Microtubule cytoskeleton involvement in muscarinic suppression of voltage-gated calcium channel current in guinea-pig ileal smooth muscle

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1 Effects of agents, which affect microtubule polymerization-depolymerization cycle, on Ba^{2+} current (I_{Ba}) flowing through voltage-gated Ca^{2+} channels and carbachol (CCh)-induced sustained suppression of I_{Ba} were examined in whole-cell voltage-clamped smooth muscle cells of guinea-pig ileum.

2 Colchicine (100 μM) and vinblastine (100 μM), microtubule depolymerizers, increased the amplitude of I_{Ba}. Lumicolchicine (100 μM), an inactive analogue of colchicine, had no effect on I_{Ba}.

3 Taxol (1–100 μM), a microtubule polymerizer, decreased I_{Ba} in a concentration-dependent manner and accelerated the rate of inactivation of I_{Ba}. Baccatin III (100 μM), an inactive analogue of taxol, had no effect on I_{Ba}.

4 Colchicine (100 μM) and vinblastine (100 μM), but not lumicolchicine (100 μM), decreased or abolished the sustained component of CCh (10 μM)-induced I_{Ba} suppression.

5 Pretreatment with taxol (10–100 μM) resulted in a concentration-dependent decrease in I_{Ba} and the action of CCh on I_{Ba}. The inhibitory effects of taxol and CCh on I_{Ba} were not additive.

6 Colchicine (100 μM) or taxol (100 μM) had no effect on voltage-gated K^+ channel current or CCh-induced non-selective cationic channel current.

7 These results suggest that polymerization of microtubules leads to suppression of Ca^{2+} channel activity, and that muscarinic sustained suppression of Ca^{2+} channel current is mediated by a signal transduction element which involves microtubule cytoskeleton.

Keywords: Carbachol; Ca^{2+} channel current; microtubule cytoskeleton; colchicine; taxol; smooth muscle; guinea-pig ileum

Abbreviations: BAPTA, 1, 2-bis (2-aminophenoxy) ethane-N, N, N′, N′-tetraacetic acid; CCh, carbachol; EGTA, ethylene glycol-bis (β-aminoethyl ether) N, N, N′, N′-tetraacetic acid; G protein, GTP binding protein; I_{Ba}, Ba^{2+} current; I_{Ca}, Ca^{2+} current; I_{CCh}, non-selective cationic channel current; I_K, K^+ current; I_{KCa}, Ca^{2+}-activated K^+ current; IP$_3$, inositol 1,4,5,-trisphosphate; PIP$_2$, inositol bisphosphate; PLC, phospholipase C; PSS, physiological salt solution; PTX, pertussis toxin; VGCCs, voltage-gated Ca^{2+} channels

Introduction

In guinea-pig ileal smooth muscle, muscarinic receptors couple with at least two types of GTP-binding protein (G protein) (Komori *et al.*, 1992). One is pertussis toxin (PTX)-sensitive and mediates opening of non-selective cationic channels leading to the membrane depolarization (Benham *et al.*, 1985; Inoue & Isenberg, 1990). The other is PTX-insensitive and activates phospholipase C (PLC), which leads to production of inositol 1,4,5,-trisphosphate (IP$_3$) to release Ca^{2+} from intracellular stores (Komori & Bolton, 1991). We have shown in the same type of smooth muscle that muscarinic receptor stimulation induces suppression of Ca^{2+} current (I_{Ca}) through voltage-gated Ca^{2+} channels with an initial transient component followed by a sustained component (Unno *et al.*, 1995; 1996). A PTX-insensitive G protein is involved in these two phases of I_{Ca} suppression. The initial transient phase of I_{Ca} suppression is brought about by operation of a Ca^{2+}-induced inactivation mechanism due to release of stored Ca^{2+} by IP$_3$. However, the sustained component of muscarinic action on I_{Ca} occurs independently of IP$_3$-induced Ca^{2+} release (Unno *et al.*, 1995; Beech, 1997). The signal transduction for the effect of muscarinic receptor stimulation remains unknown. No involvement of IP$_3$, protein kinase C, cyclic AMP, cyclic GMP

and arachidonic acid has been suggested, but an intermediate is likely because the sustained effect develops with a slow time course.

Recent evidence indicates that cytoskeletons, such as microfilament and microtubule, play a role not only for compartmentalization and anchoring of membrane proteins including receptors and ion channels, but also for regulation of their functions. Fukuda *et al.* (1981) suggested that in guinea-pig dorsal root ganglion neurons microtubule as well as microfilament regulates voltage-gated Na^+ channel activity and voltage-gated Ca^{2+} channel activity. Microtubule cytoskeleton has also been suggested to modulate voltage-gated Ca^{2+} channels in rat hippocampal pyramidal neurons (Johnson & Byerly, 1994) and chick embryonic ventricular myocytes (Galli & DeFelice, 1994).

A microtubule is a polymer composed of $\alpha\beta$ heterodimers of tubulin, and polymerization and depolymerization of the dimers are dynamically regulated by cytosolic Ca^{2+}, Mg^{2+}, GTP and some cytoskeleton-associated proteins (for reviews see Dustin, 1984). It has been suggested that tubulin is capable of modulating signal transduction elements including G proteins (Leiber *et al.*, 1993; Roychowdhury *et al.*, 1993; Roychowdhury & Rasenick, 1994). Ravindra *et al.* (1996) and Popova *et al.* (1997) reported that PTX-insensitive Gq protein and tubulin can interact with each other and as a result of the

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interaction, subsequent activation of PLC and polymerization-depolymerization cycle of microtubule cytoskeleton are modified. In PC12 cells, stimulation of muscarinic receptors induces a modulation of microtubule polymerization which is suggested to be due to dephosphorylation of microtubule-associated protein, τ (Sadot *et al*., 1996).

In the present study, we investigated the effects of agents which influence microtubule cytoskeleton, on muscarinic receptor-mediated suppression of voltage-gated Ca^{2+} channels in guinea-pig ileal smooth muscle. The results suggest that polymerization of microtubules or recruitment of tubulin molecule for the signal transduction is likely to occur during the sustained phase of muscarinic suppression of I_{Ca}.

Methods

Preparation of cells

Male guinea-pigs, weighing 350–450 g were stunned and killed by exsanguination. Single smooth muscle cells were isolated from the longitudinal layer of small intestine by enzymatic procedures, as previously described (Komori *et al*., 1992), and suspended in physiological salt solution (PSS; for composition, see below) containing 0.5 mM Ca^{2+}.

Pretreatment of cells with cytoskeletal agents

In general, agents, such as colchicine and taxol exert a time- and concentration-dependent action to form their complex with cytoskeletons. The action is exhibited slowly and it takes several hours to reach an equilibrium, especially, at a low concentration (1 μM or less) (Wilson *et al*., 1974; Dustin, 1984). In the present study, cells were pretreated with cytoskeletal agents at a high concentration of 100 μM for more than 1 h, unless otherwise stated.

