Acta Physiologica Sinica, April 25, 2006, 58 (2): 171-176
http://www.actaps.com.cn

Research Paper

Intraganglionic laminar endings act as mechanoreceptors of vagal afferent nerve in guinea pig esophagus

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Abstract: Intraganglionic laminar endings (IGLEs) have been supposed to be the mechanoreceptors in the gut by electrophysiological recording techniques. But the specialized morphology of IGLEs could not be displayed closely associated with this function and the mechanism that IGLEs act as the mechanotransduction sites in the gut is not yet well understood. In the present study, we used styryl dye FM1-43 combined with stretch stimulation in the guinea pig esophagus to test whether IGLEs acted as the mechano-sensitive receptors of the vagal afferent nerves. At the same time, the special structure of IGLEs displayed by FM1-43 was further confirmed by neurobiotin anterograde labeling technique. To further investigate the characteristics of IGLEs as mechanosensitive receptors, different drugs were used to block or stimulate IGLEs activation. Our results indicated that only in the stretched preparation could FM1-43 enter the IGLEs and completely display their specialized structure, which was consistent with that shown by neurobiotin. The amount of IGLEs shown by stretch-evoked FM1-43 staining was much more than that shown without stretch stimulation [(90.4±9.5)% *vs* (10.7±2.1)%, $P<0.05$]. Ca^{2+}, TTX (0.6 μmol/L), atropine (0.6 μmol/L), SKF (50 μmol/L), and gadolium (100 μmol/L) had no effect on the IGLEs activation. But for benzamil (100 μmol/L), an epithelial sodium channel blocker, activation of IGLEs by stretch stimulation was significantly blocked. The potent ATP analogue, α,β-methylene ATP (100 μmol/L) could not activate FM1-43 staining without stretch. These results indicate that IGLEs are sensitive to mechanical stimulation. This could lead to the deduction that IGLEs act as the mechanoreceptors of vagal afferent nerve. IGLEs could transmit mechanical stimuli directly through ion channels, independent of neurotransmitter release and action potential propagation. The stretch-sensitive channels on IGLEs probably belong to the epithelial sodium channel family rather than voltage-gated sodium ion channels. Furthermore, styryl dye FM1-43 is a useful activity-dependent marker to demonstrate the structure and function of IGLEs in guinea pig esophagus.

Key words: intraganglionic laminar endings; mechanotransduction; mechanoreceptors; receptor; FM1-43; anterograde; labeling

神经节内板状末梢是豚鼠食道迷走传入神经末梢的机械敏感性受体

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摘 要：电生理学研究发现迷走传入神经在胃肠道的特有结构——神经节内板状末梢(intraganglionic laminar endings, IGLEs)具有感受机械刺激的功能，推断其为迷走神经机械敏感性受体。但是电生理学方法不能将 IGLEs 的特异结构与其感受机械刺激的功能同时显示出来，而且 IGLEs 作为机械敏感性受体，其传导机械刺激的机制尚不清楚。本研究应用活性依赖性荧光染料 FM1-43 结合牵拉刺激豚鼠食道显示激活的 IGLEs 结构，以期观察 IGLEs 是否对机械刺激敏感。同时用多种药物阻断或促进豚鼠食道 IGLEs 的激活以探讨 IGLEs 传导机械刺激的机制。应用神经顺行标记技术以验证 FM1-43 显示的特异结构是否为 IGLEs。结果表明，牵拉刺激结合 FM1-43 染色显示的结构与神经顺行标记法一致， 牵拉刺激组激活的 IGLEs 数目明显多于未牵拉组 [(90.4±9.5)% *vs* (10.7±2.1)%, $P<0.05$]。 IGLEs 对牵拉刺激的敏感性，表明 IGLEs 是迷走传入神经在胃肠道内感受机械刺激的受体。TTX，阿托品和钙离子对牵拉刺激激活 IGLEs 无明显影响，表明 IGLEs 对机械刺激的传导不需要神经递质以及动作电位的传导，而是直接通过机械门控离子通道实现的。多种 TRP 通道阻断剂包括 SKF, gadolinium 对 IGLEs 的激活无影响，而上皮钠离子通道阻断剂 benzamil 可以明显阻断 IGLEs 的激活，因此推断，IGLEs 结构中传导机械刺激的离子通道可能属于上皮钠离子通道家族而非电压门控钠离子通道或 TRP 通道。

