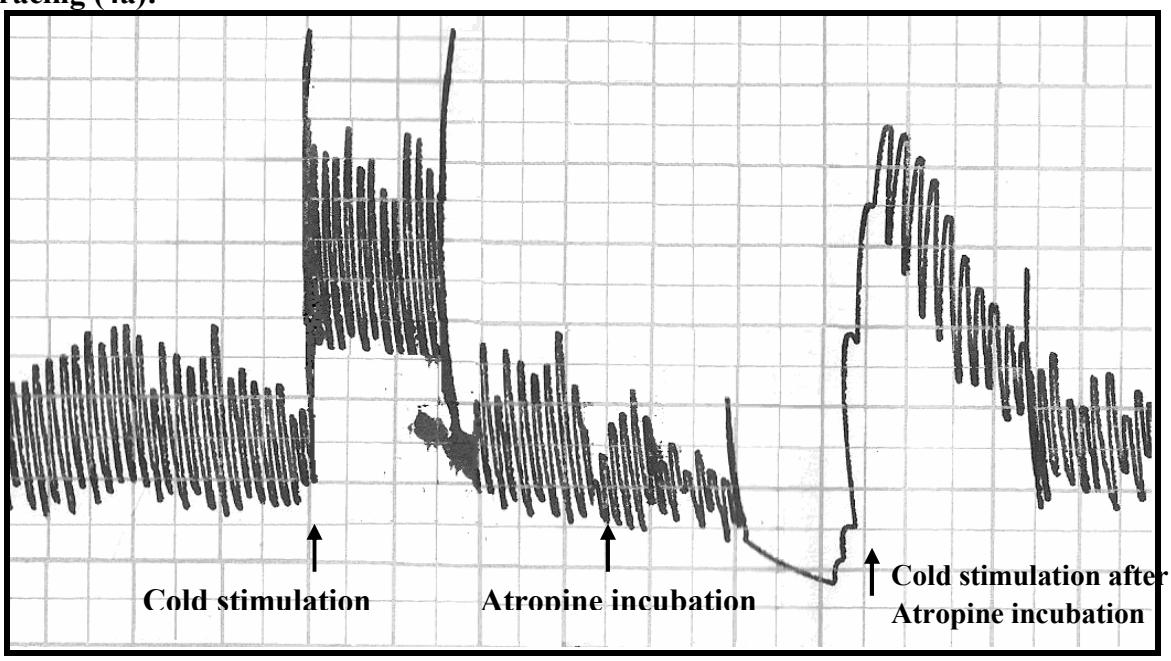
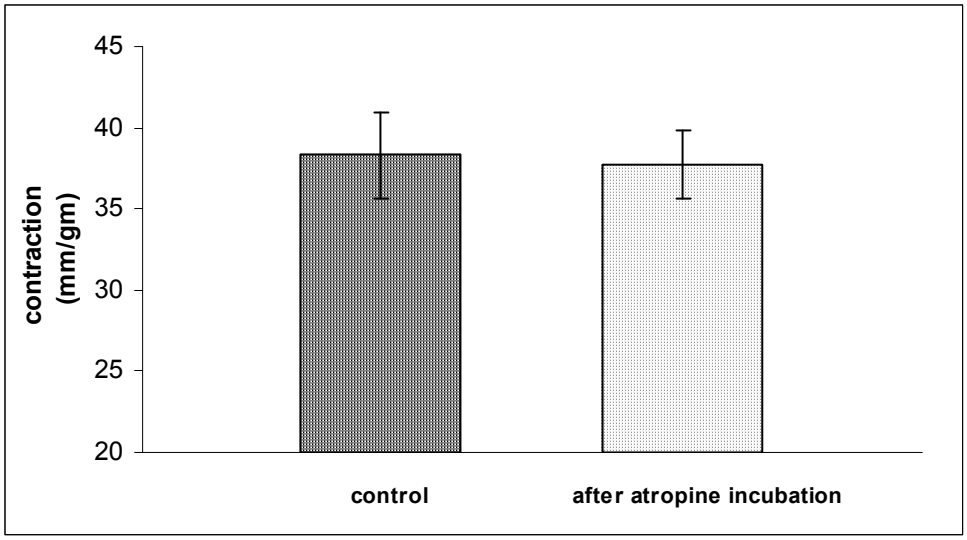