The cell suspension in PSS containing 0.5 mM Ca^{2+} was divided into two or more parts. A cytoskeletal agent was added to one part of the cell suspension to give a required concentration and each cell suspension was placed on coverslips in a small aliquot and kept in a dark room at room temperature (21–25°C) until use on the same day. To evaluate the effect of a cytoskeletal agent, cells pretreated with the agent and cells incubated without the agent, but handled in an otherwise identical way, were used for recording membrane currents.

Recording of membrane currents

Membrane currents were recorded from single cells by use of the same patch-clamp techniques as described previously (Unno *et al*., 1995) and patch pipettes filled with a Cs^+-based pipette solution (for composition, see below) with a resistance of 3–6 MΩ. For recording of membrane currents from cells pretreated with a cytoskeletal agent, the agent was added to both the bathing solution and the pipette solution and continued to be present throughout the experiments. Voltage-gated Ca^{2+} channel current (I_{Ca}) was elicited repeatedly by depolarizing pulses of a brief duration (30 ms) from the holding potential of −60 to 0 mV applied at a frequency of 0.25 Hz. After the amplitude of I_{Ca} was stabilized, the solution in the recording chamber was replaced with PSS containing Ba^{2+} to use as a charge carrier. Effects of cytoskeletal agents on I_{Ca} were evaluated using Ba^{2+} current (I_{Ba}) flowing through voltage-gated Ca^{2+} channels. Carbachol (CCh) was applied extracellularly 2 min

after the replacement of Ca^{2+} with Ba^{2+} and the effect on I_{Ba} was investigated. Application of CCh was made by replacing the solution in the recording chamber with CCh-containing solution more than five times. The amplitude of I_{Ba} was estimated as the difference from the current level ($I_{Ba}=0$) obtained by application of the depolarizing pulse to the cell in the presence of 100 μM Cd^{2+} which was applied at the end of each experiment. For calculation of current density of I_{Ba}, membrane capacitance was measured from capacity current elicited by applying a 10 mV hyperpolarizing pulse.

When measuring K^+ current (I_K) flowing through voltage-gated K^+ channels, PSS containing Mn^{2+} instead of Ca^{2+} was used as the solution in the recording chamber and K^+-based pCa 6.5 solution (for composition, see below) as the pipette solution, and depolarizing pulses (2 s duration) to −40 mV or more positive potential (up to 80 mV) in 20 mV increments from the holding potential of −80 mV were applied. The amplitude of I_K was estimated by subtracting a leakage component from the evoked outward current, as described previously (Unno *et al*., 1996).

In experiments where Ca^{2+}-activated K^+ channel current (I_{KCa}) was recorded, PSS containing 2 mM Ca^{2+} was used as the solution in the recording chamber and K^+-based solution (for composition, see below) as the pipette solution, and cells were held under voltage-clamp at 0 mV. A change in intracellular Ca^{2+} concentration brought about by Ca^{2+} release from intracellular stores induced by CCh was detected by monitoring I_{KCa} (Komori *et al*., 1998).

The values in the text are presented as means ± s.e.mean. Statistical significance was tested by Student's unpaired *t*-test and differences were considered significant when $P<0.05$.

Solutions and drugs

PSS used in the experiments had the following composition (mM): NaCl 126, KCl 6, CaCl$_2$ 2, MgCl$_2$ 1.2, glucose 14; HEPES 10.5. When Ba^{2+} and Mn^{2+} were used for extracellular solution, the CaCl$_2$ was replaced with an equimolar solution of BaCl$_2$ and MnCl$_2$, respectively. Composition of patch-pipette solutions (mM) was as follows. Cs^+-based solution: CsCl 134, MgCl$_2$ 1.2, MgATP 1, NaGTP 0.1, glucose 14, HEPES 10.5, EGTA 0.05 (titrated to pH 7.2 with CsOH). K^+-based solution: KCl 134, MgCl$_2$ 1.2, MgATP 1, NaGTP 0.1, glucose 14, HEPES 10.5, EGTA 0.05 (titrated to pH 7.2 with KOH). K^+-based pCa 6.5 solution: KCl 80, MgCl$_2$ 2.5, MgATP 1, NaGTP 0.1, glucose 14, HEPES 10.5, BAPTA 20, CaCl$_2$ 13.3 (titrated to pH 7.2 with KOH). In this solution, Ca-BAPTA buffer was used to maintain the ionized calcium concentration at a level of pCa 6.5.

Drugs and chemicals used were baccatin III, 1, 2-bis (2-aminophenoxy) ethane-N, N, N′, N′-tetraacetic acid (BAPTA), cytochalasin B (from Helminthosporium dematioideum), lumicolchicine, taxol (from Taxus brevifolia), vinblastine (all from Sigma, St Louis, MO, U.S.A.), carbachol chloride (CCh), colchicine, phalloidin (Wako, Osaka City, Osaka, Japan) and ethylene glycol-bis (β-aminoethyl ether) N, N, N′, N′-tetraacetic acid (EGTA; Dojin Kagaku, Kamimasushiro-gun, Kumamoto, Japan). Stock solutions of baccatin III, cytochalasin B, lumicolchicine, phalloidin and taxol were 100% DMSO and the final concentration of DMSO was reduced to less than 0.2%. Treatment of cells with 0.2% DMSO for up to 7 h had no effect on I_{Ba} itself and CCh-induced suppression of I_{Ba}. All other drugs were dissolved in distilled water as their stock solutions.

