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RESEARCH PAPER

Comparative studies of AJG049, a novel Ca^{2+} channel antagonist, on voltage-dependent L-type Ca^{2+} currents in intestinal and vascular smooth muscle

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Background and purpose: Antagonists of Ca^{2+} channels reduce contraction of intestinal smooth muscle but also affect vascular smooth muscle. We have therefore examined the effects of AJG049, a newly synthesized antagonist for regulation of gut motility, on voltage-dependent L-type Ca^{2+} channels, in vascular and intestinal smooth muscle, comparing AJG049 with two other Ca^{2+} channel antagonists, verapamil and diltiazem.
Experimental approach: Affinities of AJG049 for various types of voltage-dependent Ca^{2+} channels were examined by binding studies. Effects of AJG049 on voltage-dependent inward Ca^{2+} (or Ba^{2+}) currents (I_{Ca} or I_{Ba}) in dispersed smooth muscle cells from guinea-pig ileum, colon and mesenteric artery were measured using conventional whole-cell configurations.
Key results: In binding studies, AJG049 showed a high affinity for the diltiazem-binding site of L-type Ca^{2+} channels. In whole-cell configuration, AJG049 suppressed I_{Ca} in ileal myocytes, with concentration-, voltage-and use-dependencies. AJG049 shifted the steady-state inactivation curve of I_{Ca} to the left. The order of potency to inhibit I_{Ca} in ileal myocytes was AJG049 > verapamil > diltiazem. AJG049 also suppressed I_{Ba} in guinea-pig mesenteric arterial myocytes, showing concentration- and voltage-dependencies and the potency order for this action was also AJG049 > verapamil > diltiazem. For the relative ratio of K_i values between ileal and mesenteric arterial myocytes, the order was AJG049 > diltiazem ≫ verapamil.
Conclusions and implications: These results show that AJG049 inhibits L-type Ca^{2+} channels mainly through the diltiazem-binding site(s). From our results, AJG049 showed a little selectivity for these Ca^{2+} channels in intestinal smooth muscle.
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Keywords: AJG049; Ca^{2+} channel antagonists; intestinal smooth muscle; irritable bowel syndrome; L-type Ca^{2+} channels; tissue selectivity

Abbreviations: AJG049, *R*-(+)-5,11-dihydro-5-[1-(4-methoxyphenethyl)-2-pyrrolidinylmethyl] dibenzo [*b*,*e*][1,4] oxazepine hydrochloride; GF/C filters, glass microfibre filter type C; I_{Ba}, voltage-dependent Ba^{2+} currents; IBS, irritable bowel syndrome; I_{Ca}, voltage-dependent Ca^{2+} currents; PSS, physiological salt solution; TEA, tetraethylammonium

Introduction

It is well known that voltage-dependent Ca^{2+} channels in gastrointestinal smooth muscle play a significant role in regulating not only membrane excitability but also bowel motility as one of the major Ca^{2+} entry pathways (reviewed by Bolton *et al*., 1999). As several Ca^{2+} channel antagonists, such as nifedipine, diltiazem and verapamil, inhibit voltage-dependent Ca^{2+} channels in intestinal smooth muscle (Terada *et al*., 1987; Duridanova *et al*., 1993), Ca^{2+} channel antagonists reduce contraction of smooth muscle. Thus, it is generally thought that these Ca^{2+} channel antagonists may be useful to moderate the abnormal motility in patients with irritable bowel syndrome (IBS) (verapamil, Byrne, 1987; diltiazem, Johnson *et al*., 1988). However, these drugs also produce dose-dependent cardiovascular effects (e.g. hypotensive action) over similar dose ranges as those required to inhibit hypermotility in gastrointestinal smooth muscle (reviewed by Traube and McCallum, 1984). Much effort is currently being spent to synthesize selective Ca^{2+} channel antagonists for the treatment of hypermotility disorders of gastrointestinal tract smooth muscle.

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AJG049 (*R*-(+)-5,11-dihydro-5-[1-(4-methoxyphenethyl)-2-pyrrolidinylmethyl] dibenzo [*b*,*e*] [1,4] oxazepine hydrochloride) is a newly developed Ca^{2+} channel antagonist. The purpose of the present study was to investigate whether or not AJG049 may possess a tissue selectivity for intestinal smooth muscle in comparison with that for vascular smooth muscle, observing the effects of AJG049 on voltage-dependent Ca^{2+} (or Ba^{2+}) currents (I_{Ca} or I_{Ba}) in enzymatically dispersed single guinea-pig intestinal (ileum and colon) and vascular (mesenteric artery) smooth muscle cells using conventional whole-cell recording techniques. We have further compared the potency of the drug with those of other types of well-known Ca^{2+} channel antagonists (verapamil and diltiazem) in whole-cell recordings. Moreover, radioligand receptor-binding assays were performed to identify high-affinity binding sites for AJG049 in several voltage-dependent Ca^{2+} channels.

Methods

Radioligand binding assay for various voltage-dependent Ca^{2+} channels in rat cerebral cortex

