Selective labeling and isolation of functional classes of interstitial cells of Cajal of human and murine small intestine

Hui Chen,1 Doug Redelman,1,2,3 Seungil Ro,1 Sean M. Ward,1 Tamás Örđög,1 and Kenton M. Sanders1
1Department of Physiology and Cell Biology and 2Cytometry Center, University of Nevada, and 3Sierra Cytometry, Reno, Nevada

Submitted 31 March 2006; accepted in final form 16 August 2006

Selective labeling and isolation of functional classes of interstitial cells of Cajal of human and murine small intestine. Am J Physiol Cell Physiol 292: C497–C507, 2007. First published August 30, 2006; doi:10.1152/ajpcell.00147.2006.—Specific functions of interstitial cells of Cajal (ICC) have been linked to distinct classes that differ by morphology and distribution. In the small intestine, slow wave–generating ICC are located in the myenteric region (ICC-MY), whereas ICC that mediate neuromuscular neurotransmission occur either throughout the circular muscle layer (intramuscular ICC, ICC-DMP) or in association with the deep muscular plexus (ICC-DMP). Selective isolation of ICC to characterize specific properties has been difficult. Recently, neurokinin-1 receptors have been detected in murine ICC-DMP and neurons but not in ICC-MY. Here we identified and isolated ICC-DMP/IM by receptor-mediated internalization of fluorescent substance P and Kit immunofluorescence. Specificity of labeling was verified by confocal microscopy. Mouse and human ICC-DMP/IM were detected in suspension by fluorescent microscopy and harvested for RT-PCR with micropipettes. The isolated cells expressed Kit but not markers for neurons, smooth muscle, or antigen-presenting cells. ICC-DMP expressed neurokinin-1 receptor, M2 and M3 muscarinic receptors, P2Y1 and P2Y4 purinergic receptors, VIP receptor 2, soluble guanylate cyclase-1 subunits, and protein kinase G. L- or T-type Ca2+ channels were not detected in these cells. ICC-MY and ICC-DMP were simultaneously detected and enumerated by flow cytometry and sorted to purity by fluorescence-activated cell sorting. In summary, functional classes of ICC have distinct molecular identities that can be used to selectively identify and harvest these cells in their role in neuromuscular neurotransmission.

Kit; substance P; neurokinin-1 receptor; flow cytometry; RT-PCR

INTERSTITIAL CELLS OF CAJAL (ICC) develop from mesenchymal cells that are also the common precursors for smooth muscle cells (35). Specific ICC functions include the generation of electrical pacemaker activity that manifests in smooth muscles as electrical slow waves and contributes to segmenting and propagating (peristaltic) contractile activity (17, 34, 35). ICC also serve as an interface between the autonomic and enteric nervous systems and the smooth musculature by mediating efferent inputs to smooth muscle cells (15, 53) and, indirectly, to the pacemaker apparatus (15). ICC may also mediate afferent mechanical signals to vagal (9, 10) and enteric neuronal circuits (6, 11).

The functions of ICC are performed by distinct classes located in discrete regions of the gastrointestinal (GI) tunica muscularis. For example, in the small intestine pacemaker activity driving electrical slow waves is generated and propagated by multipolar ICC that form a two-dimensional network in the myenteric region (ICC-MY) (18, 22, 43, 49, 50, 52), whereas inhibitory and excitatory neurotransmission to smooth muscle cells is mediated by elongated ICC (intramuscular ICC, ICC-IM) within the circular muscle layer that form close synaptic contacts with varicose processes of enteric motor neurons (4, 15, 19, 47, 48, 51, 53). In the small bowel ICC-IM are often referred to as ICC-DMP as these cells are concentrated in the region of the deep muscular plexus (19, 48, 53). In humans, ICC-DMP and ICC-IM may coexist as separate classes (47), and it is unclear whether additional functions are provided by these cells. Mechanoreception is accomplished by ICC-IM in the stomach (54), and it is possible that these cells also contribute to afferent neural signaling in the stomach and rectum (see Refs. 6, 9, 10).

The cellular and molecular mechanisms of the functions of ICC are not fully understood. Molecular analysis of ICC is difficult because of the scarcity and widespread distribution of these cells in the tunica muscularis. Therefore, gene expression underlying specific ICC functions has either been inferred from physiological, pharmacological, and immunohistochemistry experiments or studied on a smaller scale, e.g., in a few isolated cells. Using an array of experimental approaches, we have proposed (23, 36, 52) a comprehensive model of electrical pacemaking by small intestinal ICC-MY that incorporates the dependence of slow wave activity on mitochondrial function and intracellular Ca2+ signaling. However, some aspects of this model have been challenged (1, 15, 37, 42, 44, 56), and the molecular identity of key ionic conductances associated with electrical pacemaking remains unclear. The molecular basis for the mediation of motor neurotransmission by ICC-IM and ICC-DMP is likely to involve expression of proteins that participate in the binding and transduction of neurotransmitters. For example, previous studies have shown that ICC express a variety of receptors, including peptide receptors, such as neurokinin (19, 25, 32, 46), somatostatin (41) and VIP receptors (7), M2 and M4 muscarinic receptors (7), and nucletide receptors (5). Expression of intracellular signaling intermediates such as protein kinases (30, 40) and cGMP (38, 55) is also consistent with a role for ICC-IM in mediating neuroeffector functions. Moreover, ICC-IM may also amplify the
efferent neuronal signals by producing intercellular signaling molecules, such as nitric oxide (33, 45), CO (27), and prostaglandins (31). Despite the division of labor between ICC-MY and ICC-IM (or ICC-DMP), certain receptors found in cells that mediate neurotransmission can also be found in pacemaker ICC that do not appear to be direct targets of neuroeffector signaling (7, 25).