Table (4a):

	Control	Cold stimulation after atropine incubation
Mean	+38.3	+37.7
±SD	2.6	2.1
T	0.494	
P	>0.05	

Figure (4a):



Tracing (4b):

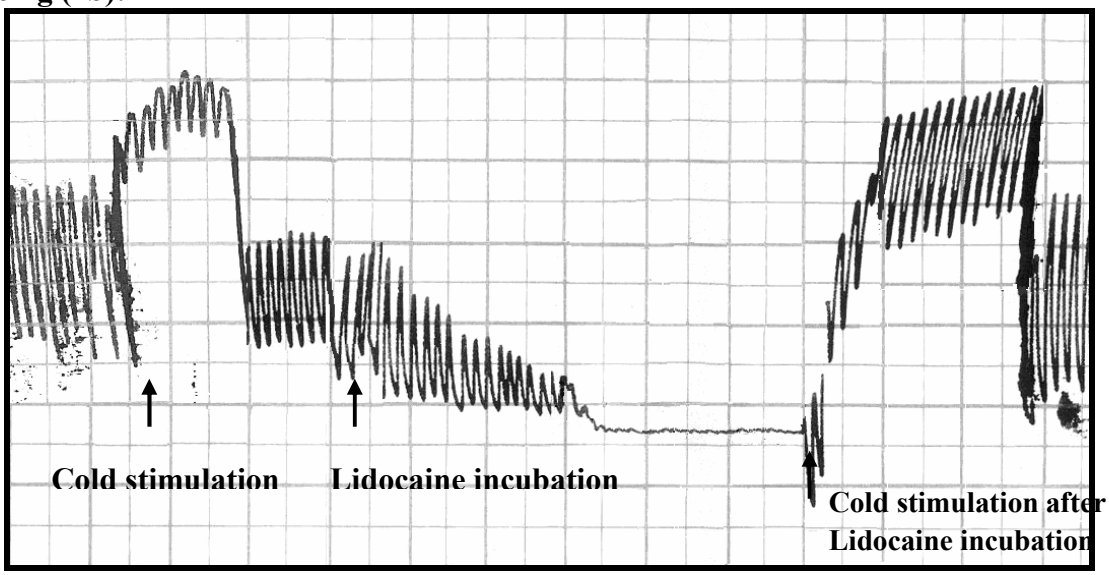
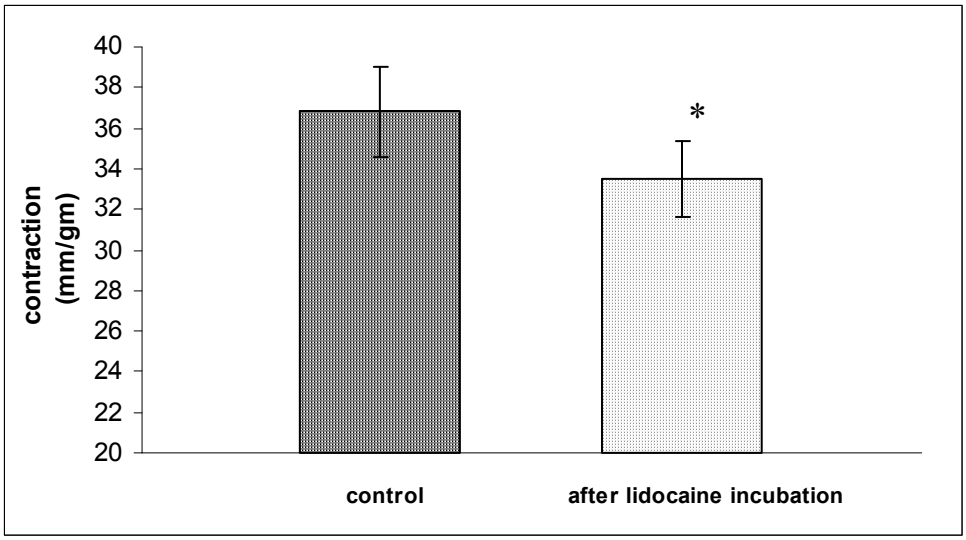