Male Sprague–Dawley rats were killed by decapitation, and the brain was quickly removed. The cerebral cortex was dissected from the rest of the brain on a Petri dish over ice. The tissue was then added to separate centrifugation tubes containing 19 volumes of 50 mM Tris-HCl (pH 7.4), and the tissue homogenized for 10 s repeated three times. The cerebral cortical homogenate was washed twice (48 000 *g* for 10 min) and re-suspended in 50 mM Tris-HCl (pH 7.4). Radioligand binding studies with *d-cis*-[^{3}H]diltiazem, (+)-[^{3}H]PN200-110, [^{3}H]desmethoxyverapamil ([^{3}H]D888) and [^{125}I]ω-conotoxin GVIA were carried out at 25°C in 50 mM Tris-HCl (pH 7.4) as described previously (Lee *et al*., 1983), with minor modifications. Radioligand concentration, protein concentration and incubation time of competition binding experiments were 2.61 nM, 5 mg tissue eq ml^{-1} and 120 min for *d-cis*-[^{3}H]diltiazem; 0.49 nM, 10 mg tissue eq ml^{-1} and 90 min for (+)-[^{3}H]PN200-110; 0.49 nM, 5 mg tissue eq ml^{-1} and 30 min for [^{3}H]D888; and 0.008 nM, 10 mg tissue eq ml^{-1} and 30 min for [^{125}I]ω-conotoxin GVIA, respectively. Nonspecific binding was measured in the presence of 100 μM diltiazem for *d-cis*-[^{3}H]diltiazem binding, 100 μM nifedipine for (+)-[^{3}H]PN200-110 binding, 100 μM D600 for [^{3}H]D888 binding and 10 μM ω-conotoxin GVIA for [^{125}I]ω-conotoxin GVIA binding. At the end of the incubation period, samples were diluted with 3 ml of ice-cold washing buffer (50 mM Tris-HCl, pH 7.4) and immediately filtered through GF/C filters (glass microfibre filter type C, Whatman International Ltd, Maidstone, UK), which had been presoaked in 0.5% polyethyleneimine. Filters were washed three times with 3 ml of ice-cold buffer. A Brandel cell harvester was used for the filtration procedure. Radioactivity on the filter was measured by liquid scintillation counting or gamma counting.

Cell dispersion

Male guinea-pigs weighing 300–400 g were killed by cervical dislocation and exsanguination. A segment of ileum, colon or mesenteric artery (first branch; 1.0–1.5 cm) was excised and rinsed thoroughly in Ca^{2+}-free physiological salt solution (PSS) (mM): Na^+ 140, K^+ 5, Mg^{2+} 1.2, glucose 5, Cl^- 151.4, HEPES 10, titrated to pH 7.40 with Tris base. A segment of the tissue was incubated at 35°C in Ca^{2+}-free PSS containing collagenase (intestinal muscle, 1.3 mg ml^{-1}, type I, Sigma-Aldrich Japan KK, Tokyo, Japan; mesenteric artery, 1 mg ml^{-1}, Wako Pure Chem. Ind. Ltd, Osaka, Japan) for 20–25 min. Thereafter, the digested segment of the thin longitudinal layer was carefully peeled off with fine forceps and scissors under a binocular microscope and stored in 0.5 mM Ca^{2+}-containing PSS containing 1 mg ml^{-1} fatty acid-free bovine serum albumin at 4–6°C. The cells were dispersed for each experiment by mincing the segments into pieces and then triturating them with a blunt-tipped Pasteur pipette. The spindle-shaped dispersed cells were stored at 4°C and normally used within 4 h for experiments.

Recording procedure

Patch-clamp experiments were performed at room temperature (21–23°C) as described previously (Hamill *et al*., 1981; Teramoto *et al*., 2001). Briefly, patch electrodes were prepared with an electrode puller (P-97, Sutter Instrument Company, Novato, CA, USA) and a heat polisher (MF-83, Narishige Sci. Inst. Lab., Tokyo, Japan). A high-resistance seal (>10 GΩ) was obtained by application of a negative pressure to the patch pipette (2–5 MΩ). Voltage-clamp recordings were obtained with the conventional whole-cell configuration. Generation of voltage pulses was performed using a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA, USA). The sampled current data were filtered at 10 kHz and stored together with potential records on videotape using a video recorder (PCM-501ES, SONY, Tokyo, Japan) for subsequent off-line analysis. Junction potentials between bath and pipette solutions were measured using a 3 M KCl reference electrode and were <2 mV, so that correction for these potentials was not made. Capacitance was kept to a minimum by maintaining the test solution in the bath as low as possible. At the beginning of each experiment, the series resistance was compensated, adjusting the transient cancellation control to provide the best cancellation.

Solutions

The following solutions were used for whole-cell recording. PSS (mM): Na^+ 140, K^+ 5, Mg^{2+} 1.2, Ca^{2+} 2, glucose 5, Cl^- 151.4, HEPES 10 and was titrated to pH 7.35–7.4 with Tris base. The pipette solution contained (mM) Cs^+ 130, tetraethylammonium (TEA^+) 10, Mg^{2+} 4, Cl^- 148, creatine phosphate 5, EGTA 5, ATP 5, HEPES 10/Tris (pH 7.35–7.4). For recording I_{Ba} in whole-cell configuration, Ba^{2+} 10 mM bath solution contained (mM) Ba^{2+} 10, TEA^+ 135, Cl^- 155, glucose 10, HEPES 10/Tris (pH 7.35–7.40).

Data analysis and statistics

Whole-cell current data were low-pass filtered at 500 Hz by an eight-pole Bessel filter, sampled at 1 ms intervals and

analysed on a computer (Macintosh PowerPC 7100/80AV, Apple Computer Japan, Tokyo, Japan) by commercial software 'MacLab 3.4.2' (ADInstruments Pty Ltd, Castle Hill, Australia). The amplitudes of whole-cell currents were measured at peak height. Measurements of the amplitude of I_{Ca} were corrected for background membrane current by subtraction of currents obtained when voltage command pulses were applied to the cells in the presence of extracellular Cd^{2+} (50 μM).

The peak amplitude of the Ca^{2+} (or Ba^{2+}) current elicited by a step pulse to 0 mV (+10 mV for the Ba^{2+} current) from the holding potential just before application of a drug was normalized as one. The curves were drawn by fitting the following equation using the least-squares method.

Relative amplitude of $I_{Ca} = 1/\{1 + ([D]/K_i\}n_H$, where K_i, [D] and n_H are the inhibitory dissociation constant, concentration of drug (nM) and Hill's coefficient, respectively.