Understanding how ICC accomplish specialized functions may be aided by large-scale analysis of gene expression. ICC are only a minor component of GI muscles, so general molecular analyses of the tunica muscularis are obscured by the expression patterns of other cell types. We recently reported techniques to identify murine ICC in cell suspensions (29). This technique is based on the detection of Kit, which is the receptor for stem cell factor and is an established immunohistological marker for ICC. Our approach permits enumeration of ICC by flow cytometry (FCM) from any part of the GI tract of purity by fluorescence-activated cell sorting (FACS) (16, 29).

Animals and tissue preparation. Adult and 6- to 14-day-old BALB/c mice were obtained from breeder pairs purchased from Charles River Laboratories (Wilmington, MA). The animals were anesthetized with isoflurane (AErrane; Baxter Healthcare, Deerfield, IL) inhalation and killed by decapitation. Mice were maintained and the experiments were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and the American Physiological Society’s “Guiding Principles in the Care and Use of Animals.” All protocols were approved by the Institutional Animal Care and Use Committee at the University of Nevada, Reno. The jejunum and ileum were excised and opened along the mesentery, and their contents were washed away with ice-cold Krebs Ringer buffer solution (KRB) (see Solutions). The mucosa and submucosa were removed by peeling, and only the tunica muscularis of the entire jejunum and ileum was used.

Human tissues. Human jejunal segments were obtained as surgical waste during gastric bypass surgeries for morbid obesity. The protocol was approved by the University of Nevada, Reno and the University of California, Davis Human Subjects Research Committees. The studies were conducted according to Declaration of Helsinki principles. Tissue strips 15 mm in length were cut parallel to the circular muscle fibers with a knife consisting of a pair of parallel scalpel blades set 1.5 mm apart. The tunica mucosa was removed, and the circular muscle layer and the myenteric region were separated by sharp dissection.

Vital labeling with fluorescent substance P. NK1R-expressing cells in murine and human tissues were labeled by receptor-mediated internalization of fluorescent substance P (SP) (19, 25). Murine tunica muscularis tissues were exposed to SP or Oregon Green 488-SP (OG488-SP, 1 μM; Molecular Probes, Eugene, OR), initially at 4°C for 1 h to allow the agonist to bind to cell surface receptors, followed by incubation at 37°C for 20 min to facilitate the internalization of the agonist-receptor complexes (19). Vital labeling of human tissues was performed similarly except that the jejunal tissue strips were incubated at 4°C for 4 h and at 37°C for 30 min. In some of these experiments, SP conjugated with Alexa Fluor 488 (AF488; Molecular Probes) was used instead of OG488-SP.

Immunohistochemistry. After vital labeling with OG488-SP, murine and human tissues were stretched to 110% of their resting length, fixed with 4% paraformaldehyde-saline (pH 7.40; 10 min at room temperature), and permeabilized with 0.3% (vol/wt) Triton X-100 (Sigma, St. Louis, MO; 1 h at 4°C). Nonspecific antibody binding was reduced by incubating the tissues in 1% (wt/vol) bovine serum albumin (BSA; Sigma; 1 h at room temperature). ICC in the murine and human tissues were labeled with monoclonal anti-Kit antibodies (anti-mouse Kit: clone 2B8, eBioscience, San Diego, CA; anti-human Kit: clone YB5.8, BD Pharmingen, San Diego, CA). The primary antibodies were applied at 5 μg/ml for 48 h at 4°C and visualized with Alexa Fluor 594-conjugated secondary antibodies (goat anti-rat IgG; Molecular Probes; 10 μg/ml, 1 h at room temperature). Specificity was verified by omitting either the primary or the secondary antibodies. Whole mounts were examined with a Zeiss LSM 510 META confocal microscope (Carl Zeiss Microimaging, Thornwood, NY).

For examination of NK1R internalization, whole mounts (5 × 10 mm) were pinned to Sylgard elastomer panels (1 × 10 × 15 mm) at 110% of resting length and width. Tissues were immersed in an organ bath and allowed to equilibrate in KRB (97% O2-3% CO2) at 37.5 ± 0.5°C for 60 min before experiments were initiated. Experiments were performed in N2-nitro-L-arginine (0.1 mM) and monensin (5 μM). After stimulation with SP (1 μM; 60 min at 4°C, followed by 20 min at 37°C), the muscles were fixed with Zamboni fixative for 1 h at room temperature, followed by wash with 0.01 M phosphate-buffered saline (PBS, pH 7.4) with 0.3% Triton X-100 overnight with several changes of the solution. Control tissues were not exposed to SP before fixation. Nonspecific antibody binding was reduced by incubation of the tissues in BSA (1% in PBS, Sigma) for 1 h at room temperature. Tissues were incubated with antibody to NK1R (rabbit polyclonal antiseraum; 1:2,000; Sigma S8305) diluted with PBS containing Triton X-100 (0.3%) for 48 h at 4°C.

