Preservative solution for freeze-storage of surgically excised human colon to enable study of smooth muscle function in vitro

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Abstract

We have compared the reactivity to carbachol and high potassium of circular smooth muscle isolated from segments of human colon which was freeze-stored in different preservative solutions for more than one month following surgical resection. Concentration-dependent contractions in response to carbachol were reduced in terms of both their sensitivity (pEC₅₀) and reactivity (Emax), depending on the preservative solutions used. Similar reduction of reactivity to 100 mM KCl was also observed. The best responsiveness was shown when the tissue was freeze-stored in SFM101. It is concluded that the freeze-storage of surgically excised human colon in SFM101 or phosphate buffer solution for more than one month provided the best preservation of smooth muscle function for in vitro pharmacological examination.

Key words: human colon, contraction, freeze-storage, cryopreservative solution, carbachol

Introduction

Gastrointestinal smooth muscle is widely used for pharmacological studies of its autonomic innervation, receptor systems and ion channels (Costa et al., 1986; McKirdy, 1988; Rehfeld, 1998). The distribution of autonomic nerves, receptors and ion channels, however, differs from species to species and along the length of the intestinal canal in each species (Kamikawa et al., 1985; Dressman, 1986; Büllbring and Tomita, 1987; Percy et al., 1990; Kerr et al., 1995; Glavind et al., 1997; Okamura et al., 1998; Uchida et al., 1998). Therefore, it is desirable to be able to use human intestinal tissue for the testing of newly developed therapeutic agents for the treatment of gastrointestinal disorders such as peptic ulcer, gastric distress and irritable bowel syndrome (Hoyle et al., 1990; Bennett et al., 1992; MacDonald et al., 1996; De Ponti and Malagelada, 1998).
Surgically excised human intestinal specimens need to be delivered to the pharmacological laboratory as soon as possible after immersion in a suitable preservative solution. Our previous paper demonstrated that Dulbecco’s Ca\(^{2+}\)- and Mg\(^{2+}\)-free phosphate buffer solution (PBS) is the most suitable preservative solution for cold-storage of isolated human colon in a refrigerator at 4°C (Kamikawa et al., 2000). However, this cold-storage was limited to within 3 days. Valuable human specimens need to be stored in a tissue bank to supply a number of investigators over a longer period of time. Therefore, it is desirable to develop freeze-storage of intestinal smooth muscle tissues for longer periods of time.

The aim of the present study was to compare the motor reactivity of segments of isolated human colon which was freeze-stored in different preservative solutions for periods of a month or more.

### Materials and Methods

The study was approved by the Dokkyo University School of Medicine Ethics Committee (No. 1223). Written informed consent was obtained from all subjects before participation. Macroscopically normal segments of human distal colon, 2 cm in the longitudinal axis and 5 cm in the circular axis, were obtained immediately after removal from patients undergoing resection for colon cancer (n=48, 28 male and 20 female patients with an age averaging 66.8 ± 1.5 years). The tissue was pre-washed with and immersed into PBS (4°C) and quickly delivered to the laboratory. The mucosa was removed and a muscle strip approximately 3 mm wide and 12 mm long was cut parallel to the circular muscle. Three to five strips were taken from each human colon segment. The fresh circular muscle strip thus obtained was immersed in a 2 ml cryotube filled with one of the preservative solutions.