Results

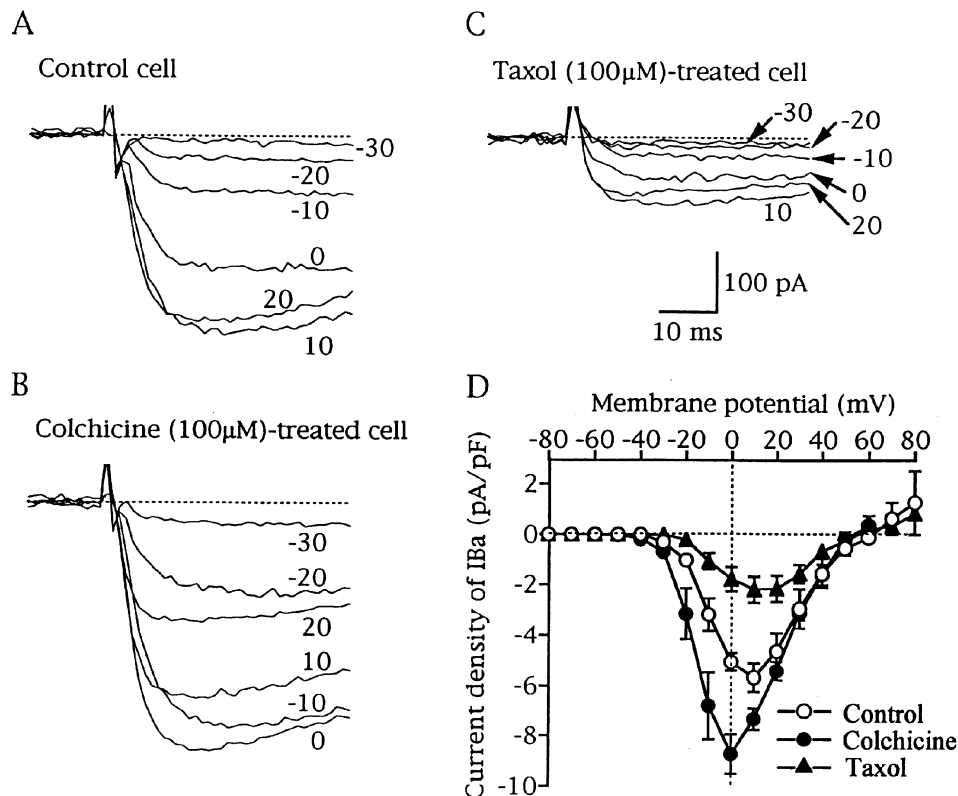*Effects of colchicine, vinblastine and taxol on voltage-gated Ca^{2+} channel current*

Cells were pretreated for 1–7 h with or without colchicine (100 μM), which binds to tubulin to inhibit polymerization of microtubules resulting in the disruption of the cytoskeleton. The mean treatment time was 3.9 ± 0.4 h ($n=20$) for colchicine-treated cells and 4.1 ± 0.4 h ($n=18$) for control cells. The pretreatment with colchicine increased somewhat the amplitude of I_{Ba} elicited by depolarizing pulses to 0 mV (Figures 1 and 2). Using the peak amplitude of I_{Ba}, recorded 10–15 min after achieving the whole-cell clamp configuration, and cell-capacitance measurement, the current density of I_{Ba} was estimated (see Methods). The current density of I_{Ba} varied between 4.5 and 14.6 pA•pF^{-1} among colchicine-treated cells with a mean of 8.2 ± 0.6 pA•pF^{-1} ($n=20$), and between 3.5 and 9.0 pA•pF^{-1} among control cells with a mean of 5.3 ± 0.4 pA•pF^{-1} ($n=18$). The difference between the two means was statistically significant, although the mean cell capacitance of 39.8 ± 1.8 pF ($n=20$) in colchicine-treated cells was not different from that (40.7 ± 1.9 pF, $n=18$) in control cells. When colchicine (100 μM) was applied extracellularly without pretreatment, the amplitude of I_{Ba} remained almost unaffected even 10 min after the application of colchicine (data not shown), indicating that colchicine takes a longer time to exhibit the action of a stimulant on I_{Ba} amplitude.

Pretreatment of cells with lumicolchicine (100 μM; mean treatment time: 4.2 ± 0.5 h, $n=8$), an inactive analogue of colchicine, did not change the current density of I_{Ba}. The mean current density of I_{Ba} of 6.0 ± 0.6 pA•pF^{-1} (mean cell capacitance: 42.6 ± 2.7 pF) was not significantly different from that in control cells, but it was significantly smaller than that in colchicine-treated cells. In cells pretreated with another microtubule disrupter, vinblastine (100 μM; mean treatment time: 2.9 ± 0.5 h, $n=7$), the mean current density of I_{Ba} (8.0 ± 0.9 pA•pF^{-1}; mean cell capacitance: 37.1 ± 3.6 pF) was also increased with a statistically significant difference from that in control cells.

The finding that disruption of microtubule cytoskeleton resulted in an increase of the activity of voltage-gated Ca^{2+} channels suggests that microtubule cytoskeleton may contribute to an inhibitory regulation of the Ca^{2+} channels.

Pretreatment of cells with taxol (100 μM), which polymerizes microtubule and stabilizes it, reduced markedly the amplitude of I_{Ba} (Figure 1C). The inhibitory effect of taxol on I_{Ba} was dependent on its treatment time. The pretreatment with 100 μM taxol for 30 min did not significantly change the mean current density of I_{Ba} (6.8 ± 1.2 pA•pF^{-1} ($n=4$) in taxol-treated cells and 5.3 ± 1.1 pA•pF^{-1} ($n=5$) in time-matched control cells). The reduction of I_{Ba} amplitude was evident when taxol was applied for 1 h and the inhibitory effect was maintained invariably up to 7 h. In cells pretreated with 1, 10 and 100 μM taxol for 1–7 h (mean treatment time: 3.7 ± 0.6 h for 1 μM, 4.2 ± 0.7 h for 10 μM, 4.4 ± 0.4 h for 100 μM), the mean current