The dissociation constant for drug binding to the channel to the inactivated state could be estimated from the shift of the voltage-dependent inactivation curve and the concentration response curve obtained at the resting state by use of the following equation (Bean *et al*., 1983; Teramoto, 1993),

$$-\Delta V_{half} = k^* \ln\{(1 + [D]/K_{inact}/(1 + [D]/K_{rest})\}$$

where ΔV_{half} is the amplitude of the shift of the voltage-dependent inactivation curve, k is a slope factor for the inactivation curve and [D] is the concentration of drug applied. K_{inact} and K_{rest} are dissociation constants of AJG049 for the inactivated and the resting states of voltage-dependent Ca^{2+} channels, respectively.

Statistical analyses were performed with Student's *t*-test for paired values. Changes were considered significant at $P<0.01$. Data are expressed as mean with s.e.m.

Materials

The following chemicals were used: AJG049 (Pharmaceutical Research Laboratories of Ajinomoto Co., Tokyo, Japan), prepared daily as a 10 mM stock solution in ethanol and diluted just before use. The final concentration of ethanol was less than 0.1%. Other drugs and chemicals were obtained from Sigma Chemicals (Sigma Chemical KK, Tokyo, Japan).

Results

Affinities of AJG049 for voltage-dependent Ca^{2+} channels in rat cerebral cortex

In order to investigate the affinities of AJG049 in several voltage-dependent Ca^{2+} channels, the affinities of AJG049 for two kinds of voltage-dependent Ca^{2+} channels (L-type and N-type) were evaluated by use of radioligand receptor binding assays in rat cerebral cortex. The values were determined for AJG049 by nonlinear regression analysis of their competition curves (see Methods). AJG049 possessed the highest affinity for the diltiazem-binding site of L-type Ca^{2+} channels ($IC_{50} = 79$ nM), compared with other binding sites of L-type Ca^{2+} channels (verapamil site, $IC_{50} = 245$ nM; dihydropyridine site, $IC_{50} \gg 10$ μM) and N-type Ca^{2+} channels ($IC_{50} \gg 10$ μM).

The effects of AJG049 on I_{Ca} in guinea-pig intestinal smooth muscle

To investigate the effects of AJG049 on I_{Ca}, conventional whole-cell recordings were performed in guinea-pig ileal myocytes. When the recording pipette was filled with a Cs^+-TEA^+ solution containing 5 mM EGTA and the bath solution was physiological saline solution (PSS) containing 2 mM Ca^{2+}, application of a depolarizing step to −40 mV from a holding potential of −60 mV produced an inward Ca^{2+} current (Figure 1a). This current increased slightly with time after establishing the whole-cell configuration, reaching a steady level approximately 4 min after rupture of the membrane patch ($n=10$). This peak value was then maintained at least for 15 min, whereas test depolarization pulses (1 s duration) were applied at 30 s intervals (the peak amplitude of I_{Ca} at 15 min being 99±2% ($n=25$) of the value determined 4 min after the establishment of whole-cell recording). Consequently, all experiments were performed within a 15 min period.

As shown in Figure 1a, AJG049 (10 nM) inhibited the peak amplitude of I_{Ca} evoked by depolarizing pulses (1 s duration) from a holding potential of −60 mV at levels more positive than −20 mV. Figure 1b shows the current–voltage relationships in the absence and presence of AJG049 (10 nM, 1 and 10 μM), and the inhibition showed concentration-dependent behaviour. AJG049 (10 μM) suppressed the peak amplitude of I_{Ca} (Figure 1b). In Figure 1c, the AJG049 (10 nM)-induced inhibition showed a voltage dependency ($n=5$). The peak amplitude of I_{Ca} was also suppressed by 10 μM nifedipine, a selective L-type Ca^{2+} channel blocker (data not shown). Figure 2a shows the relationships between the relative peak amplitude of I_{Ca} and concentrations of AJG049 at two different holding potentials (−60 and −90 mV). I_{Ca} was evoked by a depolarizing pulse to 0 mV applied every 30 s. AJG049 inhibited the peak amplitude of I_{Ca} in a concentration-dependent manner (−60 mV, $K_i = 66.5$ nM; −90 mV, $K_i = 739.1$ nM). To compare the inhibitory potency of AJG049 ($K_i = 66.5$ nM) with that of other Ca^{2+} channel antagonists at −60 mV, verapamil and diltiazem were applied (Figure 2a). These Ca^{2+} channel antagonists reduced the peak amplitude of I_{Ca} in a concentration-dependent manner as reported previously. In guinea-pig colonic myocytes, AJG049 also suppressed the peak amplitude of I_{Ca} in a concentration-dependent manner, showing less potency than in guinea-pig ileal myocytes (Figure 2b). The voltage dependency was investigated before and after the application of 300 nM AJG049 using the experimental protocol shown in Figure 2c (conditioning pulse duration, 10 s; holding membrane potential, −90 mV). In the absence of AJG049 (control), inactivation of I_{Ca} occurred with depolarizing pulses positive to −50 mV in guinea-pig ileal myocytes. After the application of 300 nM AJG049 (approximately 5 min later), the voltage-dependent inactivation curve in the same cells was significantly shifted to the left by 6±1 mV ($n=4$; Figure 2c).