Manual harvesting of ICC-DMP/IM identified by receptor-mediated uptake of fluorescent SP. NK1R-expressing cells in murine and human tissues were labeled by receptor-mediated internalization of OG488-SP and AF488-SP, respectively. Murine jejunum and ileum tunica muscularis tissues were equilibrated with cold nominally Ca2+- and Mg2+-free Hanks’ solution (see Solutions), transferred into collagenase-containing enzyme solution (see Solutions), and incubated, without stirring, at 37°C for 30 min. Tissue strips prepared from human jejunal circular muscles were incubated in enzyme solution overnight at 4°C and then at 37°C for 20–25 min. After three washes, the murine and human tissues were triturated through a series of three blunt pipettes of decreasing tip diameter. The resultant cell suspensions were counterstained with monoclonal antibodies against the common leukocyte marker CD45 tagged with R-phycoerythrin (PE)-conjugated (1:2,000; Sigma S8305) diluted with PBS containing Triton X-100 (0.3%) for 48 h at 4°C. Epifluorescent imaging was performed with a TILL Photonics (Gräfelfing, Germany) system consisting of a Polychrome IV monochromator, an Imago QE camera, and TILLvisION 4.1 software. For manual harvesting of labeled ICC, freshly dispersed cells were placed in glass-bottom dishes on a Nikon
Diaphot 2 inverted microscope equipped with fluorescence and phase-contrast optics. Cells with ICC-DMP/IM-like morphology and green fluorescence were sucked into large-diameter, fire-polished micropettes made from borosilicate capillaries and mounted in a micromanipulator (7). Fifty to eighty cells were pooled for each RT-PCR experiment.

**FCM and FACS.** ICC in murine jejunum and ileum tunica muscularis tissues were labeled vitally by incubating with R-PE-conjugated rat monoclonal anti-mouse Kit antibody (clone ACK2, 1 μl/ml KRB; eBioscience) at 4°C for 3 h. NK1R-expressing cells were labeled by receptor-mediated internalization of OG488-SP as described above. After equilibrating with cold nominally Ca²⁺- and Mg²⁺-free Hanks’ solution (see Solutions), the tissues were transferred into collagenase-containing enzyme solution (see Solutions) and incubated, without stirring, at 37°C for 30 min. After three washes, the tissues were triturated through a series of three blunt pipettes of decreasing tip diameter. The resultant cell suspensions were sedimented by centrifugation (300 g; 5 min), resuspended in 1 ml of Ca²⁺- and Mg²⁺-free Hanks’ solution containing 5% FBS (GIBCO Invitrogen, Grand Island, NY), and filtered through a polyester filter with 30-μm mesh size (Miltenyi Biotec, Auburn, CA) to obtain single-cell suspensions. Labeling of ICC in these suspensions was reinforced with 0.2 μg of PE-ACK2 (eBioscience). In some experiments, PE-ACK2 was replaced with PE-2B8 (0.5 μg), which reacts with a different extracellular epitope of Kit but recognizes the same complement of Kit⁺ cells (unpublished observation). Macrophages and dendritic cells that may take up fluorescent labels nonspecifically and mast cells that also express Kit but are strongly CD45 immunopositive were identified with antibodies labeled with PC5 tandem conjugates of rat monoclonal (IgG2b) anti-mouse F4/80 (clone: CL-3; 1.5 μg; CALTAG, Burlingame, CA), rat monoclonal (IgG2b) anti-mouse CD11b (clone M1/70.15, 0.5 μg; CALTAG), and rat monoclonal anti-mouse CD45 (Ly-5 or leukocyte common antigen; clone 30-F11; 0.2 μg; eBioscience) (16, 29). Fibroblasts and endothelial cells that may also contaminate sorted ICC populations were labeled with biotin-anti-CD34 (clone RAM34, 0.5 μg; BD Pharmingen). The cells were incubated with the above antibodies for 30 min at 4°C and then washed, centrifuged (300 g; 5 min), resuspended in 1 ml of Ca²⁺ and Mg²⁺-free Hanks’ solution: 125 mM NaCl, 5.9 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 15.5 NaHCO₃, 1.2 NaH₂PO₄, 11.5 glucose, pH 7.3–7.4 when bubbled with 97% O₂ and 3% CO₂; Ca²⁺- and Mg²⁺-free Hanks’ solution: 125 mM NaCl, 5.36 KCl, 15.5 NaHCO₃, 0.336 NaH₂PO₄, 0.44 KH₂PO₄, 10 glucose, 2.9 sucrose, and 11 HEPES adjusted to pH 7.2 with NaOH; and enzyme solution: collagenase (1.3 mg/ml, Worthington type II; Worthington Biochemical, Freehold, NJ), BSA (2 mg/ml), trypsin inhibitor (2 mg/ml), and ATP (0.27 mg/ml) (all from Sigma) in Ca²⁺- and Mg²⁺-free Hanks’ solution.