Figure 1 Effects of colchicine and taxol on current-voltage relationships of Ba²⁺ current (I_{Ba}). I_{Ba} was elicited by depolarizing pulses (2 s duration) to various potentials ranging from -70 to 80 mV in 10 mV increments from the holding potential of -80 mV. (A, B and C) Superimposed current traces recorded from a drug-untreated cell (control cell), a colchicine (100 μM)-treated cell and a taxol (100 μM)-treated cell, respectively. The attached figures near current traces represent the potentials attained by the depolarizing pulses. Leak currents were subtracted and interrupted lines indicate zero I_{Ba} levels. (D) current-voltage relationships of I_{Ba} obtained from control cells ($n=7$), colchicine-treated cells ($n=8$) and taxol-treated cells ($n=7$). The peak currents normalized to cell capacitance were averaged, and the mean current densities were plotted against membrane potentials attained by depolarizing pulses.
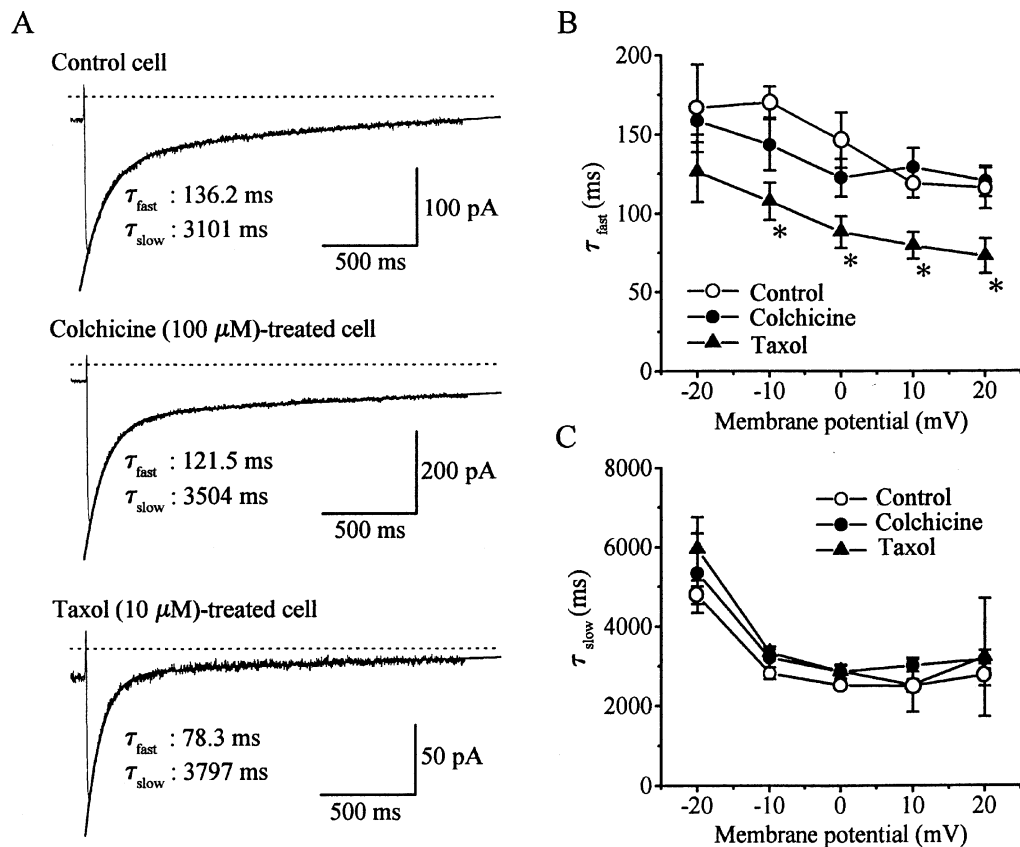
Figure 2 Effects of colchicine and taxol on the inactivation of I_{Ba}. I_{Ba} was elicited by depolarizing pulses (2 s duration) to various potentials ranging from -20 to 20 mV from the holding potential of -80 mV. (A) Current traces of I_{Ba} elicited by depolarizing pulses to 0 mV in a drug-untreated cell (control cell), a colchicine (100 μM)-treated cell and a taxol (10 μM)-treated cell. The smooth curves in the inactivation phase of I_{Ba} are the fitted two-exponential function and the time constants for the fast component (τ fast) and the slow component (τ slow) are shown. Note different calibration for each trace. (B and C) Voltage-dependence of τ fast and τ slow, respectively. Mean time constants averaged from seven control cells, eight colchicine-treated cells and seven taxol-treated cells were plotted against voltages attained by depolarizing pulses. *Statistically different ($P < 0.05$) from the corresponding mean value for control. Taxol pretreatment decreased the time constants of inactivation for the fast component.

density of I_{Ba} was 5.2 ± 0.6 pA•pF^{-1} ($n = 5$), 3.0 ± 0.6 pA•pF^{-1} ($n = 8$) and 1.5 ± 0.3 pA•pF^{-1} ($n = 17$), respectively. The latter two mean values were significantly smaller than the control (5.3 ± 0.4 pA•pF^{-1}, $n = 18$), although the mean cell capacitances were unchanged by any concentrations of taxol. The concentration of taxol to produce 50% inhibition was estimated as 16.0 μM (Hill coefficient: 0.8) by fitting of the data with a modified Hill equation: $P_{cont}/\{1 + ([taxol]/IC_{50})^n\}$, where P_{cont}, [taxol], IC_{50} and n are mean current density of I_{Ba} in control cells, taxol concentration, the concentration required 50% inhibition and the Hill coefficient, respectively.

When baccatin III (100 μM), a taxol analogue which has little effect on microtubules, was used instead of taxol (mean treatment time: 3.9 ± 0.6 h, $n = 9$), the mean current density of I_{Ba} (5.3 ± 0.4 pA•pF^{-1}; mean cell capacitance: 36.7 ± 1.9 pF, $n = 9$) was comparable to that in control cells.

These results suggest that polymerization of microtubule cytoskeleton may decrease the activity of voltage-gated Ca^{2+} channels.

Effects of colchicine and taxol on current (I)-voltage (V) relationships and the inactivation of voltage-gated Ca^{2+} channel current

I_{Ba} was elicited by depolarizing pulses from the holding potential of -80 mV to various membrane potentials ranging

between -70 and 80 mV in 10 mV increments. In colchicine-treated cells, the amplitude of I_{Ba} increased over a range of membrane potentials from -20 to 10 mV (Figure 1A and B). As shown in Figure 1D, peak potential in the I-V curve in colchicine (100 μM)-treated cells was shifted by some 10 mV in the negative direction without changing the apparent reversal potential of I_{Ba} (around 60 mV). Such effects on I_{Ba} were not observed in lumicolchicine (100 μM)-treated cells (data not shown). In taxol (100 μM)-treated cells, the current density of I_{Ba} was reduced by the same extent over a wide range of membrane potentials (Figure 1D), indicating that the inhibitory effect of taxol on I_{Ba} was voltage-independent.

As can be seen from Figure 1B and C, colchicine and taxol had little or no effect on the activation time course of I_{Ba}. The time to peak of I_{Ba} at 0 mV of 16.7 ± 1.6 ms ($n = 7$) in colchicine-treated cells and that of 16.6 ± 3.5 ms ($n = 7$) in taxol-treated cells were not statistically different from 18.3 ± 2.3 ms ($n = 7$) in control cells.

Effects of colchicine and taxol on the inactivation kinetics of I_{Ba} elicited by depolarizing pulses of a long duration of 2 s were investigated. The decay time course of I_{Ba} could be approximated well by the sum of two exponentials (fast and slow components) (Figure 2A), as previously reported by Unno *et al.* (1996). The time constants for fast and slow components in control cells decreased as the stepping potential was increased over a range of potentials between -20 and

10 mV (Figure 2B and C). This was also true for cells pretreated with colchicine (100 μM). However, pretreatment with taxol (10 μM) resulted in a decrease in the time constant for the fast component but not the slow component (Figure 2B and C).

Figure 3 shows effects of colchicine and taxol on voltage-gated K$^+$ channel current (I_K). The I_K was considered to represent the opening of both Ca^{2+}-dependent and -independent K$^+$ channels as described previously (Unno *et al.*, 1996). Pretreatment of cells with colchicine (100 μM) or taxol (100 μM) did not change the current density of I_K and the current-voltage relationship, indicating the agents have no effect on voltage-gated K$^+$ channels.