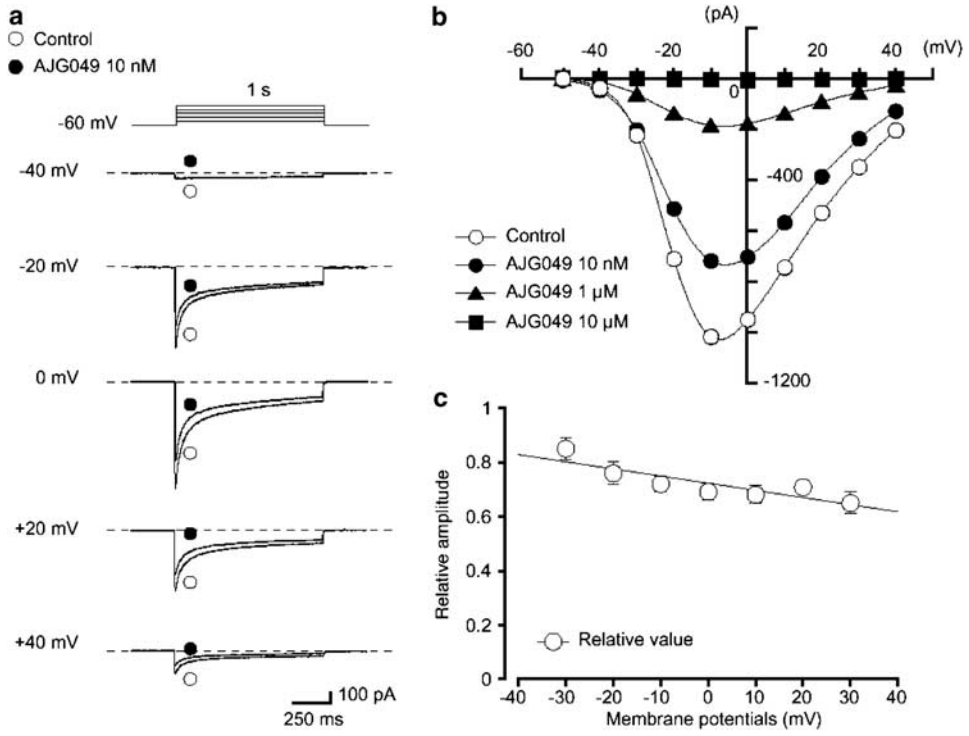

Figure 1 Effects of AJG049 on I_{Ca} at a holding membrane potential of −60 mV in guinea-pig ileal myocytes. Whole-cell recording, pipette solution Cs^+-TEA^+ solution containing 5 mM EGTA and bath solution PSS. (**a**) Original current traces before (control) and after the application of 10 nM AJG049. (**b**) Current–voltage relationships obtained in the absence (control) or presence of AJG049 (10 nM, 1 and 10 μM). The current amplitude was measured as the peak amplitude of I_{Ca} in each condition. The lines were drawn by eye. (**c**) Relationship between the test potential and relative value of I_{Ca} inhibited by 10 nM AJG049, expressed as a fraction of the peak amplitude of I_{Ca} evoked by depolarizing pulses of various amplitudes in the absence of AJG049. Each symbol indicates the mean of five observations with ±s.e.m. shown by vertical lines. Some of the s.e.m. bars are smaller than the size of the symbol. The line was drawn by eye.

Figure 3a shows the time course of the effects of AJG049 (50 nM) on I_{Ca} evoked by a depolarizing pulse to 0 mV from −60 mV. The depolarizing pulses were applied every 30 s. When the peak amplitude of I_{Ca} just before application of AJG049 (control) was taken as one, AJG049 reduced the peak amplitude of I_{Ca} to approximately 60% ($n=5$; Figure 3b). On removal of AJG049, the peak amplitude gradually recovered, but not to the control level (Figure 3a and b). As shown in Figure 3c, when a depolarizing pulse was applied after an interval of 3 min in the presence of 30 nM AJG049, the peak amplitude of I_{Ca} was smaller than that observed before the application of AJG049 but consistently larger than that recorded at 4 min with repetitive application of the depolarizing pulses (single pulse, 1.0±0.01, $n=5$ versus repetitive pulse 0.8±0.05, $n=5$; $P<0.01$).

Voltage-dependent inhibitory effects of AJG049 on I_{Ba}s in guinea-pig mesenteric arterial myocytes

To further study whether or not AJG049 possesses tissue selectivity between gut and vascular smooth muscles, the effects of AJG049 on I_{Ba} were investigated in guinea-pig mesenteric arterial myocytes, as it has previously been reported that the peak amplitude of nifedipine-sensitive I_{Ca} in guinea-pig mesenteric arterial myocytes was too small (less than 20 pA at +10 mV) to estimate precisely (Ohya *et al*., 1997). Thus, in the present experiments, Ba^{2+} (10 mM) was used as a charge carrier in the bath solution in order to enhance the amplitude of the inward currents for analysis and to isolate I_{Ba} by inhibiting other Ca^{2+}-activated mechanisms (such as Ca^{2+}-activated K^+ currents and Ca^{2+}-activated Cl^- currents). The recording pipette was filled with a Cs^+-TEA^+ solution containing 5 mM EGTA. Figure 4a shows the time course of the effects of AJG049 (100 nM) on I_{Ba} (20 s interval). Application of 100 nM AJG049 gradually reduced the peak amplitude of the inward current, nearly halving it within a few minutes ($n=10$; Figure 4b). On removal of AJG049, the peak amplitude of I_{Ba} gradually recovered, but did not return to control level (0.71±0.04, $n=5$; Figure 4b). Subsequent application of nifedipine (10 μM) completely suppressed the currents (data not shown). Figure 4c shows the relationship between the relative peak amplitude of I_{Ba} evoked by a depolarizing pulse to +10 mV from two different holding membrane potentials (−60 and −90 mV) applied every 20 s and concentrations of AJG049. AJG049 inhibited the peak amplitude of I_{Ba} in a concentration- and voltage-dependent manner (−60 mV, K_i, 190.3 nM; −90 mV, $K_i=1.9$ μM). Similarly, other Ca^{2+} channel antagonists (verapamil and diltiazem) reduced the peak amplitude of I_{Ba} in a concentration-dependent manner at a holding potential of −60 mV (K_i, verapamil, 300.8 nM; diltiazem, 2.4 μM; Figure 4c). The rank order of potency of the K_i values for I_{Ca} (or I_{Ba}) was AJG049 > verapamil > diltiazem in guinea-pig ileal and mesenteric arterial myocytes, although the K_i values were different between two tissues. As shown in Figure 4d, AJG049 inhibited the peak amplitude of