Table (4b):

	Control	Cold stimulation after lidocaine incubation
Mean	+36.8	+33.5
±SD	2.2	1.9
T	2.806	
P	<0.05	

Figure (4b):



*P<0.05.

DISCUTION

The data in the present study showed that cold-induced contractions were inversely proportional to the temperature with a maximum contraction at 17 ºC in rabbit small intestinal smooth muscle, which were specially inhibited by TRPA1 blocker ruthenium red. Ruthenium red could block TRPA1-mediated cold-evoked response [(1)], but lacking effects on TRPM8 [(15)]. Although ruthenium red could block other TRPs such as TRPV1 [(16)], only TRPM8 and TRPA1 were activated by cold stimuli. All these results suggested that TRPA1 contributed to the cold-induced contractions of rabbit intestine. These were supported by previous reports that TRPA1, but not TRPM8, was functionally expressed throughout the mouse intestine and TRPA1 could regulate gastrointestinal motility [(8), (18)]. Moreover, TRPA1 channels are shown to play a more important role in gastrointestinal tract function than TRPM8 channels are, because TRPA1 is the major mediator of cold-evoked responses in vagal visceral neurons, but TRPM8 as a cold sensor is mainly located in the somatic ganglion neurons [(4)].

Desensitization is an important pharmacological characterization of TRPA1 channel. It was found that repeated applications of TRPA1 agonist (AITC) produced the desensitization to their contractile effects in the mouse colon or rat urinary bladder [(8), (19)]. In addition, cross-desensitization was shown to mutually occur among various TRP agonist, including AITC, icilin and capsaicin, in the mouse proximal and distal colon [(8)]. However, cross desensitization between TRPA1 chemical agonists and cold stimuli has not been investigated. The results of the present study showed that cold stimuli in rabbit intestinal smooth muscle strips did not cause desensitization of their own contractile effects, which were reported by previous researches on other animal species [(9-13)]. However, AITC was observed to cause desensitization of TRPA1 to cold-induced contractions in the rabbit smooth intestinal strips. These results indicated that the mechanism of cold-induced TRPA1 activation was not the same as that of TRPA1 activation induced by agonists (AITC) completely. In addition, TRPA1 agonist AITC might be potential drugs to prevent cold induced pathological changes including intestinal smooth muscle contraction.

In the present study, it was shown that cold-induced contraction in the rabbit small intestinal smooth muscle exhibited lidocaine-sensitive components, suggesting cold induced contraction in the rabbit small intestine included both myogenic and neurogenic components. However, muscarinic receptor was not involved in the contractions, since atropine had no effects on the cold-induced responses. So it could be suggested that neurotransmitters other than acetylcholine mediated the cold induced contraction in the rabbit small intestine.

Ca^{2+} plays a primary role in regulating smooth muscle contraction. An increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), either through Ca^{2+} influx and/or release of Ca^{2+} from intracellular stores, leads to the activation of myosin light chain kinase, and subsequently phosphorylates myosin light chain, resulting in muscle contraction [(20),(21)]. Data reported by **Yong et al** [(22)] showed that cold-induced contractions in the rat proximal and distal colon were blocked by the removal of external Ca^{2+} from the medium containing 1 mmol/L EGTA and 2- APB (the inhibitor of IP3 receptor) which suggested that IP3/Ca^{2+} release pathway was involved in cold-induced contractions in the rat proximal and distal colon. IP3 receptor in sarcoplasimic reticulum mediates the release of Ca^{2+} from calcium store. However, Ca^{2+} release from calcium store only induces a transient contraction of the smooth muscle, so cold induced constant contraction of the smooth muscle in this experiment should mainly

be due to extra cellular Ca^{2+} influx, but not Ca^{2+} release from calcium store [22].