Effects of microtubule disrupters on carbachol-induced suppression of voltage-gated Ca^{2+} channel current

An attempt was made to see if microtubule cytoskeleton is involved in the muscarinic signal transduction responsible for carbachol (CCh)-induced suppression of I_{Ba}. CCh (10 μM), when applied extracellularly, induced a biphasic suppression of I_{Ba}, as previously reported by Unno *et al.* (1995); an initial transient component was followed by a sustained component persisting over the entire period of CCh presence in the bathing solution (Figures 4A and 5A).

In eight out of 13 colchicine (100 μM)-treated cells, CCh (10 μM) decreased I_{Ba} amplitude in the early period of 30 s, as shown in Figure 4B. After this period, I_{Ba} amplitude was only slightly reduced in five cells, remained almost unchanged in one cell, and rather increased in the remaining two cells (up to 19%). In the remaining five cells, CCh induced no I_{Ba} suppression irrespective of their transient and sustained components ($n=3$) or only a small sustained suppression which gradually developed (17.8%, $n=2$). In the seven colchicine-treated cells, which responded to CCh by a decrease in I_{Ba} during the corresponding period to the sustained phase of I_{Ba} suppression in control cells, the mean suppression of I_{Ba} was significantly smaller than the control (Table 1). Pretreatment of cells with vinblastine also decreased significantly the sustained component of CCh-induced I_{Ba} suppression (Table 1). Pretreatment of cells with lumicolchicine did not affect the action of CCh on I_{Ba} (Figure 4C and Table 1).

These results indicate that the sustained component of I_{Ba} suppression induced by CCh is reduced after disruption of microtubules. Therefore, it is possible that CCh suppresses I_{Ba} through polymerization of microtubules or by a mechanism which simply requires the existence of functional microtubule cytoskeleton. In addition, the inhibitory effects of colchicine and vinblastine on the transient component of I_{Ba} suppression (Table 1) suggest a possible involvement of microtubule cytoskeleton in the signalling process for this action as well.

Effect of taxol on carbachol-induced suppression of voltage-gated Ca^{2+} channel current

Pretreatment of cells with 10 μM taxol had little or no effect on the transient component but significantly reduced the sustained component of I_{Ba} suppression induced by CCh (10 μM) (Figure 5B and Table 1). When taxol was used at 100 μM, the amplitude of I_{Ba} was reduced to one-third or less of the control and the sustained component of CCh action on I_{Ba} was abolished in seven out of ten cells. In the remaining three cells, a slight sustained I_{Ba} suppression was observed (Table 1). The initial transient phase of I_{Ba} suppression was still induced by CCh, although the extent was significantly smaller than the control (Figure 5C and Table 1). Baccatin III (100 μM) was without effect on the transient and sustained components of I_{Ba} suppressions induced by CCh (Table 1).

The inhibitory effects of taxol (100 μM) on the CCh action on I_{Ba} as well as I_{Ba} itself were not observed in cells pretreated for 30 min: These cells responded to CCh (10 μM) by I_{Ba} suppression with transient (49.2$\pm$17.4%, $n=3$) and sustained (58.4$\pm$10.9%, $n=4$) components, which were not significantly different from mean values in time-matched control cells (52.8$\pm$10.9%, $n=4$ and 41.7$\pm$9.1%, $n=5$). Thus, the inhibitory effects of taxol on the CCh action on I_{Ba} seemed to develop with a similar time course to that on I_{Ba} itself.

It is, therefore, likely that the CCh action on I_{Ba} is reduced in time when microtubule cytoskeleton has already been polymerized.
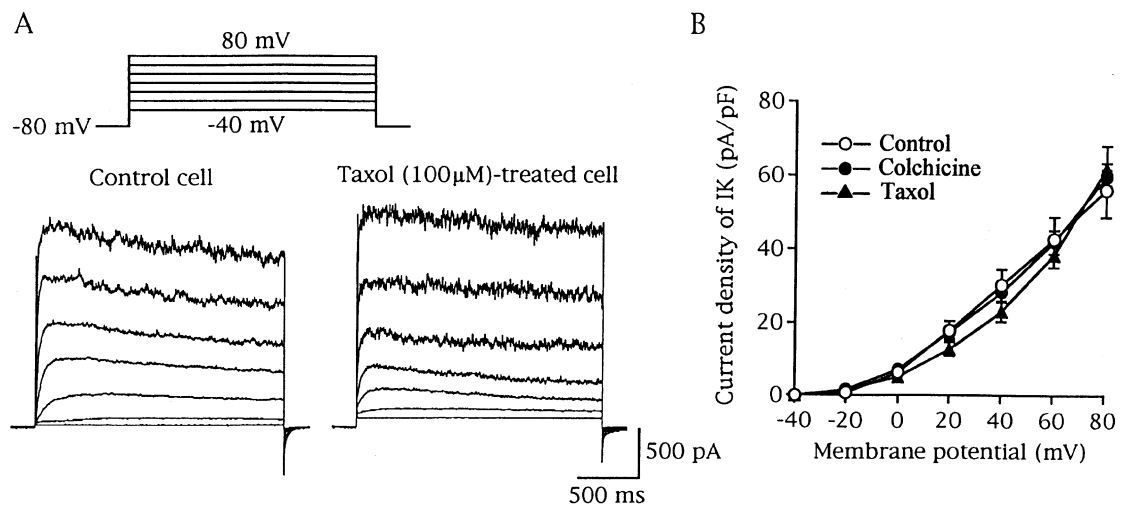
Figure 3 Effects of colchicine and taxol on K$^+$ current (I_K). I_K was elicited by depolarizing pulses (2 s duration) to test potentials of -40 to 80 mV in 20 mV increments from the holding potential of -80 mV. (A) Superimposed current traces recorded from a drug-untreated cell (control cell) and a taxol (100 μM)-treated cell. (B) Current-voltage-relationships of I_K obtained from control cells ($n=8$), colchicine (100 μM)-treated cells ($n=7$) and taxol (100 μM)-treated cells ($n=7$). The mean current densities were plotted against voltages attained by depolarizing pulses.
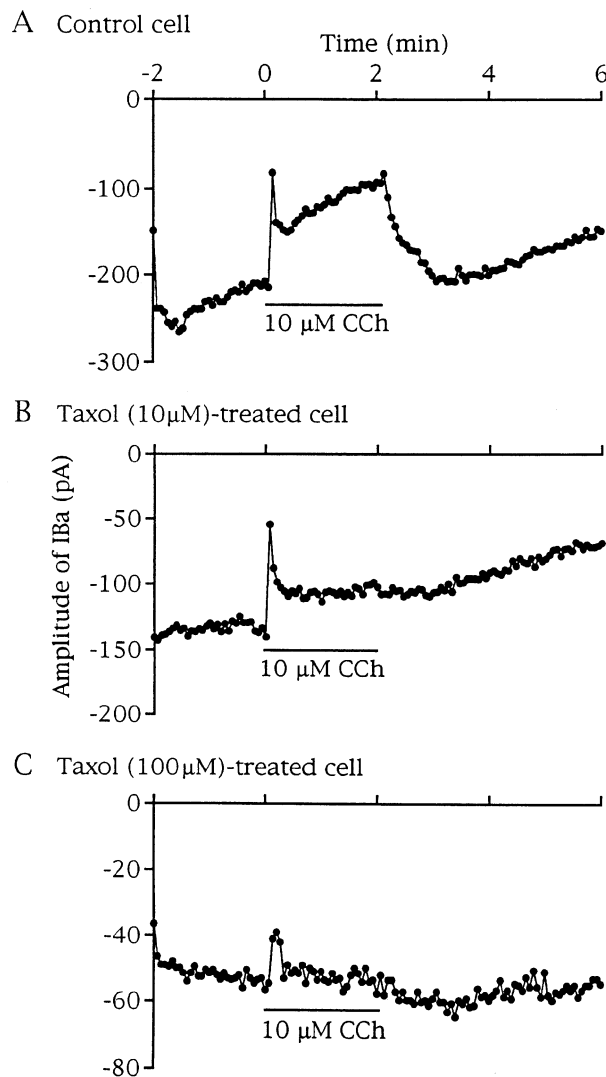
A   Control cell