Received 2005-12-09 Accepted 2006-01-11
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关键词：神经节内板状末梢；机械传导；机械受体；受体；FM1-43；顺行；标记
中图分类号：R338

The specialized afferent vagal terminal endings in the gut have been described as intraganglionic laminar endings (IGLEs). The morphology of IGLEs has been described by anterograde labeling and immunohistochemistry techniques as specific flattened, leaf-like structure circling around the myenteric ganglia. By using electrophysiological recording technique to find the "hot spot", which is sensitive to von Frey hair stimulation[1,2], Zagorodnyuk and Brooks supposed that IGLEs act as the transduction sites of vagal mechanoreceptors in guinea pig esophageal and stomach tissue. However, the specialized morphology of IGLEs could not be displayed closely associated with this function. Moreover, there are many other ion channels besides IGLEs that can modulate the action potentials being recorded. This means that electrophysiology recording is an indirect study and the mechanism that IGLEs transmit the mechanical stimulation in the gut couldn't be well understood by this technique.

FM1-43 is a cationic styryl dye that provides fluorescent signal visible under fluorescein optics when it is in a hydrophobic environment[3]. To date the FM family of dyes has been used primarily to investigate membrane trafficking, as they are taken up by endocytosis[4-6]. However, FM1-43 could also be used to display the mechanoreceptors in vertebrate hair cells by a novel mechanism for entry into sensory cells and neurons, namely permeation through mechanosensitive, non-selective ion channels[7,8]. FM1-43 labeling is activity-dependent, which means that closed or nonfunctional channels do not pass FM1-43. More importantly, for FM1-43 taken up by endocytosis, membrane trafficking and exocytosis could keep balance in a rather long time *in vitro* (21 d). So the quantification for FM1-43 loading through ion channels could be made by this persistence. Another significant advantage of FM1-43 is its specificity for sensory cells. It presents an opportunity to visualize sensory cells and sensory transduction under normal conditions with minimal disruption to the cells. As IGLEs have been supposed to mediate their action via mechanosensitive channels[9], it was hypothesized that exposure to FM1-43 under appropriate conditions would result in the uptake of the dye through mechanosensitive channels and thus the specialized morphology of IGLEs could be displayed.

Neurobiotin anterograde labeling technique has been proved to be a useful method to display the structure of IGLEs[1]. In the present study, we used it to confirm whether the structure displayed by FM1-43 was IGLEs. To further investigate the characteristics of IGLEs as the mechanoreceptors of vagal afferent nerves, different drugs were used to block or stimulate the IGLE activatioin.

1 MATERIALS AND METHODS

1.1 *Animals*

Guinea pigs, weighing 210~380 g, were killed humanely by stunning and exsanguination in a manner approved by the Animal Welfare Committee of Shandong University. The thoracic esophagus was removed and maintained in Krebs solution (mmol/L: NaCl 118, KCl 4.75, NaH_2PO_4 1.0, $NaHCO_3$ 2.5, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11, bubbled with 95% O_2-5% CO_2), before being opened into a flat sheet and having the mucosa carefully removed.

1.2 *Exposure to FM1-43*

Small adjacent squares of tissue were removed from the esophageal preparation in pairs. One of these squares was attached to an array of hooks, which were in turn attached to a mechanical transducer. The opposite end of this tissue was firmly pinned in a small organ bath lined with Sylgard. The second piece of tissue was then pinned loosely next to the first piece. The tissue was then maintained at approximately 36 °C and left to equilibrate in a warmed perfusion of bubbled Krebs for at least 30 min. The Krebs perfusion was then replaced with a warmed perfusion of a 1 μmol/L FM1-43 (Molecular Probes, Eugene, OR) solution. Both pieces of tissue were exposed to this solution for 30 min. The piece of tissue attached to the hooks was stretched with a 4-gram weight for the duration of the exposure, with 1 min of stretch, 1 min of slack alternately. The dye solution was then removed from the bath and the Krebs perfusion re-started for 30 min to wash out dye that hadn't penetrated the tissue. Both pieces of tissue were then firmly pinned out in a small dish before being fixed in 4% paraformaldehyde for at least 60 min. The tissue was then rinsed in phosphate buffered saline before removal of excess connective tissue from both the mucosal and serosal sides of the preparations. The tissue was then placed in sterile 100% buffered glycerol prior to mounting on glass slides.