**RESULTS**

We first examined whether NK1R-mediated internalization of fluorescent SP could be utilized for the selective vital labeling of functional ICC classes in the murine jejunum and ileum and in the human jejunum (Figs. 1 and 2). ICC in these tissues were identified by Kit immunohistochemistry. Consistent with previous reports (17, 34, 35, 53), Kit-specific immunofluorescence in the murine small intestines (n = 12) was detected in two distinct classes of ICC, namely, ICC-DMP and ICC-MY. ICC-DMP were identified as elongated, occasionally branching cells with ovoid cell bodies and little perinuclear cytoplasm. Two main processes occasionally divided into fork-like secondary branches. ICC-DMP lay in parallel to the axis of the circular smooth muscle cells at the level of the deep muscular plexus (Fig. 1A). ICC-MY were identified as multipolar cells forming dense two-dimensional networks in the myenteric region, enveloping ganglia and nerve trunks (Fig. 1, D and G). In both the jejunum and ileum, ICC-DMP uniformly internalized OG488-SP, and all cells with ICC-like morphology that displayed OG488-SP fluorescence were positive for Kit (Fig. 1, B and C). In addition to ICC-DMP, OG488-SP was also detected in nerve fibers (arrows in Fig. 1, B and C) running parallel to circular smooth muscle cells and ICC-DMP and establishing close appositions with the latter. Rarely, we also detected OG488-SP uptake by ICC-DMP in any part of the jejunum (Fig. 1, D–F) or ileum (Fig. 1, G–I). OG488-SP was also internalized by ICC-MY.
ized by a small population of cells in the myenteric ganglia (Fig. 1, H and I). Figure 1, J and K, show images of NK1R labeling before and after stimulation with SP. Before stimulation NK1R-like immunoreactivity (NK1R-LI) was concentrated mostly along the peripheries of ICC-DMP. As previously shown (19), stimulation with SP caused internalization of NK1R and receptor labeling was concentrated in granular structures within cells. A similar pattern of labeling in ICC-DMP was observed after incubation of tissues with OG488-SP (Fig. 2).

In the human small intestine, Kit+ ICC occur in the myenteric region (ICC-MY) and within the smooth musculature (ICC-DMP/IM), including longitudinal and circular muscle layers, intramuscular septa, and near the submucosal border of the circular muscle layers (47). Some authors have reported that ICC-DMP (ICC at the inner border of the circular muscle layer) do not express Kit (47); however, we and others (discussed in Ref. 47) have been successful at labeling ICC-DMP with Kit antibodies. In whole mounts prepared from tissue strips containing the bulk of the circular muscle layer (n = 5), we detected Kit immunofluorescence in ICC with two main processes occasionally dividing into forklike branches (Fig. 2A). These cells may have been ICC-IM, ICC-DMP, or ICC in intramuscular septa. In the tissue strips containing the myenteric region (n = 5), Kit immunofluorescence was present in multipolar, network-forming ICC (ICC-MY; Fig. 2D). Similar to murine ICC, human ICC-DMP/IM, but not ICC-MY, internalized OG488-SP (compare Fig. 2, B and C, with Fig. 2, E and F). Unlike murine ICC-DMP, not all cells in the human jejenum contained resolvable amounts of OG488-SP fluorescence (Fig. 2, B and C). Uptake of OG488-SP was also detected in some nerve fibers (Fig. 2, E and F).

We next attempted to identify ICC-DMP/IM in cell suspensions prepared from murine tunica muscularis (n = 8; Fig. 3, A–C) and human circular muscle strips (n = 3; Fig. 3, D–F). Cells with functional NK1R were labeled in whole mount preparations by receptor-mediated internalization of OG488-SP (murine tissues) or AF488-SP (human tissues). In both preparations, cells with SP-associated fluorescence were slender and bipolar (presumed ICC-DMP/IM; Fig. 3, A and D) or oval shaped (presumed to be neurons; Fig. 3, B and E). Neither type of cell was labeled with antibodies against the common leukocyte marker CD45 (not shown). We harvested 50–80 OG488-SP-positive cells from each suspension and analyzed them for the expression of cell-specific markers by RT-PCR. Kit mRNA was detected in each cell population.
examined, but we did not detect mRNAs for the neuronal marker protein gene product 9.5, the macrophage/dendritic cell marker CD68, or the smooth muscle marker myosin heavy chain 11 (Fig. 3, C and F). These tests identify the OG488-SP-positive cells as ICC-DMP/IM.

We examined the expression of various neurotransmitter receptors, intracellular signaling intermediates, and Ca\(^{2+}\) channels considered important for normal GI motility in three of the murine ICC-DMP populations with verified purity by RT-PCR and compared the results with the expression pattern of whole small intestinal muscles (Fig. 4). RNA extracted from mouse brain was used as positive control. Of the neurotransmitter receptors examined, purified ICC-DMP expressed mRNA encoding for NK1R, M\(_2\) and M\(_3\) muscarinic acetylcholine receptors, P2Y\(_1\) and P2Y\(_4\) purinergic receptors, and VIP receptor 2. Of the other receptors present in whole muscles, ICC-DMP did not express neurokinin-2 or -3 receptors, M\(_4\) muscarinic, or P2Y\(_2\) purinergic and P2Y\(_6\) pyrimidinergic receptors at detectable levels. We found no evidence of mRNA for M\(_5\) muscarinic receptors or VIP receptor 1 in whole small intestinal muscles or in ICC-DMP. ICC-DMP also expressed the nitrergic signaling intermediates soluble guanylate cyclase-1 α\(_3\)-and β3-subunits, and both type I and type II cGMP-dependent protein kinase. Importantly, we did not detect mRNA encoding for either L- or T-type Ca\(^{2+}\) channels in ICC-DMP populations despite abundant expression in whole muscles.

