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Inherent pacemaker function of duodenal GIST

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ABSTRACT

Gastrointestinal stromal tumours (GIST) are thought to derive from interstitial cells of Cajal (ICCs), which are putative pacemaker cells for gut motility. Isolated cells were obtained by enzymatic treatment of human duodenum GIST tissue having a frequent gain-of-function gene mutation. After cell culturing, c-Kit immunoreactivity was preserved and the cells developed long processes. Whole cell patch clamp recordings revealed voltage-dependent outward currents, without transient inward currents. Intracellular Ca^{2+} measurements showed oscillation-like spontaneous activity in some GIST cells. RT-PCR revealed expression of ion channels (Kv1.1, Kv1.6 and KCNH2; IP3R1, and IP3R2; TRPC1, 3, 6 and 7; Cx43), which have been suggested to play important roles in pacemaker activity. However, SCN5A, a TTX-resistant Na^+ channel known to be expressed in human ICCs, was below detectable levels. These data suggest that GIST cells appear to preserve some, but not all ionic mechanisms underlying pacemaker activity in ICC.

1. Introduction

There are small populations of cells which play important roles in numerous systems, and tumours of these cells would provide excellent opportunities to investigate the mechanisms underlying their function. Gastrointestinal stromal tumours (GIST), the most common mesenchymal tumours of the human gastrointestinal tract are thought to derive from interstitial cells of Cajal (ICC) [1], the putative pacemakers for gastrointestinal motility. A network of these cells is found in the myenteric plexus.

GIST are induced by mutation of the receptor tyrosine kinase, c-Kit [2,3]. In mice and guinea-pigs, c-Kit-immunoreactivity studies have identified the pacemaker activity of ICC [4,5]. However, only 'supporting' evidence has been presented for human ICC [6,7]. For example, in patients with diabetes mellitus, it has been reported that the number of ICC tends to decrease in the stomach, which might account for impaired gastric motility. Furthermore, in humans, the contribution of TTX-resistant Na^+ channels, in addition to voltage-operated Ca^{2+} channels, has been suggested for ICC pacemaking activity [8]. For clinical treatment of gastrointestinal dismotility

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including human, it is important to elucidate and/or confirm the mechanisms underlying gastrointestinal pacemaker activity. In the present study, using a duodenal GIST specimen, we have examined possible involvement of several mechanisms that have been proposed for ICC pacemaker activity.

2. Materials and methods

2.1. Cultured GIST cells

A duodenal specimen was obtained, under informed consent, from a 47-year-old male patient with duodenal GIST and synchronous multiple hepatic metastases. The GIST specimen was cut into small strips (1–2 mm in width, 10 mm in length). The strips were incubated overnight at 4 °C in a Ca^{2+}/Mg^{2+}-free solution containing digestive enzymes [collagenase (1–2 mg/ml; Wako Chemical, Osaka, Japan), disperse (500 PU/ml; Yakult, Tokyo, Japan); bovine albumin (16 mg/ml; Sigma, St. Louis, MO, USA)]; and then triturated with glass pipettes into an enzyme-free solution. The resultant isolated GIST cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% foetal bovine serum (Sigma) and antibiotics (streptomycin 30 μg/ml and penicillin 30 IU/ml; Sigma) at 37 °C for up to 3 weeks. Changes in cell shape were observed until day 16 of culture. In some preparations, expression of KIT was checked by staining with phycoerythrin (PE)-conjugated anti-human CD117 antibody (YB5.B8; eBioscience, San Diego, CA, USA) in 1/100–1/200 v/v.

2.2. Whole cell patch clamp and $[Ca^{2+}]_i$ measurements

Membrane current recordings and measurements of intracellular Ca^{2+} ($[Ca^{2+}]_i$) were carried out in cultured GIST cells using essentially the same methods as previously described for cultured cell cluster preparations [9,10]. Briefly, whole cell membrane currents were measured at room temperature in voltage-clamp mode using a patch clamp amplifier (Axopatch 200A: Axon Instruments, Foster City, CA, USA), and digitised through an AD/DA converter (TL-1; Axon Instruments). A cut-off frequency of 2 kHz was applied to reduce noise. The resistance of the patch pipette was 2.5–5 MΩ, when a K^+-rich pipette solution was used. For $[Ca^{2+}]_i$ measurements, the cultured GIST cells were incubated for 3–4 h in 'normal' solution containing 8 μM fluo-3/AM and detergents (0.02% Pluronic F-127, Dojindo; 0.02% cremophor EL; Sigma). A CCD camera system (Argus HiS-CA; Hamamatsu Photonics, Hamamatsu, Japan) combined with an inverted microscope (Axiovert S100TV; Zeiss, Germany) was used to continuously monitor digital images of fluo-3 emission light (excitation at 488 nm; emission light of 515–565 nm). GIST cells were maintained at 35 °C on a micro-warm plate (MP10DM; Kitazato Supply, Fujinomiya, Japan).