The commercially available preservative solutions for freeze-storage tested here were Cell Banker 2 (NZK Biochemicals, Fukushima, Japan), Cellvation (Celox Laboratories, MN, USA), Cryoprotective (Bio Whittaker, MD, USA), SFM101 (Nissui Pharmaceuticals, Tokyo, Japan), OPTE-MEM (Invitrogen Corporation, NY, USA) and Dulbecco’s Ca\(^{2+}\)- and Mg\(^{2+}\)-free phosphate buffer solution (Nissui Pharmaceuticals, Tokyo, Japan). Some of the cryopreservative solutions were supplemented with 5–20% dimethyl sulphoxide (DMSO). The cryotube was transferred to the program freezer (Nihon Freezer TNP 87S, Tokyo, Japan) and frozen down to –80°C over a period of 12 hours. Then the frozen cryotube was stored in an ultra-low temperature freezer (Nihon Freezer CL-322U, Tokyo, Japan) at –80°C for a period of between 30 and 63 days. Upon removal from the freezer, the frozen cryotube was quickly warmed in a water bath at 37°C. The colonic strip was mounted in a 10 ml organ bath filled with Krebs bicarbonate solution at 37°C and oxygenated with 95% O\(_2\) and 5% CO\(_2\). The composition (mM) of Krebs solution was: NaCl 120, KCl 4.7, CaCl\(_2\) 2.5, MgCl\(_2\) 1.2, NaHCO\(_3\) 25, KH\(_2\)PO\(_4\) 1.2 and glucose 11 (pH 7.4).

To measure the isometric tension response of the colonic smooth muscle, the circular strips were suspended under a load of 2 g and connected to an isometric transducer (Nihon Kohden TB-651T, Tokyo, Japan) and a Nihon Kohden polygraph (RJG-4124) as described in our previous paper (Uchida et al., 1997). The tissues were then equilibrated for 60 min and washed with fresh Krebs solution every 15 min. At the end of equilibration, all preparations were exposed to
10 µM carbachol or 100 mM KCl to obtain maximum contraction. Following washout, the preparations were allowed to equilibrate for a further 30 min. The preparations were then exposed to cumulatively increasing concentrations of carbachol (10 nM–30 µM). The values of pEC50 (negative logarithm for molar concentration eliciting 50% of the maximum contraction) were calculated from individual concentration-response curves. Differences between the contractile responsiveness of male and female segments were not observed (data not shown).

Data are expressed as the mean ± S.E. P values less than 0.05 were considered to be significant.

Drug used was carbamylcholine chloride (carbachol, Sigma, St Louis, MO, USA). Carbachol was dissolved in and diluted with 0.9% NaCl solution (saline). The molar concentrations of drugs in this paper refer to the final bath concentrations.

### Results

Circular smooth muscle isolated from the human distal colon responded well to the application of carbachol (10 nM–30 µM) with concentration-dependent contractions. The pEC50 value for carbachol in fresh preparations was 6.10 (Table 1). The amplitude of the maximally developed tension (6.36 g) induced by 10 µM carbachol was larger than that (4.17 g) induced by 100 mM KCl (Table 1). The contractile response to carbachol was reproduced after freeze storage of strips for a month or more, but the responsiveness was reduced in each of the preservative solutions tested. Any preparation freeze-stored in Cell Banker2, Cellvation, Cryoprotective or OPTE-MEM showed a significant reduction of both pEC50 and Emax values for carbachol contracture (Table 1). The preparations freeze-stored in SFM101 or PBS showed a significant reduction of pEC50 for carbachol, but their maximum contractions were not significantly different from that in fresh preparations (Table 1). The amplitudes induced by 100

### Table 1. Contractile responsiveness to carbachol or 100 mM KCl of the human isolated colon freeze-stored in different preservative solutions

<table>
<thead>
<tr>
<th>Preservative solution</th>
<th>n</th>
<th>Carbachol contraction</th>
<th>KCl contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pEC50</td>
<td>Emax (g)</td>
</tr>
<tr>
<td>(Fresh preparation)</td>
<td>13</td>
<td>6.10±0.1</td>
<td>6.36±1.9</td>
</tr>
<tr>
<td>Cell Banker 2+5–20% DMSO</td>
<td>10</td>
<td>5.70±0.07*</td>
<td>3.08±0.64**</td>
</tr>
<tr>
<td>Cellvation- DMSO-free</td>
<td>10</td>
<td>5.32±0.09***</td>
<td>1.76±0.40***</td>
</tr>
<tr>
<td>Cryoprotective +15% DMSO</td>
<td>10</td>
<td>5.44±0.09***</td>
<td>2.39±0.76***</td>
</tr>
<tr>
<td>SFM101+10% DMSO</td>
<td>10</td>
<td>5.70±0.11*</td>
<td>4.66±0.82</td>
</tr>
<tr>
<td>OPTE-MEM +10% DMSO</td>
<td>10</td>
<td>5.61±0.09**</td>
<td>2.95±0.45**</td>
</tr>
<tr>
<td>PBS+10% DMSO</td>
<td>10</td>
<td>5.52±0.08***</td>
<td>4.44±1.13</td>
</tr>
</tbody>
</table>