We also attempted to identify and enumerate murine ICC-DMP and ICC-MY in suspension by FCM and investigated the feasibility of harvesting these cells by FACS in large quantities for detailed expression profiling (Fig. 5). Cells with functional NK1R were identified by receptor-mediated internalization of OG488-SP. ICC were labeled with PE-conjugated anti-Kit monoclonal antibodies (PE-ACK2). Macrophages and dendritic cells that may take up fluorescent labels nonspecifically, mast cells that also express Kit but are strongly CD45 positive, and fibroblasts and endothelial cells that may also contaminate the sorted populations were identified by a cocktail of monoclonal antibodies tagged with PC5 tandem conjugates. After exposure to SP (1 μM; 60 min at 4°C, followed by 20 min at 37°C; E), NK1R-LI was concentrated into small granular structures within the cytoplasm of ICC-DMP (arrowheads). Fluorescence labeling was similar in intestinal muscles exposed to OG488-SP (1 μM; 60 min at 4°C, followed by 20 min at 37°C; F). These observations suggest that OG488-SP was taken up by and internalized with NK1R. Scale bar in J applies to panels A–I; scale bar in L applies to J–L.

Fig. 1. Identification of substance P (SP)-internalizing cells in the murine small intestinal tunica muscularis. In a previous study (19) we showed that deep muscular plexus interstitial cells of Cajal (ICC-DMP) internalize SP receptors when the cells are stimulated with SP or neurally released neurokinins. Here we used internalization of functional neurokinin-1 receptors (NK1R) as a means of identifying ICC-DMP in cell suspensions. Cells with NK1R were identified in whole mounts by receptor-mediated internalization of Oregon Green 488-SP (OG488-SP, green color; B, E, and H). After paraformaldehyde fixation, ICC were labeled with monoclonal antibodies to Kit (red color; A, D, and G). C, F, and I are digital composites in which shades of yellow-orange signify colocalization of OG488-SP and Kit-like immunoreactivity. A–C: representative confocal images of the deep muscular plexus region of the murine jejunal tunica muscularis. Note colocalization of intracellular OG488-SP and Kit in ICC-DMP. OG488-SP-positive cells are stimulated with SP or neurally released neurokinins. Cells of Cajal (ICC-DMP) internalize SP receptors when the peripheries of ICC-DMP (arrows). After exposure to SP (1 μM; 60 min at 4°C, followed by 20 min at 37°C; K), NK1R-LI was concentrated into small granular structures within the cytoplasm of ICC-DMP (arrowheads). Fluorescence labeling was similar in intestinal muscles exposed to OG488-SP (1 μM; 60 min at 4°C, followed by 20 min at 37°C; L). These observations suggest that OG488-SP was taken up by and internalized with NK1R. Scale bar in J applies to panels A–I; scale bar in L applies to J–L.
lacking hematopoietic and fibroblast markers could be selectively identified by the presence and absence, respectively, of OG488-SP fluorescence. ICC-DMP detected in this manner represented 21.1 ± 3.9% (n = 8) of all ICC. Median PE-ACK2 fluorescence intensities of ICC-DMP were significantly higher than those of ICC-MY (19.8 ± 7.6 vs. 10.6 ± 4.3 fluorescence units; P = 0.009, n = 8). However, this difference was due to a greater heterogeneity of Kit expression in ICC-MY compared with ICC-DMP rather than a true separation of these two ICC subsets in terms of their Kit expression. For example, ICC-MY included many cells with Kit expression comparable to that measured on ICC-DMP in addition to the cells with considerably lower levels of Kit producing lower overall median fluorescence intensity values (Fig. 5C). The clusters of ICC-

Fig. 2. Identification of SP-internalizing cells in the human small intestinal tunica muscularis. A–C: representative confocal images of a circular muscle strip from human jejunal tunica muscularis. Most, but not all, ICC-DMP/intramuscular ICC (ICC-IM) contained detectable amounts of OG488-SP. D–F: representative confocal images of human jejunal muscle strip containing the myenteric region. Only intramuscular nerve fibers, but not ICC-MY, were positive for OG488-SP. Scale bar in F applies to all images.

Fig. 3. Identification by fluorescent microscopy and manual harvesting of murine ICC-DMP and human ICC-DMP/IM in suspension. Cells expressing functional NK1R were identified in whole mounts by receptor-mediated internalization of OG488-SP (in murine tissues) or Alexa Fluor 488-SP (AF488-SP; in human tissues) before enzymatic dissociation. A–C: murine cells. A: an elongated, OG488-SP+ cell (presumed ICC-DMP) in suspension. B: an oval-shaped, OG488-SP+ cell (presumed neuron). Both cells were negative for the hematopoietic marker CD45 (not shown). C: RT-PCR analysis of presumed ICC-DMP (50 ≤ n ≤ 80) harvested by sucking the cells into capillary micropipettes. In each of the 8 independently performed experiments we could only detect mRNA for the housekeeping gene β-actin (Actb) and the ICC marker Kit but not for the neuronal marker protein gene product 9.5 (UCHL1), the macrophage/dendritic cell marker CD68 (C68), or the smooth muscle marker myosin heavy chain 11 (MYH11). D–F: human cells. D: a bipolar, AF488-SP+ cell (presumed ICC-DMP/IM). E: an oval-shaped, AF488-SP+ cell (presumed neuron). Both cells were negative for the hematopoietic marker CD45 (not shown). F: RT-PCR analysis of presumed ICC-DMP/IM (50 ≤ n ≤ 80) harvested by sucking the cells into capillary micropipettes. Similar to the results obtained with murine cells, we could only detect mRNA for the housekeeping gene GAPDH and the ICC marker KIT but not for the neuronal marker protein gene product 9.5 (UCHL1), the macrophage/dendritic cell marker CD68, or the smooth muscle marker myosin heavy chain 11 (MYH11). A representative of 3 independent experiments is shown. Scale bars in B and E also apply to A and D, respectively.
**Isolation of Functional Classes of ICC**