TRPA1, as a nonselective cation channel, is permeable to Ca^{2+}. The activation of TRPA1 could induce extra cellular Ca^{2+} influx [23]. Additionally, it was reported that TRPA1 in over expression systems was activated by elevation of $[Ca^{2+}]_i$ during cooling rather than directly by cold [24-26]. Taken together, this indicated that cold stimuli induced Ca^{2+} release from calcium store by stimulating IP3 pathway, and then the following elevation of $[Ca^{2+}]_i$ activated TRPA1. The Ca^{2+} influx mediated by TRPA1 further increased $[Ca^{2+}]_i$, which induced and maintained the constant contraction of smooth muscle [22]. So, further studies should be done to clarify role of Ca^{2+} in cold induced contraction in rabbit intestinal smooth muscle.

In conclusion, this study proved that TRPA1 was involved in cold-induced contractions in the rabbit small intestinal smooth muscle. Neural mechanism might be involved in that response. Further studies should be done to clarify role of Ca^{2+} in cold induced contraction.

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دور مستقبل الجهد المؤقت انكيرين-١ في الإنقباض المحدث بالبرودة في قطع الأمعاء المعزولة في الأرانب.

مستقبل الجهد المؤقت انكيرين-١ احد افراد عائلة قنوات مستقبلات الجهد كما أنه ينشط بواسطة البرودة المؤذية.

الهدف من البحث

تحديد إذا ما كان مستقبل الجهد المؤقت انكيرين-١ مساهم في الإنقباض المحدث بالبرودة في قطع الأمعاء المعزولة في الأرانب وكذلك محاولة استكشاف الْآليات المحتملة.

مواد و طرق البحث

أجريت هذه الدراسة علي عدد مناسب من الأرانب الذكور يكفي لتحليل البيانات إحصائيا.

وتم تسجيل التأثير الإنقباضي لدرجات برودة مختلفة علي قطع الأمعاء المعزولة وكذلك تأثير مغلق مستقبل الجهد المؤقت انكيرين-١ ومغلق مستقبلات مسكرين ومغلق قنوات الصوديوم علي هذا التاثير. كما تم دراسة خاصية سلب حساسية مستقبل الجهد المؤقت انكيرين-١ للإستجابة الإنقباضية المحدثة بالبرودة.

نتائج البحث.

وجد أن الإنقباضات المحدثة بالبرودة تتناسب عكسيا مع درجة الحرارة وأن الحد الاقصي للإنقباض حدث عند درجة حرارة ١٧ درجة مئوية وأن هذا الإنقباض قد انخفض انخفاضا ذا دلالة إحصائية عند استعمال مغلق مستقبل الجهد المؤقت انكيرين- ١ ومغلق قنوات الصوديوم ولم يتأثر بإستخدام مغلق مستقبلات مسكرين. وأن البرودة لا تسبب سلب حساسية مستقبل الجهد المؤقت انكيرين- ١ لتأثيرها الإنقباضي و لكن هذا التأثير انخفض انخفاضا ذا دلالة إحصائية عند استعمال منشط مستقبل الجهد المؤقت انكيرين- ١.

المستخلص من البحث:

- مستقبل الجهد المؤقت انكيرين- ١ مشترك في الإنقباضات المحدثة بالبرودة في قطع الامعاء المعزولة في الأرانب. وان آلية عصبية يمكن أن تكون مشتركة في هذه الاستجابة للبرودة.
- إن هناك حاجة لمزيد من الدراسات لتوضيح دور الكالسيوم في استجابة الأمعاء الإنقباضية للبرودة.