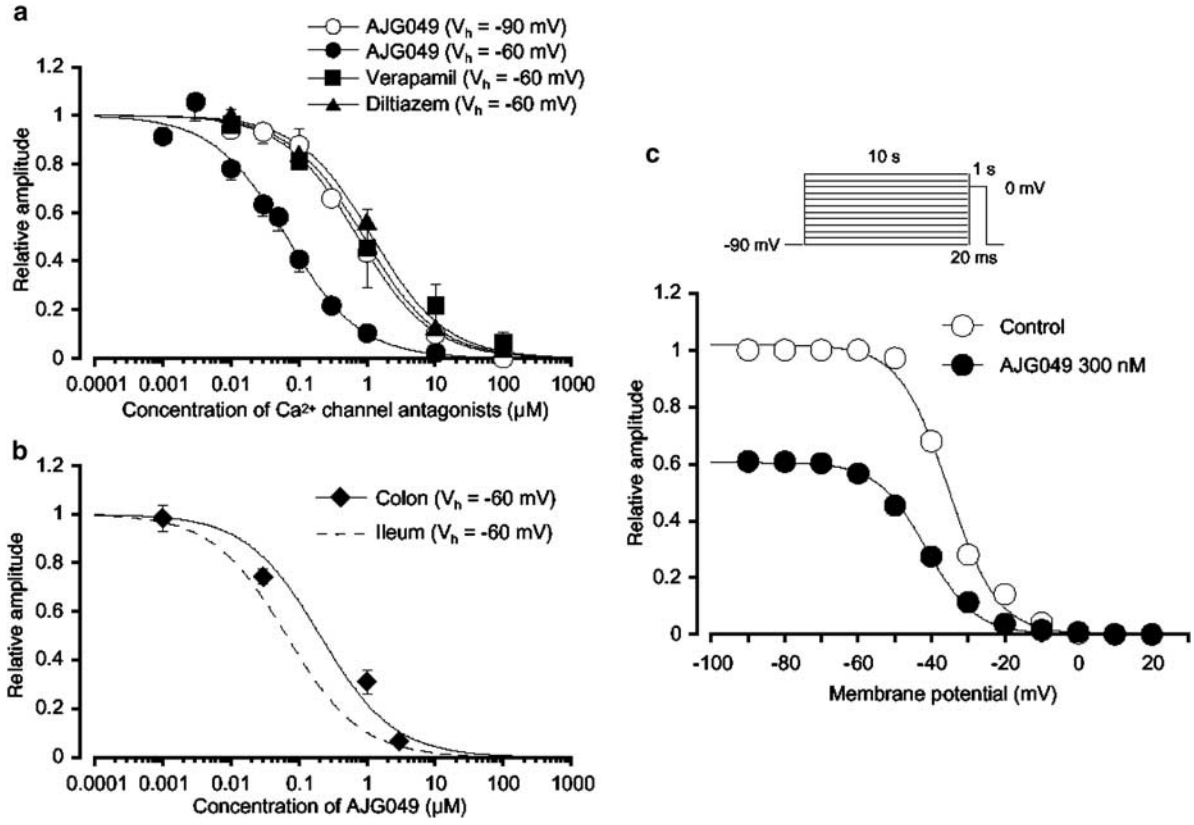

Figure 2 Effects of AJG049 on I_{Ca} in guinea-pig ileal myocytes. Whole-cell recording, pipette solution Cs^+-TEA^+ solution containing 5 mM EGTA and bath solution PSS. (**a**) Concentration–response curves for AJG049 at two holding potentials (−60 and −90 mV) and other Ca^{2+} channel antagonists (verapamil and diltiazem) at −60 mV on I_{Ca} in guinea-pig ileal myocytes. The peak amplitude of I_{Ca} elicited by a step pulse to 0 mV from the holding potential just before the application of AJG049 was normalized as one. The curves were fitted and the following values were used: AJG049, −60 mV, $K_i = 66.5$ nM, $n_H = 0.8$; −90 mV, $K_i = 739.1$ nM, $n_H = 0.8$; verapamil, $K_i = 901.6$ nM, $n_H = 0.8$; diltiazem, $K_i = 1.2$ μM, $n_H = 0.8$. Each symbol indicates the mean of 4–8 observations with s.e.m. shown by vertical lines. Some of s.e.m. bars are smaller than the size of the symbol. (**b**) Concentration–response curve for AJG049 on I_{Ca} in guinea-pig colonic myocytes at a holding potential of −60 mV ($K_i = 207.8$ nM, $n_H = 0.8$). The curve with the broken line was obtained from (**a**) regarding the inhibitory effects of AJG049 on I_{Ca} in guinea-pig ileal myocytes at −60 mV. Each symbol indicates the mean of 4–8 observations with ± s.e.m. shown by vertical lines. Some of s.e.m. bars are smaller than the size of the symbol. (**c**) At −90 mV, conditioning pulses of various amplitudes were applied (up to +30 mV, 10 s duration) before the application of the test pulse (to 0 mV, 1 s duration). An interval of 20 ms was allowed between these two pulses to allow estimation of possible contamination by capacitive current. The peak amplitude of I_{Ca} evoked by each test pulse was measured before and after the application of 300 nM AJG049. The lines were drawn by fitting the data to the following equation in the least-squares method, $I = I_{max}/\{1 + \exp[(V - V_{half})/k]\}$ where I is the peak amplitude of I_{Ca} observed at various amplitudes of the conditioning pulse and I_{max} is the response to the application of a conditioning pulse from −90 mV, V is the amplitude of the conditioning pulse and V_{half} the amplitude of the conditioning pulse that evokes I_{Ca} of half-maximum amplitude and k is the slope factor. The curves in the absence or presence of AJG049 were fitted using the following values: control, $I_{max} = 1.0$, $V_{half} = -35.2$ mV and $k = 6.7$, $n = 4$; AJG049, $I_{max} = 0.6$, $V_{half} = -41.3$ mV and $k = 6.7$, $n = 4$.