**Fig. 4.** Detection by RT-PCR of mRNA encoding for neurotransmitter receptors, intracellular signaling intermediates, and Ca\(^{2+}\) channels considered important for normal gastrointestinal motility in murine ICC-DMP. Cells were identified in suspension on the basis of their internalization of OG488-SP and elongated or bipolar morphology and sucked into capillary micropipettes (right). Left: results obtained in whole small intestinal muscles used as control. Representative results from 3 independent experiments are shown. Purified ICC-DMP expressed mRNA encoding for NK1R (Tacr1), M3 muscarinic acetylcholine receptors (Chrm2 and Chrm3), P2Y1 and P2Y4 purinergic receptors (P2ry1 and P2ry4), and VIP receptor 2 (Vipr2). ICC-DMP also expressed soluble guanylate cyclase-1 α3-and β3-subunits (Gucy1a3 and Gucy1b3) and both type I and type II cGMP-dependent protein kinase (Prkg1 and Prkg2). ICC-DMP did not express either L- or T-type Ca\(^{2+}\) channels.

**DISCUSSION**

ICC lie within and between smooth muscle layers throughout the GI tract and are usually categorized according to their location within the tunica muscularis (e.g., ICC-MY, ICC-IM, ICC-DMP) (34, 35). Functionally, ICC can be classified as cells that generate electrical pacemaker activity (slow waves) (15, 17, 22, 23, 36, 43) or cells that serve as an interface between the enteric nervous system and the smooth musculature by mediating motor neurotransmission (17, 53). ICC-IM also transduce mechanical signals such as stretch (54) and may also provide input to afferent neural pathways (6, 9–11). Assignment of specific functions to distinct morphological classes has been accomplished by studying tissues that lack specific types of ICC either naturally (e.g., the gastric fundus lacks ICC-MY) or as a result of mutations in the stem cell factor/Kit signaling pathway (6, 9–11, 15, 17, 18, 34, 35, 49, 50, 53). From such experiments it is now generally accepted that pacemaker ICC are primarily located between, or on the surface of, smooth muscle layers, e.g., in the myenteric region, in intramuscular septa, or on the submucosal surface of the circular muscle layer. On the other hand, ICC that mediate communication between enteric motor neurons and smooth muscle cells (i.e., ICC-IM) are closely associated with neural processes and lie within muscle bundles (34, 35, 53). However, despite differences in morphology, distribution, and function, members of ICC classes share many common characteristics including some ultrastructural features and expression of Kit, neurotransmitter receptors, signaling intermediates, and ion channels (7, 25, 27, 30, 40, 45). The division of labor concept is further blurred by recent findings suggesting that ICC-IM may generate pacemaker activity in the guinea pig and murine gastric antrum (8, 13, 14). The latter findings may suggest that spontaneous activity may be a feature of ICC-IM or ICC-DMP, and the irregular, nifedipine-sensitive Ca\(^{2+}\) action potentials that replace and partially compensate for the lack of slow waves in W/W\(\text{v}\) and Sl/Sl\(\text{d}\) mice lacking ICC-MY might originate in ICC-DMP (17, 18, 49). Understanding how ICC classes perform their specialized functions and what distinguishes the various ICC classes will ultimately require large-scale analysis of gene expression. Although we have recently developed a FACS-based approach to identify and harvest murine ICC in great numbers and at a high degree of purity sufficient for genomics analyses, this technique, which is based on the detection of Kit (16, 28, 29), is not able to distinguish between ICC classes from the same tissue. In this study we demonstrated a technique that allows specific labeling of ICC-DMP/IM and ICC-MY and enumeration and purification of these cells from murine and human muscles. This technique will allow more exacting studies to determine phenotypic differences between ICC-MY and intramuscular classes of ICC.

Our approach was based on previous findings that ICC-DMP and a subpopulation of myenteric neurons express NK1R, whereas expression of this receptor was not detected on ICC-MY (19, 46). NK1R is the preferred receptor for the excitatory enteric neurotransmitter SP, and SP-containing enteric neurons have been shown to functionally and preferentially innervate ICC-DMP in both mice and guinea pigs (19, 25, 32, 46). Thus it is likely that a portion of the noncholinergic excitatory response to SP is mediated via ICC-DMP. Exposure of murine small intestinal muscles to exogenous SP caused NK1R internalization in myenteric neurons and ICC-DMP.

**Fig. 5.** Tunica muscularis and ICC-DMP. (A) Tunica muscularis; (B) ICC-DMP; (C) Tunica muscularis; (D) ICC-DMP. FACS (n = 3) in high-purity mode resulted in up to ~12,000 ICC-DMP and ~55,000 ICC-MY per small intestine. The sorted populations had excellent purity, which was verified by ICC-DMP and FACS (Fig. 5) by FACS using a sort mode that favors recovery over purity and then reanalyzing the sorted cells (Fig. 5D). Final sorting of Kit\(^{+}\)OG488-SP\(^{+}\) and Kit\(^{+}\)OG488-SP\(^{-}\) cells by FACS (n = 3) in high-purity mode resulted in up to ~12,000 ICC-DMP and ~55,000 ICC-MY per small intestine. The sorted populations had excellent purity, which was verified by FCM analysis (Fig. 5, E and F) and/or RT-PCR (Fig. 5G). For example, the cells analyzed in the experiment shown in Fig. 5G only expressed mRNA for Kit (ICC-MY) or Kit and NK1R (ICC-DMP) but not markers for smooth muscle cells, glia, neurons, mast cells, macrophages/dendritic cells, and fibroblasts/endothelial cells.