The composition of 'normal' bathing solution was as follows (mM): NaCl, 125; KCl, 5.9; $CaCl_2$, 2.5; $MgCl_2$, 1.2; glucose, 11.8; Hepes, 11.8; and pH adjusted to 7.4 with Tris base. The Ca^{2+}/Mg^{2+}-free solution, used for cell isolation, was made by iso-osmotic substitution with NaCl. The composition of the pipette solution was (mM): K-aspartate, 110; KCl, 20; $MgCl_2$, 4; EGTA (ethyleneglycol-bis-(β-aminoethylether) *N*,*N*,*N*′,*N*′-tetraacetic acid), 0.1; ATP, 4; GTP, 0.1; Hepes 20 (pH 7.2). Chemicals used were ATP (disodium salt) and GTP (trisodium salt) from Seikagakukogyo (Tokyo, Japan) and EGTA (free acid) from Sigma (St. Louis, MO, USA).

2.3. RT-PCR and sequencing

Total RNA was extracted from patient GIST, as previously reported [11]. ™II RNase H^- (Invitrogen, Carlsbad, CA, USA) and 200 μg/ml of random hexamer were used to reverse transcribe the RNA sample.

Real-time quantitative PCR was performed using Syber Green chemistry on an ABI 7000 sequence detection system (PE Biosystems). For PCR primers, see on-line Supplemental Table. Standard curves were drawn by regression analysis of the mean values for multiplex RT-PCR products of the $\log_{10}$-diluted cDNA. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as an endogenous standard. All experiments were performed in triplicate. The specificity of each PCR product (amplicon in each band) was confirmed by sequencing with the chain termination method (ABI PRIZM model 3100) (PE Biosystems, Hayward, CA, USA).

In separate examinations to determine the mutation site, PCR products for exon 11 of c-Kit were cloned and sequenced as previously described by [12]. The primers used were as follows: forward: 5′-CCA GAG TGC TCT AAT GAC TG-3′; reverse: 5′-ACT CAG CCT GTT TCT GGG AAA CTC-3′.

2.4. Statistics

Numerical data are expressed as means ± standard deviation (SD).

3. Results

Isolated cells were prepared by triturating duodenal GIST tissue after treating with digestive enzymes, and then kept in culture medium for 3 weeks. RNA was also extracted from the duodenum GIST, and cDNA was reverse transcribed. Sequencing of the PCR products revealed that the c-Kit proto-oncogene underwent in-frame deletion of 6 bp in the juxtamembrane (JM) domain of exon 11 (Table 1), which is known to be the most frequent gain-of-function mutation [2,12,13].

Fig. 1A shows changes in the shape of isolated GIST cells during culture. On day 1 after isolation only round cells were observed (Fig. 1A(a)), although thin short processes were observed on the cell surface just after isolation (not shown). The diameter was 20–40 μm (26.7 ± 6.9 μm, $n = 50$). After 1 week, isolated GIST cells became oval-shaped with longer and shorter axes with some having processes as long as the major axis of the cells (Fig. 1A(b) and (c)). After further culturing, GIST cells developed long and multiple processes (Fig. 1A(d)–(f)), and fusion of the processes between neighbouring GIST cells (Fig. 1A(e)) like the ICC network in the myenteric plexus. Most GIST cells showed c-Kit-immunoreactivity (Fig. 1B) even after day 12.

Whole cell membrane currents were recorded from isolated GIST cells cultured for 8–16 days, using a K^+-rich solution in the pipette. The capacitance of the GIST cell membrane was 30.4 ± 4.1 pF ($n = 6$). Fig. 2A shows an example of membrane currents elicited by rectangular voltage steps of