I_{Ba} evoked by depolarizing pulses (1 s duration) from a holding potential of −60 mV at levels more positive than −30 mV. Figure 4d shows the current–voltage relationships in the absence and presence of 100 nM AJG049. In Figure 4e, the AJG049 (100 nM)-induced inhibition showed a voltage dependency ($n = 5$). The value of the tissue selectivity was calculated as a relative ratio of the K_i values between guinea-pig ileal (Figure 2a) and mesenteric arterial myocytes (Figure 4c), indicating that the rank order of a relative ratio was AJG049 (2.9) > diltiazem (2.0) ≫ verapamil (0.3).

Discussion

These experiments have demonstrated that AJG049 is a potent inhibitor of voltage-dependent L-type Ca^{2+} channels in guinea-pig ileal, colonic and vascular smooth muscle cells, and appears to inhibit the peak amplitude of voltage-dependent L-type Ca^{2+} currents in ileal myocytes with greater efficacy than those in vascular or colonic myocytes.

Inhibitory effects of AJG049 on voltage-dependent Ca^{2+} channels in smooth muscle

In ileal and mesenteric arterial myocytes, the inward I_{Ca} mainly flows through voltage-dependent L-type Ca^{2+} channels (Bean *et al*., 1986; Worley *et al*., 1986; Terada *et al*., 1987; Setoguchi *et al*., 1995). In the present study, nifedipine, a selective inhibitor of voltage-dependent L-type Ca^{2+} channels, suppressed Ca^{2+} (or Ba^{2+}) currents in guinea-pig ileal and mesenteric arterial myocytes. We have shown that IC_{50}

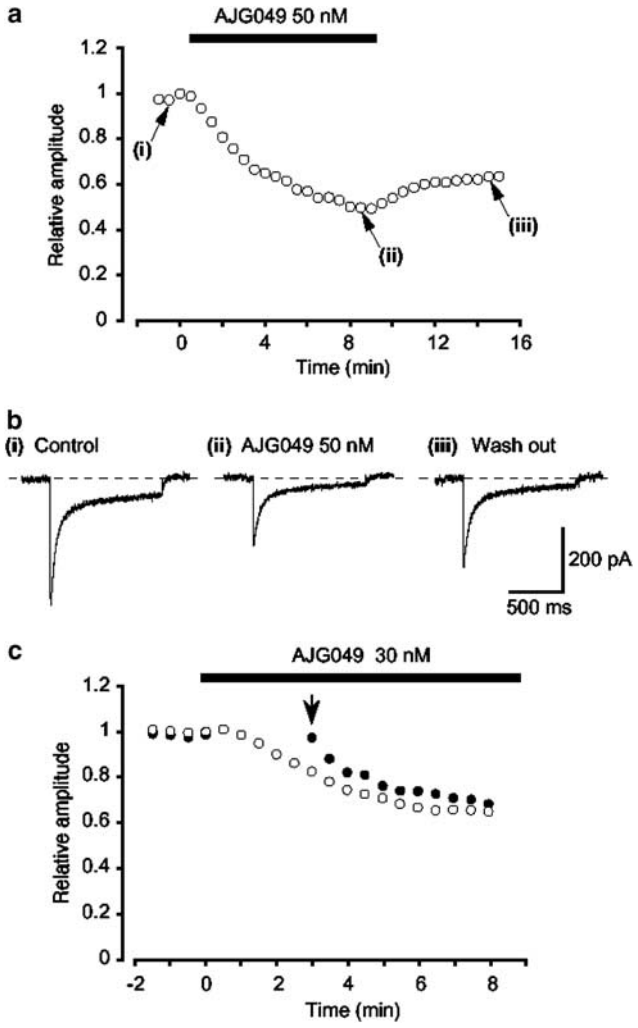

Figure 3 The time course of changes in the peak amplitude of I_{Ca} evoked by repetitive depolarizing pulses to 0 mV from a holding potential of −60 mV before and after the application of AJG049. The abscissa shows the time (min). Time 0 indicates the time at which AJG049 was added to the bath. The ordinate shows the relative size of the peak amplitude of I_{Ca} when the peak amplitude of I_{Ca} in the absence of AJG049 (i.e. control) was normalized as one. (**a**) The typical time course of application of 50 nM AJG049 on the peak amplitude of I_{Ca}. (**b**) Original current traces before (control (i)) and after application of 50 nM AJG049 (ii), as indicated in (**a**). (iii) Original current trace indicates a current trace following removal of AJG049. (**c**) No pulses were applied for the initial 3 min after application of 30 nM AJG049. The peak amplitude of I_{Ca} just before the application of AJG049 was normalized as one (control).

values for verapamil and diltiazem were very close to those of previous reports (Terada *et al*., 1987; Setoguchi *et al*., 1995).

In the present experiments, by use of radioligand receptor binding assays, AJG049 was shown to exhibit a high affinity for the diltiazem-binding site of voltage-dependent L-type Ca^{2+} channels. In conventional whole-cell recording, AJG049 suppressed both I_{Ca} in guinea-pig ileal myocytes and I_{Ba} in guinea-pig mesenteric arterial myocytes, showing concentration- and voltage-dependent characteristics. On removal of the drug, neither I_{Ca} in guinea-pig ileal myocytes nor I_{Ba} in guinea-pig mesenteric arterial myocytes recovered to control level. Furthermore, the rank order of the potency for inhibiting the peak amplitude of I_{Ca} (or I_{Ba}) coincides in guinea-pig ileal and mesenteric arterial myocytes (AJG049 > verapamil > diltiazem) when conventional whole-cell recordings were made. These results strongly suggest that the target channel for AJG049 is likely to be the voltage-dependent L-type Ca^{2+} channel in guinea-pig ileal and mesenteric arterial myocytes, and that AJG049 exhibits a long-lasting inhibitory effect.