1.3 *Different drug treatment on the tissue*

A modified Krebs solution (Ca^{2+}-free, 2.5 mmol/L $MgCl_2$), 0.6 μmol/L TTX, 0.6 μmol/L atropine sulphate, 100 μmol/L α,β-methylene ATP, 100 μmol/L benzamil, 50 μmol/L SKF and 100 μmol/L gadolium (all drugs from Sigma

Chemical Company) were made separately. The preparation was exposed to each solution via a warmed perfusion for 15 min before FM1-43 was added to the solution to the final concentration of 1 μmol/L. Except for the α,β-methylene ATP group and the control group which were not stretched in the experiment, one of the pieces of tissue in other groups was then stretched for 30 min as per the protocol for FM1-43 exposure before rinsing, fixing and mounting.

1.4 *Anterograde labeling*

Anterograde labeling technique was used to display the structure of IGLEs in guinea pig esophagus as the following[1]. A fine vagal nerve trunk innervating the distal esophagus was chosen and dissociated carefully in Krebs solution. Then the nerve trunk was placed in a paraffin-filled chamber and one drop of 5% biotinamide (Molecular Probes, Eugene, OR) in an artificial intracellular medium (mmol/L: monopotassium *L*-glutamic acid 150, $MgCl_2$ 7, glucose 5, EGTA 1, HEPES 20, disodium adenosine-triphosphate 5, 0.02% saponin, 1% dimethyl sulfoxide), was placed on the nerve trunk. The main chamber, containing the esophageal preparation, was filled with sterile supplemented culture medium [DME/F12 with 10% fetal bovine serum, 1.8 mmol/L $CaCl_2$, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 20 μg/ml gentamicin (Cytosystems, NSW), pH 7.4]. Note that the two chambers must be separated completely by coverslip and silicon grease. The tissue was incubated in humidified incubator with 5% CO_2 in air at 37 °C. After 5~6 h, preparations were put in the O_2/CO_2 bubbled Krebs solution and stretched in 1 μmol/L FM1-43 according to the above protocol. After being fixed for 1 h in 4% paraformaldehyde, the picture for IGLEs stained by FM1-43 was taken under the fluorescent microscope. Then the preparation was cleared in 100% glycerol overnight and rinsed in phosphate buffered saline (PBS: 150 mmol/L NaCl in 10 mmol/L sodium phosphate buffer, pH 7.2). The neurobiotin-labelled nerve fibres were visualised with streptavidin CY5 (Molecular Probes, Eugene, OR, 1:4 000, 4 h).

1.5 *Data analysis*

The amount of IGLEs was expressed as the percentage of IGLEs displayed by FM1-43 to the total number of the myenteric ganglia of the preparation[10]. This method has the advantage of objectivity for its independence of the size and shape among different preparations. Results were expressed as means±SEM, with *n* referring to the number of animals. Statistical analysis was performed by Student's two-tailed *t* test for paired data. Differences were considered significant if $P<0.05$.

2 RESULTS

2.1 IGLEs activation displayed by FM1-43

In the stretched esophageal preparation, FM1-43-filled IGLEs were visible on microscopic examination. The flattened, leaf-like structure of IGLEs was shown very clearly. On 1 cm^2 of tissue, an average of (90.4±9.5)% myenteric ganglia were supplied with IGLEs. They were highly fluorescent compared to the background tissue. In addition to IGLEs, fine muscle fibers were also noted by fluorescence. Comparatively, the no-stretch control tissue displayed relatively fewer IGLEs (an average of (10.7±2.1)% of the ganglia, $P<0.01$ *vs* the stretch group) in addition to the fluorescent muscle fibers noted in the stretched tissue (n=10, Fig.1). It could be deduced that the FM1-43-filled IGLEs were stretch-sensitive and the staining was stretch-dependent.