B   Colchicine (100μM)-treated cell

C   Lumicolchicine (100μM)-treated cell
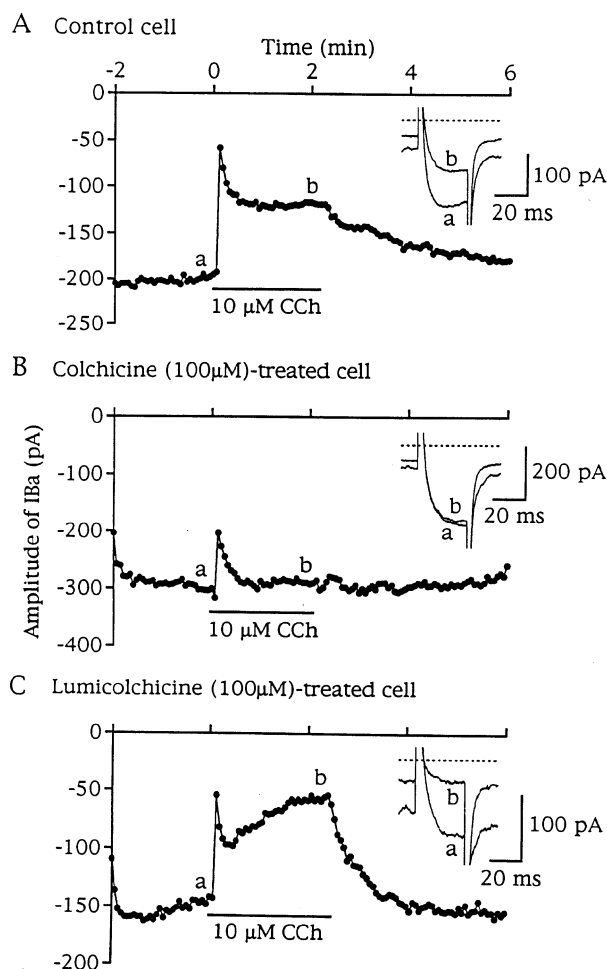
Figure 4 Effects of colchicine and lumicolchicine on transient and sustained components of carbachol (CCh, 10 μM)-induced suppression of I_{Ba}. I_{Ba} was repeatedly elicited by depolarizing pulses (30 ms duration) to 0 mV from a holding potential of -60 mV at 0.25 Hz. (A, B and C) Time-series plots for peak I_{Ba} amplitude (the beginning of CCh application was taken as zero) in a drug-untreated cell (control cell), a colchicine (100 μM)-treated cell and a lumicolchicine (100 μM)-treated cell, respectively. Examples of actual I_{Ba} traces for before (a) and during CCh application (b) are inserted in each plot. The downward deflection of holding current reflects the induction of non-selective cationic channel current induced by CCh. Pretreatment with colchicine, but not lumicolchicine, caused inhibition of the CCh-induced sustained suppression of I_{Ba}.

Effects of colchicine and taxol on carbachol-induced non-selective cationic channel current and Ca^{2+} release from intracellular stores

CCh not only suppressed I_{Ba} but also induced non-selective cationic channel current (I_{CCh}) in a biphasic manner (Unno *et al.*, 1995). The mean peak current densities of I_{CCh} in the transient and sustained components were 3.3 ± 0.6 pA•pF^{-1} ($n=8$) and 1.4 ± 0.2 pA•pF^{-1} ($n=13$) in colchicine (100 μM)-treated cells and 5.5 ± 1.9 pA•pF^{-1} ($n=6$) and 1.1 ± 0.2 pA•pF^{-1} ($n=10$) in taxol (100 μM)-treated cells, respectively. These mean values were not significantly different from the corresponding mean values in control cells (4.7 ± 0.6 pA•pF^{-1}, $n=11$ and 1.3 ± 0.2 pA•pF^{-1}, $n=12$, respectively).

The transient component of I_{CCh} is associated with IP$_3$-induced Ca^{2+} release from intracellular stores (Unno *et al.*, 1995). Therefore, both colchicine and taxol may not prevent

A   Control cell

B   Taxol (10μM)-treated cell

C   Taxol (100μM)-treated cell

Figure 5 Effect of taxol on transient and sustained components of CCh (10 μM)-induced suppression of I_{Ba}. (A, B and C) Time-series plots for peak I_{Ba} amplitude in a drug-untreated cell (control cell), a 10 μM taxol- and a 100 μM taxol-treated cell, respectively. Taxol pretreatment resulted in concentration-dependent inhibition of CCh-induced sustained suppression of I_{Ba}.

this pathway for Ca^{2+} release. In fact, transient activation of Ca^{2+}-activated K$^+$ channel current (I_{KCa}) induced by CCh, which can be used as an indicator of massive release of stored Ca^{2+} (Komori *et al.*, 1998), remained unchanged after treatment with colchicine and taxol. The mean peak current density of CCh (10 μM)-induced I_{KCa} was 100.1 ± 15.0 pA•pF^{-1} ($n=10$) in colchicine (100 μM)-treated cells and 119.6 ± 51.1 pA•pF^{-1} ($n=5$) in taxol (100 μM)-treated cells, which were not significantly different from 97.5 ± 16.8 pA•pF^{-1} ($n=9$) in control cells.

Thus, the cytoskeletal agents do not prevent activation of non-selective cationic channels and release of Ca^{2+} from intracellular stores induced by CCh.