Kinetic studies concerning the actions of AJG049 on I_{Ca}

According to the receptor modulated hypothesis, each voltage-dependent Ca^{2+} channel is either in the resting, open or inactivated state (Hille, 1992). In the present study, the same amplitude of I_{Ca} was produced by the application of depolarizing pulses from a holding membrane potential of −90 mV or more negative, suggesting that all voltage-dependent Ca^{2+} channels at these potentials may be in the resting state (data not shown). The ability of AJG049 to suppress the peak amplitude of I_{Ca} evoked by a depolarizing pulse from two different holding potentials (−90 and −120 mV) was not significantly different, suggesting that at these negative holding potentials, AJG049 may inhibit I_{Ca} in a voltage-independent manner (AJG049: resting state block). When the holding potential was elevated to −60 mV from −90 mV, voltage-dependent inhibition by AJG049 was observed and the concentration–response curve was shifted to the left. The voltage-dependent inactivation curve was also shifted to the left after the application of AJG049. These results suggest that voltage-dependent inhibitory actions of AJG049 on voltage-dependent Ca^{2+} channels also occurred in the inactivated state of voltage-dependent Ca^{2+} channels (AJG049: voltage-dependent block). In the present experiments, the K_{rest} value was estimated to be 739.1 nM from the concentration–response curve at a holding potential of −90 mV. When ΔV_{half} value was obtained from the results using 10 s conditioning pulses, the estimated K_{inact} value was 120.4 nM (see Methods). Given this, we suggest that AJG049 may bind to the inactivated state with approximately six times higher affinity than to the resting state.

Tissue selectivity of AJG049 for voltage-dependent L-type Ca^{2+} *channels in smooth muscle*

The value of the selectivity ratio for AJG049 is the largest in the Ca^{2+} channel antagonists examined (AJG049 (2.9) > diltiazem (2.0) ≫ verapamil (0.3)), although diltiazem, possessing profound cardiovascular effects in clinical field, also has a selectivity ratio of 2.0. Thus, it is somewhat difficult to categorize AJG049 as specific for intestinal Ca^{+} channels on this basis.

The observations also suggest that the binding affinity of AJG049 for inhibiting voltage-dependent Ca^{2+} channels may be different for vascular and intestinal smooth muscle.

Recently, molecular biological studies have revealed that the pore-forming structure of voltage-dependent Ca^{2+} channels is the α_1 subunit and that the α_1 subunit is a potential target for Ca^{2+} channel antagonists (reviewed by Slish *et al*., 1992). It is generally thought that the α_{1C} subunit appears to be a pore-forming structure of L-type Ca^{2+} channels. Several isoforms of the α_{1C} subunit of the L-type