2.2 Different drug treatment on IGLEs activation

IGLEs were still visible with the same amount in stretched esophageal tissue despite exposure to 0.6 μmol/L TTX, 0.6 μmol/L atropine, 50 μmol/L SKF, 100 μmol/L gadolium and Ca^{2+}-free Krebs solution, respectively. The amount of IGLEs being displayed was (86.3±6.8)% for TTX, (87.9±7.3)% for atropine, (89.1±9.1)% for Ca^{2+}, (83.9±6.6)% for SKF, (80.3±7.2)% for gadolinium, respectively. Exposing no-stretch esophageal tissue to a 100 μmol/L α,β-methylene ATP solution for 30 min did not result in any specific staining in the preparation compared with the no-stretch control group (12.6±2.8%). But on the 100 μmol/L benzamil-treated preparation, the specific staining of IGLEs was significantly blocked (25.3±3.6%) (n=6, Fig.2).

2.3 Neurobiotin anterograde labeling

The morphology of IGLEs demonstrated by neurobiotin anterograde labeling was completely consistent with that displayed by the stretch-evoked FM1-43 staining (n=4, Fig.3). After 5~6 h of culture, the tissue was not as healthy as the fresh tissue so that the structure of IGLEs displayed by FM1-43 was not as complete as that in neurobiotin labeling. Even in some preparation IGLEs displayed by neurobiotin could not be shown by FM1-43. This indicated that IGLEs displayed by FM1-43 were closely related to the activity of the mechanosensitive channels.

3 DISCUSSION

The study for mechanotransduction pathway in the gut is always a mystery because the gut is supplied with both the

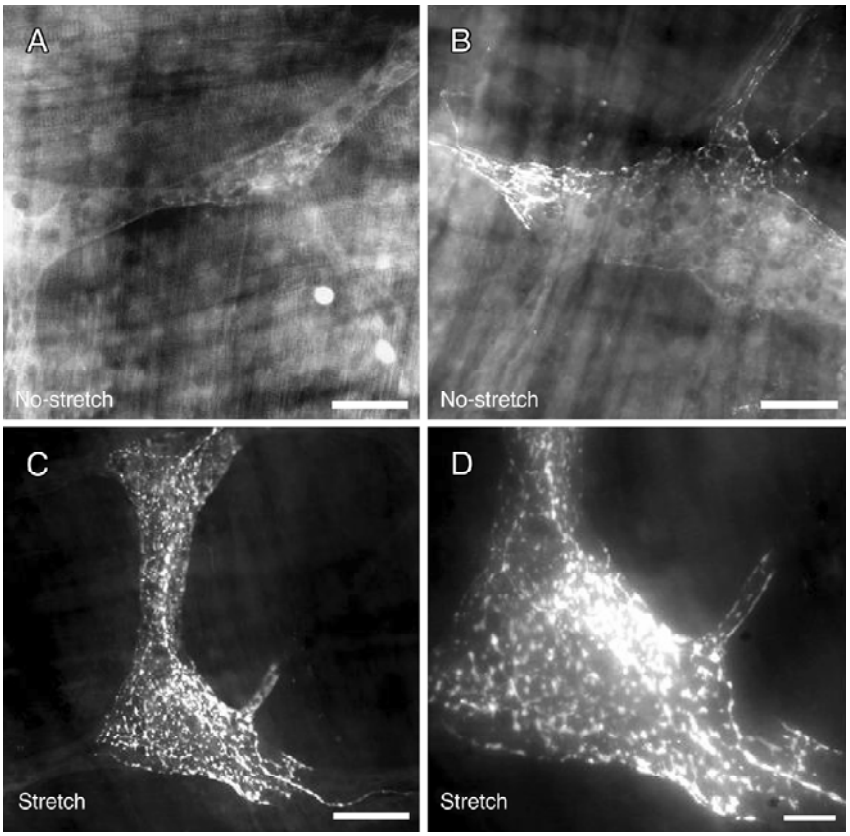

Fig. 1. Morphology of IGLEs displayed by FM1-43 staining. *A*: In no-stretch esophageal tissue IGLEs were not displayed in the myenteric ganglia. *B*: IGLEs could be shown in some no-stretch preparations with incompletes structure and less fluorescence intensity. *C*: IGLEs shown by stretch-evoked FM1-43 staining had specific structure and strong fluorescence intensity. *D*: The specific flattened, leaf-like structure of IGLEs with high magnification. Scale bar in *A*, *B*, *C*=50 μm, *D*=20 μm.