Effects of cytochalasin B and phalloidin on carbachol-induced suppression of voltage-gated Ca^{2+} channel current

Pretreatment of cells with cytochalasin B (100 μM; mean treatment time: 3.7 ± 0.7 h, $n=6$) or phalloidin (100 μM; it was

Table 1 Effects of cytoskeletal agents on the transient and sustained components of carbachol (CCh)-induced suppression of Ba^{2+} current (I_{Ba})

	Transient suppression (%)		Sustained suppression (%)	
Control	58.0 ± 6.0	(11/12)	49.2 ± 4.2	(12/12)
Colchicine (100 μM)	32.7 ± 4.3	(8/13)*	8.6 ± 2.6	(7/13)*
Lumicolchicine (100 μM)	48.9 ± 6.8	(6/6)	50.2 ± 4.0	(6/6)
Vinblastine (100 μM)	30.8 ± 8.0	(4/6)*	13.7 ± 3.5	(6/6)*
Taxol (1 μM)	55.5 ± 8.9	(5/5)	46.5 ± 6.3	(5/5)
(10 μM)	56.0 ± 5.7	(8/8)	22.6 ± 6.9	(8/8)*
(100 μM)	30.9 ± 4.5	(6/10)*	9.1 ± 2.9	(3/10)*
Baccatin III (100 μM)	45.3 ± 4.8	(5/6)	48.8 ± 5.9	(6/6)
Cytochalasin B (100 μM)	63.4 ± 7.8	(6/6)	48.3 ± 10.3	(6/6)
Phalloidin (100 μM)	53.8 ± 10.9	(5/5)	48.3 ± 7.9	(5/5)

The mean percentage suppressions of I_{Ba} in the initial transient component (transient suppression) and the sustained component (sustained suppression) in response to CCh (10 μM) are shown. The numbers in parentheses are the number of cells where CCh produced a suppression of I_{Ba} compared to the total number of cells tested. The mean values for both components were obtained at the respective peaks in the responding cells. Each value represents the mean $\pm$ s.e.mean. *Indicates statistically significant differences ($P < 0.05$) from the control.

treated intracellularly for 10–15 min *via* the patch pipette), a disrupter and a stabilizer of actin microfilaments, respectively, did not reduce the current density of I_{Ba} and transient and sustained components of I_{Ba} suppression induced by CCh (Table 1). These results suggest that actin microfilaments are not involved in the muscarinic suppression of Ca^{2+} channel current.

Discussion

In the present study, we attempted to demonstrate a contribution of microtubule cytoskeleton to modulation of the activity of voltage-gated Ca^{2+} channels (VGCCs) in smooth muscle cells of the small intestine and to explore its possible involvement in suppression of the current through VGCCs induced by muscarinic receptor stimulation.

Pretreatment with colchicine, which inhibits tubulin assembly and dissociates microtubules into tubulin, increased VGCC current (I_{Ba}). Similar result was also obtained by another microtubule disrupter, vinblastine, which binds to tubulin at a different site from colchicine. Colchicine and vinblastine have been reported to increase intracellular cyclic AMP levels (Leiber *et al.*, 1993) which possibly affect VGCC activity. However, adenylate cyclase activators and membrane permeable analogues of cyclic AMP are incapable of increasing VGCC activity in intestinal smooth muscle cells (Beech, 1997). This makes it difficult to explain the effects of colchicine and vinblastine on I_{Ba} by their action to increase cyclic AMP levels. In contrast to the action of microtubule disrupters, taxol, which promotes tubulin assembly and stabilizes microtubules, decreased I_{Ba}. The inhibitory effect of taxol on I_{Ba} is not attributable to its action to depolymerize actin microfilament (Antin *et al.*, 1981), since cytochalasin B, an actin depolymer-

izer, had no effect on I_{Ba}. Colchicine and taxol appeared to be selective for VGCCs because the agents did not affect voltage-gated K channels and non-selective cationic channels induced by CCh (I_{CCh}). Given these findings, it is likely that the VGCC activity is accelerated under disruption of microtubule cytoskeletons, but it is reduced under polymerization of them. The findings that inactive analogues of the cytoskeletal agents had no effect on I_{Ba} may support this view.

Microtubules, in addition to microfilaments, have been suggested to play a role for compartmentalization and anchoring of some membrane proteins, including receptors and ion channels (Froehner, 1993). Therefore, a conformational change of microtubule cytoskeleton is expected to have an influence on ion channel activity. In fact, it has been demonstrated in rat myotubes and neurons that the depolymerization of cytoskeleton decreased receptors and ion channels in number (Tilson *et al.*, 1989; Froehner, 1993). However, this is not directly applicable to the present findings that the cytoskeletal depolymerization increased I_{Ba}. Furthermore, the current-voltage relationship and the inactivation curve of I_{Ba} were modified by the cytoskeletal agents, indicating that the observed modulation of the kinetics of I_{Ba} cannot be explained merely by changes in the number of VGCCs. Single channel recordings of VGCC activity in chick cardiac cells revealed that colchicine and taxol modulate inactivation kinetics of the VGCCs without changing the number of the channels (Galli & DeFelice, 1994).

A disruption of microtubule cytoskeleton might cause cell swelling as suggested in some cell types (Horie *et al.*, 1983; Häussinger *et al.*, 1993) and modulate ion channel activity. The activities of Ca^{2+}-activated K^+ channels in rabbit pulmonary artery (Kirber *et al.*, 1992) and of VGCCs in guinea-pig stomach (Xu *et al.*, 1996) are facilitated by cell inflation with a positive pressure applied *via* the patch pipette or a hypotonicity. Waniishi *et al.* (1997) demonstrated in the same type of smooth muscle cells as used in the present studies that a hypotonic cell swelling increased the amplitude of muscarinic non-selective cationic channel current as well as VGCC current. We have not observed increase in either Ca^{2+}-activated K^+ channel current or I_{CCh}, which is more sensitive to the hypotonicity-induced cell swelling than VGCC current (Waniishi *et al.*, 1997), but observed increase in VGCC current after treatment with colchicine and vinblastine. If the agents caused cell swelling, it would be expected that I_{CCh} was also facilitated noticeably, so that the data are not positive to a role of cell swelling in the observed increase in VGCC current.