**Legend**

**A** Tunica muscularis

**B** ICC-DMP

**C** Tunica muscularis

**D** ICC-DMP

**E** Tunica muscularis

**F** ICC-DMP

**G** Tunica muscularis

**H** ICC-DMP

**I** Tunica muscularis

**J** ICC-DMP

**K** Tunica muscularis

**L** ICC-DMP
Fig. 5. Detection by flow cytometry (FCM) and purification by fluorescence-activated cell sorting (FACS) of ICC-MY and ICC-DMP of the murine jejunum and ileum. See MATERIALS AND METHODS for details on labeling and preparation of small intestinal cell suspensions. A: gates used for selecting cells with light scatter properties characteristic of live cells (LS cells) for further analysis. B: gates used for the isolation of cells that do not express hematopoietic markers (the macrophage markers F4/80 and CD11b and the general hematopoietic marker CD45) or the fibroblast/endothelial marker CD34 on their surface (PC5− cells). C: identification of Kit SP+ presumed ICC-DMP and Kit SP+ presumed ICC-MY in PC5− LS cells on the basis of their OG488-SP uptake and Kit immunofluorescence detected with PE-ACK2. Diagonal line separates Kit ICC and the remaining Kit− cells (mainly smooth muscle cells, neurons, and glia). The numbers of ICC-DMP and ICC-MY identified in this projection were divided by the number of LS cells in A to obtain cell frequencies. D: enrichment of ICC populations by sorting Kit+ ICC and the remaining Kit− cells (i.e., cells right of diagonal line) with a sort mode that favors recovery over purity. Note improved definition of Kit SP+ and Kit SP− clusters. E and F: verification by FCM of the purity of ICC-MY (Kit SP− cells; E) and ICC-DMP (Kit SP− cells; F) sorted in high-purity mode by FACS using the gates shown in D. G: verification by RT-PCR of the purity of ICC-MY (Kit SP− cells) and ICC-DMP (Kit SP+ cells) sorted as shown in A–F. Note that ICC-MY only expressed Kit, while ICC-DMP only expressed Kit and NK1R (Tacr1) but not markers for smooth muscle (Myh11), glia (Gfap), neurons (Uchl1), mast cells (Mcpt6), macrophages/dendritic cells (Cd68), or fibroblasts/endothelial cells (Cd34). Size markers (from bottom up): 100, 200, 300 bp. Note that the sequence of Kit and Tacr1 was accidentally reversed in the case of the Kit SP+ cells; dashed horizontal line indicates the position of the Kit band.
In contrast, SP exposure did not reveal NK1R-like immunoreactivity in ICC-MY. Here we have used receptor internalization to load ICC-DMP (and possibly ICC-IM in the human) with a fluorescent tag, and among Kit-positive cells we could detect internalization of fluorescent SP only in ICC-DMP and not in ICC-MY. Identical results were obtained for jejunal cells (Fig. 1). In both parts of the small intestine, myenteric neuromuscular and intramural nerve fibers, and, very rarely, smooth muscle cells also internalized the fluorescent SP but they could be easily distinguished from ICC by their lack of Kit immunoreactivity. The use of receptor-mediated uptake of fluorescent SP coupled with Kit immunolabeling permitted the selective and quantitative detection of ICC-DMP/IM.

Little is known about ICC in human GI muscles, and most of the functional conclusions about the role of ICC have been deduced from studies of animal models. Thus we attempted to extend the validity and usefulness of our experiments to develop techniques that might make it possible in the future to assess the molecular apparatus of human ICC. SP is also a major excitatory neurotransmitter in the human gut (12). Cells with ICC-like morphology have been shown to express NK1R or bind radioactive SP throughout the human GI tract (24, 26, 39). In circular muscles of the human jejunum we observed internalization of fluorescent SP in ICC-DMP/IM and nerve fibers but not in ICC-MY or smooth muscle cells (Fig. 2). At the present time we are unable to report that uptake of SP was exclusively by ICC-DMP, and we do not yet have a good means to separate ICC-IM and ICC-DMP from human muscles. More work will be necessary to develop a technique to label these cells specifically. Another problem encountered with human cells was incomplete labeling of all ICC-DMP/IM. This may have been due to technical problems, such as poor penetration of the labeled SP, or nonuniform expression of NK1R on ICC-DMP/IM. Thus, while it is possible to achieve selective identification of some of the ICC-DMP/IM from human jejunum, these findings suggest that the technique we have developed for separation of murine ICC is unsuitable, under present experimental conditions, for the selective detection and harvesting of human ICC-MY. Thus we concentrated molecular studies and further development and refinement of selection techniques on studies of murine cells.

As a first step toward selective harvesting of small intestinal ICC for molecular analyses, we collected murine ICC-DMP and human ICC-DMP/IM from cell suspension with the aid of fluorescent microscopy (Fig. 3). Cells were selected on the basis of their internalization of fluorescent SP and elongated or bipolar morphology. Without exception, each batch of 50–80 cells was found to express mRNA for the ICC marker Kit but not for the neuronal marker protein gene product 9.5 (Uchl1), the smooth muscle marker myosin heavy chain (Myh11), or the macrophage/dendritic cell marker CD68, indicating that receptor-mediated uptake of fluorescent SP combined with microscopic assessment of cell morphology is sufficient for the selective detection of mouse ICC-DMP and human ICC-DMP/IM in suspension.