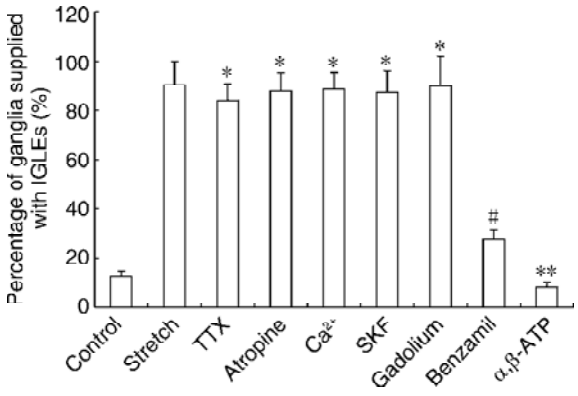

Fig. 2. Different drug treatment on IGLEs activation in guinea pig esophagus. TTX, atropine, Ca^{2+}, SKF, and gadolium treatment with stretch stimulation had no effect on IGLEs activation ($^{*}P$> 0.05 *vs* stretch group without drug treatment). Benzamil significantly decreased the IGLEs activation ($^{\#}P$<0.05 *vs* stretch group without drug treatment); α,β-methylene ATP had no effect on IGLEs activation ($^{**}P$>0.05 *vs* no-stretch control group). *n*=6.

extrinsic and intrinsic nervous system. Many factors including neural and endocrinal factors could influence the afferent pathway[11]. Vagal afferent neurons give rise to three types of specialized endings in the gut wall[12-14]. IGLEs are found on the surfaces of myenteric ganglia and are abundant throughout the gastrointestinal tract[10]. For the esophagus, the upper part isn't innervated by the vagus nerve. So we take the thoracic esophagus to study the specialized structure and function of IGLEs. The function of IGLEs studied here has previously only been investigated by electrophysiology, which is essentially an indirect study of transduction as it records action potentials, which can be modulated by the action of other ion channels. Moreover, the structure of IGLEs which was supposed to be associated with this function could only be demonstrated by combining the "hot-spot" and the nerve tracing results. However, in the present study the specialized structure that is achieved by stretch stimulation and FM1-43 looks directly at the transduction pathway without other modulation factors, therefore making it a direct reflection of the underlying function of IGLEs.

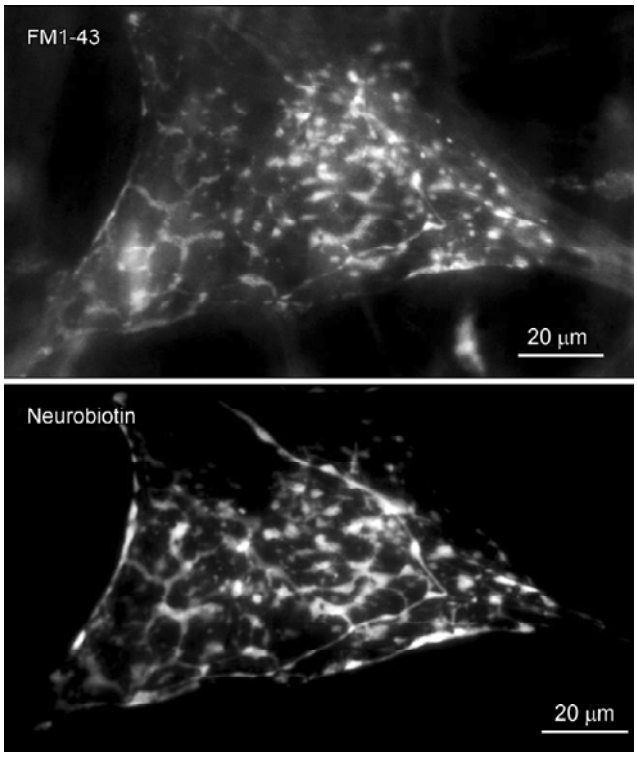

Fig. 3. Structue of IGLEs displayed by stretch-evoked FM1-43 staining and neurobiotin anterograde labeling. The structure displayed by stretch-evoked FM1-43 staining was consistent with that shown by neurobiotin anterograde labeling. *A*: Stretch-evoked FM1-43 staining showing specific laminar endings in esophagus myenteric ganglia. *B*: The same IGLEs displayed by neurobiotin anterograde labeling. Scale bar, 20 μm.