Taxol, but not colchicine, accelerated the inactivation of I_{Ba} (Figure 2A). The results were opposite to previous findings in cardiac muscle cells (Galli & DeFelice, 1994) where the inactivation of Ca^{2+} channel current was accelerated by colchicine and slowed by taxol. The explanation for the differences is not clear, but it seems likely that the molecular basis for the interaction between microtubule cytoskeleton and calcium channel protein in smooth muscle cells may be different in some way from that in cardiac muscle cells. In general, VGCC in cardiac and smooth muscle cells consists of α_1-subunit forming channel pore and other auxiliary subunits including β- and α_2/δ-subunits. The voltage-dependence of the channel is an intrinsic property of the α_1-subunit which is affected by the auxiliary subunits (for reviews see McDonald *et al.*, 1994). The β-subunit is a cytoskeleton-binding protein and directly interacts with a linker region of the α_1-subunit protein (Pragnell *et al.*, 1994) to modulate activation and inactivation kinetics. In smooth muscle cells, the type of β-subunit is thought to be different from that in cardiac muscle cells (β_3 in smooth muscle which does not contain consensus protein

kinase A phosphorylation sequences) (McDonald *et al.*, 1994). Therefore, changes in physical state of microtubule cytoskeleton might affect conformation of the β-subunit differently between cardiac and smooth muscle cells and this might be responsible for the different regulation of the inactivation kinetics by the cytoskeleton.

It is interesting to think about a possible involvement of microtubule cytoskeleton in the sustained suppression of VGCC current induced by CCh. We have previously suggested that a cytosolic factor, which is well preserved during dialysis with the pipette solution, or a membrane-bound or associated factor is involved in exerting the effect of CCh, since the CCh effect can be observed some 30 min after achieving the conventional whole-cell clamp configuration (Unno *et al.*, 1995). Microtubule cytoskeleton possesses such properties and is a potential candidate as a factor mentioned above. Pretreatment of cells with colchicine and vinblastine, but not lumicolchicine, resulted in a marked decrease or abolition of the sustained component of I_{Ba} suppression induced by CCh. In connection with the data, colchicine and vinblastine have been shown to increase the sensitivity to a muscarinic agonist in producing contraction in guinea-pig ileal tissue (Famaey *et al.*, 1977). These findings lend support to an involvement of microtubule cytoskeleton in the CCh action on I_{Ba}.

The inhibitory effects of colchicine and taxol on the CCh action are not considered to result from a decrease in the number of muscarinic receptors, since the binding studies revealed that these agents did not produce changes in the number of muscarinic receptors and in the binding of a muscarinic agonist to the receptor (McKay *et al.*, 1985; 1991). In fact, I_{CCh} and CCh-induced Ca^{2+} release from intracellular stores, mediated by $G_{i/o}$ type G protein coupled with M_2 subtype of muscarinic receptor and Gq type G protein coupled with M_3 subtype of muscarinic receptor, respectively (Zholos & Bolton, 1997; Komori *et al.*, 1998), remained unchanged after treatment with colchicine and taxol. The results also indicate that the agents do not disturb muscarinic receptor-G protein coupling in a non-specific manner.

Considering that tubulin is a common target for the cytoskeletal agents used in the present study (colchicine, vinblastine and taxol), it is conceivable that tubulin-related mechanisms may be involved in the muscarinic signalling linked to VGCCs. Tubulin molecule exists in membrane-bound and cytosolic forms, and recent evidence provides the molecule with variety of roles in different signal transduction pathways (Roychowdhury *et al.*, 1993; Roychowdhury & Rasenick, 1994; Popova *et al.*, 1997). Tubulin has GTPase activity which enables its interaction with several signal transduction elements, such as receptor-coupled G proteins and protein kinases (Rasenick *et al.*, 1989; Huby *et al.*, 1995; Ravindra *et al.*, 1996; Garcia-Rocha *et al.*, 1997). If muscarinic signal transduction utilizes tubulin as an intermediate, the cytoskeletal agents could interact directly with tubulin resulting in perturbation of the signal transduction. It has been demonstrated that colchicine and taxol are capable of inhibiting GTPase activity of tubulin molecule independently of their ability to depolymerize or polymerize microtubules (Ravindra & Aronstam, 1993; and the references therein). Therefore, the finding that both colchicine and taxol, which have opposite effects on microtubule cytoskeleton, inhibit the CCh action on I_{Ba}

may be explained by such a dual action of the agents on tubulin molecule itself and microtubules.

Another possible interpretation of the present findings is that CCh polymerizes microtubule to suppress I_{Ba}. The inhibitory effect of taxol on I_{Ba} was accompanied by acceleration of the channel inactivation. This is similar to that obtained with CCh (Unno *et al.*, 1996). Furthermore, microtubule requires low but certain levels of $[Ca^{2+}]_i$ ($<1~\mu$M) for its polymerization (Dustin, 1984), as does the inhibitory effect of CCh on I_{Ba} (Unno *et al.*, 1996). The effects of CCh and taxol on I_{Ba} were not additive, and after treatment with taxol at 100 μM high enough to produce the maximal effect on I_{Ba}, CCh could no longer suppress I_{Ba}. The results are explained if CCh and taxol are working through the same mechanism. Colchicine and vinblastine may act to prevent the polymerization induced by CCh. There are some reports which lead us to suppose signalling pathways between muscarinic receptors and microtubule polymerization. It has been suggested that Gq type G protein can interact directly with tubulin and stimulate its assembly to microtubules (Ravindra *et al.*, 1996). Popova *et al.* (1997) have reported that phosphatidyl inositol bisphosphate (PIP_2) can bind to tubulin molecule and modulate tubulin assembly. If so, activation of Gq type of G protein by muscarinic receptor stimulation produces a reduction of PIP_2 levels resulting from activation of phospholipase C and thereby free tubulin is released from PIP_2-bound tubulin, which in turn may facilitate formation of microtubules leading to a decrease in VGCC activity. As suggested in other cell types (Sadot *et al.*, 1996; Fromm *et al.*, 1997; Schmidt *et al.*, 1997) muscarinic receptor stimulation might activate a small molecular weight G protein or a phosphatase which are both known to promote polymerization of cytoskeletons (Murthy & Flavin, 1983; Yamamoto *et al.*, 1988; Takai *et al.*, 1995). Thus, it seems likely that microtubule polymerization is involved in the signal transduction of the muscarinic inhibitory effect on the VGCC activity.

The transient component of I_{Ba} suppression induced by CCh, which is brought about by Ca^{2+} release from intracellular stores, was also inhibited by the cytoskeletal agents to a smaller extent, compared with the sustained component. This may result from a role of cytoskeleton in the Ca^{2+}-induced inactivation process of VGCCs, as suggested in hippocampus pyramidal neurons of adult rats (Johnson & Byerly, 1994) and cardiac cells of chick embryos (Galli & DeFelice, 1994).

In summary, the present studies suggest that microtubule cytoskeleton may modulate the activity of VGCCs in ileal smooth muscle cells of the guinea-pig and that muscarinic receptor stimulation induces sustained suppression of the VGCC current through a process, which is probably polymerization of microtubules or recruitment of tubulin molecule for the signal transduction. To determine the full significance of our results, more direct evidence, such as visualization of the state of microtubule, or a biochemical measurement of tubulin assembly, may be needed.

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