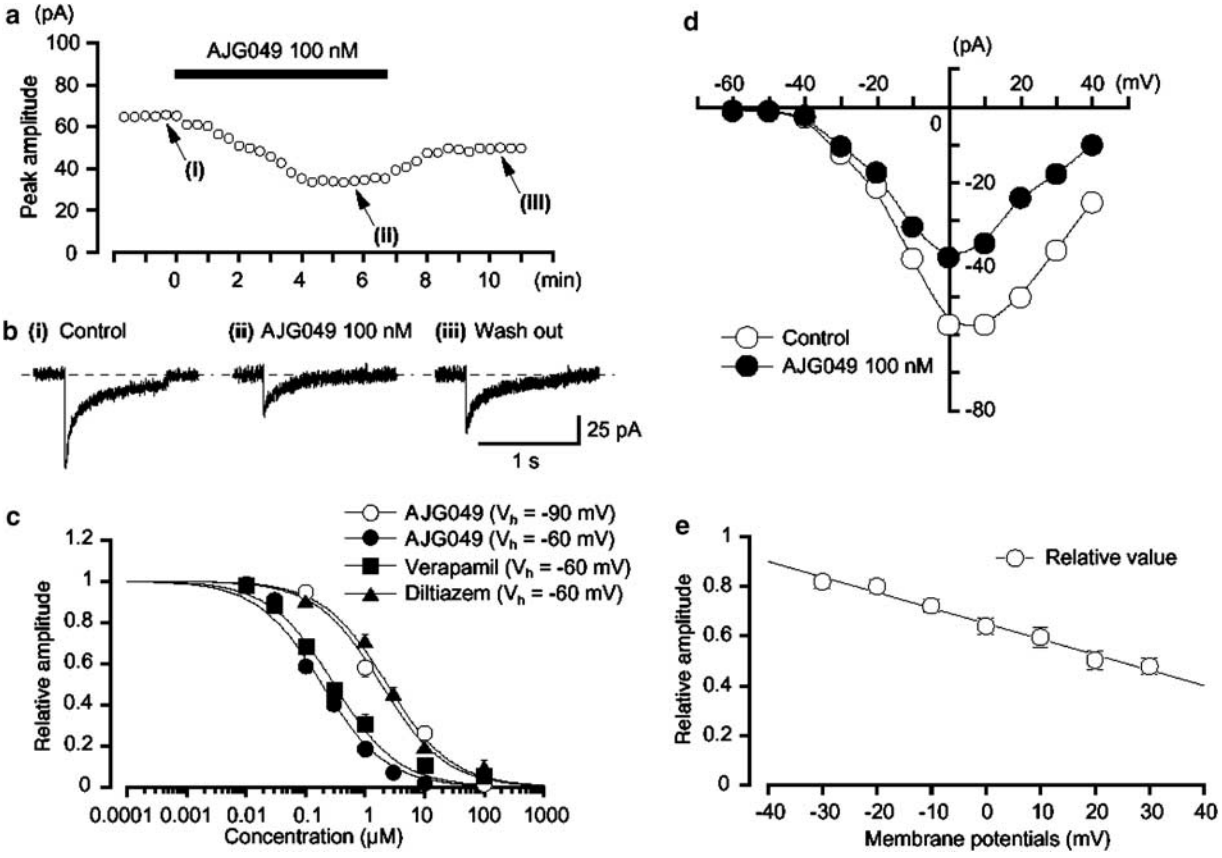

Figure 4 Effects of AJG049 on I_{Ba} in dispersed smooth muscle cells from guinea-pig mesenteric artery. Whole-cell recording, pipette solution Cs^+-TEA^+ solution containing 5 mM EGTA and bath solution 10 mM Ba^{2+} containing 135 mM TEA^+. (**a**) The time course of the effects of application of AJG049 (100 nM) on the peak amplitude of I_{Ba} evoked by repetitive depolarizing pulses to +10 mV from a holding potential of −60 mV. Time 0 indicates the time when AJG049 was applied to the bath. (**b**) Original current traces before (control (i)) and after the application of 100 nM AJG049 (ii), as indicated in (**a**). (iii) Original current trace indicates a current trace following the removal of AJG049. (**c**) Effects of AJG049 and other Ca^{2+} channel antagonists (verapamil and diltiazem) on I_{Ba} at a holding membrane potential of −60 mV in guinea-pig mesenteric arterial myocytes. The concentration–response curve for AJG049 on I_{Ba} at −90 mV was also shown. The peak amplitude of I_{Ba} elicited by a step pulse to +10 mV from the holding potential (−60 or −90 mV) just before the application of drugs was normalized as one. The following values were used for curve fitting: AJG049, −60 mV, K_i = 190.3 nM, n_H = 0.8; AJG049, −90 mV, K_i = 1.9 μM, n_H = 0.8; verapamil, K_i = 300.8 nM, n_H = 0.8; diltiazem, K_i = 2.4 μM, n_H = 0.8. Each symbol indicates the mean of 4–8 observations with s.e.m. shown by vertical lines. Some of the s.e.m. bars are smaller than the size of the symbol. (**d**) Current–voltage relationships obtained in the absence (control) or presence of 100 nM AJG049. The current amplitude was measured as the peak amplitude of I_{Ba} in each condition. The lines were drawn by eye. (**e**) Relationship between the test potential and relative value of I_{Ba} inhibited by 100 nM AJG049, expressed as a fraction of the peak amplitude of I_{Ba} evoked by various amplitudes of depolarizing pulse in the absence of AJG049. Each symbol indicates the mean of five observations with ±s.e.m. shown by vertical lines. The line was drawn by eye.

Ca^{2+} channel are expressed from different genes and produced by various splicing processes (Perez-Reyes and Schneider, 1995). Their relative expression is tissue-dependent and the relative expression of the major isoforms of α_{1C} subunits, $\alpha_{1C\text{-}a}$ (IVS3A, 46%) and $\alpha_{1C\text{-}b}$ (IVS3B, 54%), in intestinal smooth muscles is different from that in vascular smooth muscles where the expression of the $\alpha_{1C\text{-}a}$ (IVS3A) isoform is decreased to 14% of α_{1C} subunits (Feron *et al*., 1994). Welling *et al*. (1997) reported that the tissue selectivity of nisoldipine was likely to be due to the difference in the affinities for the cardiac $\alpha_{1C\text{-}a}$ subunit and the vascular $\alpha_{1C\text{-}b}$ subunit. Similarly, it has been reported that nifedipine is a more potent inhibitor of the $\alpha_{1C\text{-}b}$ subunit than the $\alpha_{1C\text{-}a}$ subunit, although the potency of verapamil was identical with both isoforms (Morel *et al*., 1998). These results suggest that the diversity of α_{1C} subunits might contribute to the diversity of responses of Ca^{2+} channels to Ca^{2+} channel blockers. Interestingly, different sensitivities of intestinal and vascular smooth muscles were commonly observed with several types of Ca^{2+} channel antagonists (Triggle *et al*., 1988; Godfraind *et al*., 1992; Zhang and Bolton, 1995). In the present experiments, AJG049 possessed an inhibitory action on L-type Ca^{2+} currents (diltiazem-binding site). Thus, we suggest that AJG049 readily binds with a high affinity to L-type Ca^{2+} channel isoforms.

Implications for gut-selective Ca^{2+} channel antagonists

Contractile activity of intestinal as well as vascular smooth muscle is related to the entry of Ca^{2+} from the extracellular fluid. As the bowel has a high rate of spontaneous activity under normal conditions, one of the main Ca^{2+} entry pathways is thought to be via voltage-dependent Ca^{2+} channels. In the clinical field, excessive postprandial non-

propulsive contractions are commonly observed in IBS patients who have diarrhoea- and constipation-predominant disorders. Any blockade arising during spontaneous activity would be only slowly dissipated during a rest period; during hypermotility, its blocking action would be expected to develop strongly. Thus, as a wide variety of Ca^{2+} channel antagonists reduce abnormal motility of intestinal smooth muscle, Ca^{2+} channel antagonists are thought to be potentially useful drugs for the treatment of IBS. However, in patients with IBS, the activation of the autonomic nervous system induced by the hypotensive response associated with these drugs may adversely influence the symptoms (Aggarwal *et al*., 1994). In the present experiments, AJG049 showed some bowel selectivity, which may have been due to its use-dependent and persistent action on voltage-dependent L-type Ca^{2+} channels. We suggest that bowel-selective Ca^{2+} channel antagonists might be expected to be useful in conditions such as IBS, which is characterized by hypermotility of gut smooth muscle.

In conclusion, the present direct evidence indicates that AJG049 inhibits I_{Ca} (or I_{Ba}) in a long-lasting and voltage-dependent manner mainly through the diltiazem-binding site(s) of voltage-dependent L-type Ca^{2+} channels, and that any measurable gut selectivity exists only in certain regions of the digestive tract.

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Conflict of interest

The authors state no conflict of interest.

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