The findings of this study provide clear evidence that IGLEs displayed by stretch-evoked FM1-43 labeling was stretch-sensitive. It could be deduced that IGLEs act as the mechanosensitive receptors of the vagal afferent nerves. This result is compatible with the findings of Zagorodnyuk by electrophysiological recording[2]. The consistence of the morphology of IGLEs displayed by stretch-evoked FM1-43 with that by neurobiotin anterograde labeling indicated that FM1-43 was a good candidate marker for IGLEs. Furthermore, there are some defects with anterograde labeling. Only 1~2 extrinsic nerves could be traced in one experiment and it is not sure whether the structure being labeled is IGLEs. To the worse is that IGLEs being labeled is not the IGLEs on the hot spot. These factors give more blindness and much greater work for the experiment. So compared with Zagorodnyuk's research, the present study has more advantages because it could illustrate the morphology and function of IGLEs at the same time in a more direct and easier way.

Different drug treatments were done to further investigate the characteristics of IGLEs as the mechanosensitive receptors. Calcium is required for neurotransmitter release from synaptic vesicles, and TTX blocks action potential propagation by preventing sodium entry through voltage-gated sodium channels. According to Carr *et al*[15], Some effects of benzamil (100 μmol/L) have previously been attributed to blockade of voltage-gated sodium channels. In the present study, calcium, high concentration of TTX (0.6 μmol/L) and atropine (0.6 μmol/L) had no effect on IGLEs activation by stretch stimulation. This indicates that neurotransmitter (such as cholinergic) and action potential are not involved in the mechanotransduction pathway of the gut while the blocking effect of benzamil on the IGLEs activation may act through the epithelial sodium ion channels rather than the voltage-gated sodium channels existing on IGLEs.

TRPA1 has been supposed to be the mechanoreceptors in vetebrate hair cells[8]. Unfortunately up to date there is no specific TRPA1 blocker. To investigate the relationship between TRPA1 and IGLEs, we used several non-specific TRP channel blockers such as SKF and gadolinium[16]. The reason for that SKF and gadolinium had no effect on IGLEs activation may be their non-specificity to TRPA1 or that TRP channels are not involved in the mechanotransduction induced by IGLEs in the gut.

Apart from neurobiotin anterograde labeling, one kind of ATP receptors—$P2X_2$ is another useful marker for IGLEs because ATP could excite vagal mechanoreceptors via P2X receptors[17,18]. But in the present study, α,β-methylene ATP had no effect on the IGLEs activation. This may due to the heteromeric character of ATP receptors including P2X and P2Y, both of which have many subtypes. But it hasn't been identified which subtype is responsible for the mechanical transduction.

In the no-stretch preparation there were some IGLEs visible in reduced number and fluorescence intensity. This presence reflected the baseline activity of the IGLEs, which was first identified by Zagorodnyuk and Brookes[1]. This spontaneous firing was clearly adequate to allow the permeation of small amounts of FM1-43 into some IGLEs. On the other hand, by loosely pinning the control preparation out into a flat sheet, it was also ensured that there was little in the way of a diffusion barrier, which would also play a role in the permeation of the dye.

Styryl dye FM1-43 permeation through ion channels provides a novel mechanism for labeling of mechanoreceptors and has many advantages over other vital labeling techniques. For its activity-dependence and persistence in the sensory cells, the fluorescence intensity of the dye is closely related with the amount and sensitivity of the mechanoreceptors being activated.

In conclusion, IGLEs displayed by stretch-evoked FM1-43 staining confirmed that IGLEs act as the mechanorecep-

tors in guinea pig esophagus. The transduction mechanism of IGLEs is directly through some kind of epithelial sodium ion channels rather than voltage-gated sodium ion channels. And neurotransmission and action potential propagation are not involved in this process.

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