In three independently harvested populations of murine ICC-DMP we examined mRNA encoding for neurotransmitter receptors, intracellular signaling intermediates, and Ca$^{2+}$ channels considered important for normal GI motility (Fig. 4). Consistent with previous reports (7, 19, 25, 32, 46), we detected expression of M2 and M3 muscarinic receptors (Chrm2 and Chrm3) and NK1R (Tacr1), i.e., receptors for enteric excitatory neurotransmitters in the GI tract. We did not, however, detect mRNA for tachykinin-3 receptors, as previously reported for ICC-IM and ICC-MY harvested from dispersed murine fundic and small intestinal muscles, respectively (7). Previous reports have also identified ICC as targets of inhibitory neuromuscular neurotransmission (53). For example, ICC have been reported to express VIP receptor 2 (7) and cGMP (38, 55), a second messenger synthesized in response to binding of nitric oxide to soluble guanylyl cyclase (21). In the present study we detected mRNA for purinergic P2Y4 and pyrimidinergic P2Y4 receptors in isolated ICC-DMP. To our knowledge, this is the first report of P2Y receptor isoforms in ICC, and the functional significance of these findings may be linked to the initial component of the inhibitory junction potentials in GI muscles. In contrast with results of Epperson et al. (7), we could detect only VIP receptor 2, but not VIP receptor 1, in either ICC-DMP or whole small intestinal muscles. The reason for this discrepancy is unclear, but it is important to mention that murine colonic muscles also appear to express type 2, rather than type 1, VIP receptors (2). Consistent with a role for ICC in nitricergic signaling (53), we detected mRNA encoding isoforms of soluble guanylyl cyclase-1 (Gucy1a3, Gucy1b3) and cGMP-dependent protein kinase (Prkg1, Prkg2) in isolated ICC-DMP. In contrast, and despite abundant expression in whole muscles, we found no evidence of expression by ICC-DMP of either T-type Ca$^{2+}$ channels (Cacna1g, Cacna1h, and Cacna2d2a subunits), which play a role in electrical pacemaking by ICC-MY (20), or L-type Ca$^{2+}$ channels (Cacna1c and Cacna1d subunits), which are important for smooth muscle contractile activity. Thus our observations are consistent with a role for ICC-DMP as mediators of excitatory and inhibitory neuromuscular neurotransmission (51) but make it very unlikely that ICC-DMP could be the origin of nifedipine-sensitive Ca$^{2+}$ action potentials in W/WV and Sl/Sld mice lacking ICC-MY (see Refs. 17, 18, 49).

We reported previously (3) that ICC-MY of the fundus can generate basic electrical rhythmicity (called unitary potentials), but these events do not regenerate and organize into slow waves. In this study we speculated that ICC-MY may lack the voltage-dependent means of coordinating pacemaker activity, which occurs via voltage-dependent Ca$^{2+}$ channels. A lack of voltage-dependent Ca$^{2+}$ channels in ICC-DMP is consistent with the inability of these cells to generate (or organize) electrical rhythmicity in the murine small intestine (50).

Using the new separation technique for ICC-DMP and ICC-MY, we attempted to detect, quantify, and purify these two classes of murine ICC in cell suspensions. FCM analysis indicated that after dead and potentially contaminating cells (macrophages, dendritic cells, mast cells, fibroblasts, and endothelial cells) were excluded Kit$^+$ cells could be separated into Kit$^+$/SP$^-$ and Kit$^+$/SP$^+$ populations. Kit$^+$ ICC represented 7.1 ± 1.2% of all cells in suspensions of cells from the tunica muscularis. Consistent with immunohistochemical observations, ICC-DMP represented a considerably smaller fraction of total ICC (21.1 ± 3.9%) than ICC-MY. Although ICC-MY had significantly lower median Kit fluorescence intensities than ICC-DMP, this difference was due to a marked heterogeneity of Kit expression by ICC-MY and the two ICC subsets could not be distinguished by Kit immunofluorescence alone. The relative fractions of ICC classes varied little between experi-
ments (n = 8), indicating that quantitative assessment of ICC populations can be considered a reliable tool for assessing pathological changes in ICC populations in disease models (see Ref. 29 for review), and this might become a diagnostic tool for the assessment of the involvement of ICC in human motility disorders. In addition to FCM analysis, we have also purified ICC-MY in large quantities (up to ~12,000 and ~55,000 per small intestine, respectively) and at high levels of purity necessary and sufficient for large-scale analysis of their gene expression profiles. These results verify our hypothesis that ICC-DMP and ICC-MY of the murine small intestine can be selectively identified and sorted on the basis of receptor-mediated uptake of labeled SP and Kit immunofluorescence. This may help in the development of genetic fingerprints for specific classes of ICC that might be adversely affected in various human motility disorders.

ACKNOWLEDGMENTS
We thank Dr. Neal Fleming, Dept. of Medicine, University of California, Davis Medical Center, for providing human jejunal segments from gastric bypass surgeries.

GRANTS
This work was supported by NIH Program Project Grant DK-41315. T. Ördo¨g and D. Redelman also received support from Grant DK-58185. Core laboratories (i.e., immunofluorescence and FACS) were supported by Grant DK-41315. The University of Nevada, Reno Cytometry Center was supported, in part, by Nevada Biomedical Research Infrastructure Network Grant P20/RR-16464 from the NIH.

REFERENCES