Each value represents mean ± S.E. pEC50 values show negative logarithm of the concentration effective in evoking contractile response equal to 50% of the maximum contraction. Emax values show the maximum amplitude of contraction as a developed tension (g). *P<0.05; **P<0.01; ***P<0.001. These were compared the values between fresh preparation and freeze-stored preparation using one way analysis of variance and the Bonferroni multiple comparisons test. DMSO, dimethyl sulfoxide.
mM KCl were significantly reduced in all preparations freeze-stored in any of the preservative solutions. When the tissue was freeze-stored in DMSO-free medium (Cellvation), both contractile responses to carbachol and KCl were strongly inhibited.

**Discussion**

The present experiments were designed to compare the pharmacological responsiveness to carbachol or high potassium of the human colon freeze-stored in different preservative solutions. When the surgically excised human colon was tested within 5 hours, the circular smooth muscle strips contracted on the addition of carbachol in a concentration-dependent manner. The pEC$_{50}$ values (equal to 6.10, Table 1) for carbachol observed here were ten times as high as those reported by Kerr et al. (1995). The cause of this discrepancy is unclear but different experimental conditions may contribute. The preparations freeze-stored in the different preservative solutions for a period of one month or more showed a reduced contractile response. The degree of reduction was different from medium to medium. When the tissue was freeze-stored in SFM101 or PBS with 10% DMSO, the Emax value for carbachol was not significantly different from that of fresh preparation, but the pEC$_{50}$ value was significantly smaller than that of fresh preparation (Table 1). The high potassium-induced contractions were significantly reduced even in the preparations freeze-stored in either SFM101 or PBS. This suggests that in human colonic smooth muscle the electro-mechanical coupling, but not the pharmaco-mechanical coupling, is more easily injured by the freeze-storage. The human colon freeze-stored in other preservative solutions responded poorly to both carbachol and high potassium. These results indicate that the best preservative solution for freeze-storage of human colon in pharmacological study is SFM101. Although PBS had not been used for freeze-storage of cultured cells, the present results suggest that PBS may also be useful for freeze-storage of smooth muscle organs. Our previous study demonstrated that Dulbeco’s PBS, rather than Krebs bicarbonate solution or Eagle’s minimum essential medium (MEM), was the most suitable for 4°C cold-storage of human colon (Kamikawa et al., 2000).

The cause of different responsiveness of human colon freeze-stored in different preservative solutions is not yet clear. The supplementation with 10–20% DMSO was essential for maintaining the responsiveness of smooth muscle, because the preparation freeze-stored in Cellvation without DMSO responded to spasmogens in the poorest manner (Lovelock and Bishop, 1957). In contrast, the supplementation with serum was inadequate to use the human colon for pharmacological study, because our preliminary experiments showed that the tissue freeze-stored in MEM with fetal bovine serum responded poorly to carbachol (data not shown). There are several reports indicating that serum albumin can inhibit the contractile response of smooth muscle via the release of prostaglandins and endothelium- or epithelium-derived relaxing factors (Bina et al., 1998; Wu-Wong et al., 1998; Yang et al., 2000; Boschetto et al., 2001). Therefore, only the serum-free cryopreservative solutions were used in this study. To use the surgically excised human tissues for in vitro pharmacological study, further tests are required to determine the precise protocol for the freezing and defrosting procedures, using SFM101 or PBS as a cryopreservative solution.
Preservative solution for freeze-storage of human colon

To conclude, the present study provides the first evidence of the importance of cryopreservative solutions for future in vitro studies on smooth muscle function using the human colon. The results suggest that contractile responses of circular smooth muscle appear to be best preserved in SFM101 or PBS freeze-stored human colon for a month or more after surgical resection.

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References


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