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Inhibitors of spasmogen-induced Ca^{2+} channel suppression in smooth muscle cells from small intestine

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1 Whole-cell patch-clamp recordings were made from smooth muscle cells isolated from the longitudinal muscle layer of guinea-pig ileum. Carbachol (acting at muscarinic receptors) or histamine (acting at H_1 histamine receptors) suppressed Ca^{2+} channel current. The effect of either agonist had an initial transient component followed by a sustained component.

2 Wortmannin inhibited transient and sustained components of carbachol-induced Ca^{2+} channel current suppression: half-effective inhibitory concentrations (IC_{50}) were 1.1 μM and 0.6 μM for the two components respectively. Wortmannin also inhibited the transient phase of carbachol-induced cationic current (IC_{50} 1.6 μM) and Ca^{2+}-dependent K^+-current (IC_{50} 1.7 μM). Wortmannin did not appear to produce any direct block of cationic channels or Ca^{2+} channels.

3 Intracellular application of the phospholipase inhibitor D609 (tricyclodecan-9-ylxanthogenate) inhibited transient and sustained components of histamine action on the Ca^{2+} channel current: the IC_{50} was about 130 μM for both components. Carbachol action on Ca^{2+} channels was also inhibited by D609. D609 had no significant direct blocking effect on Ca^{2+} channels, cationic channels activated by carbachol, or Ca^{2+}-activated K^+-current in response to flash-photolysis of caged-inositol 1,4,5-trisphosphate.

4 Micromolar concentrations of wortmannin and D609 are inhibitors of both components of spasmogen-induced Ca^{2+} channel suppression. The data suggest that both components are mediated by a common, or similar, signal transduction element which is a phospholipase C (PLC) or phospholipase D (PLD) isoform.

Keywords: Calcium; muscarinic receptors; histamine H_1 receptors; intestine; phospholipase C; smooth muscle cells

Introduction

Contraction is the usual net effect of acetylcholine or histamine on the guinea-pig small intestine. It is, therefore, surprising that recordings from single smooth muscle cells isolated from the tissue have revealed that both agonists induce inhibition of Ca^{2+}-current through voltage-gated Ca^{2+} channels (Beech, 1993; Unno *et al*., 1995). This inhibitory effect is partial and presumably does not prevent sufficient Ca^{2+} from entering the cell to cause contraction. The inhibition is evident in the whole tissue as histamine- or carbachol-induced relaxation of high KCl-induced contraction (Bolton *et al*., 1981; Mitsui & Karaki, 1990), and it may be a mechanism for heterologous desensitization (Himpens *et al*., 1991). Bradykinin and substance P also suppress Ca^{2+}-current in ileal smooth muscle cells, as does noradrenaline in portal vein smooth muscle cells, acetylcholine in urinary bladder and tracheal smooth muscle cells and oxytocin in myometrial smooth muscle cells (Pacaud *et al*., 1987; Inoue *et al*., 1992; Beech, 1993; Unno *et al*., 1995; Yoshino & Yabu, 1995; Wade *et al*., 1996). Similar effects of agonists on Ca^{2+} channels have also been observed in neurones (Beech *et al*., 1991; Hille, 1994). Therefore, Ca^{2+}-current inhibition by spasmogenic agonists may be a common phenomenon in smooth muscle and related effects may occur in other cell types.

The mechanisms of the inhibitory agonist actions on Ca^{2+} channels have only been partially elucidated. A key starting point in attempting to determine the mechanisms has been the fact that acetylcholine, histamine, bradykinin and substance P all stimulate phosphatidylinositol hydrolysis in ileal smooth muscle (Jafferji & Michell, 1975; Best *et al*., 1985; Ransom *et al*., 1992). Indeed, this pathway is important for the suppressive effects of the agonists on Ca^{2+} channels. The initial transient phase of Ca^{2+}-current inhibition occurs because IP_3 induces Ca^{2+}-release from intracellular stores (Komori & Bolton, 1991; Beech, 1993; Unno *et al*., 1995). The resulting rise in intracellular Ca^{2+} levels is presumed to inhibit Ca^{2+} channel current *via* the Ca^{2+}-induced inactivation phenomenon, which may involve a direct action of Ca^{2+} on the Ca^{2+} channel α_1-subunit (Zhou *et al*., 1997). The transient component of agonist action on Ca^{2+} channels is, however, followed by sustained inhibition which occurs independently of IP_3-mediated Ca^{2+}-release (Beech, 1993; Unno *et al*., 1995). The coupling mechanism for the sustained effect has not been established but a basal level of intracellular Ca^{2+} and activated G-protein are obligatory (Beech, 1993; Unno *et al*., 1995; Beech, 1997). Capacitative Ca^{2+}-entry (I_{CRAC}) is not required because the sustained effect occurs in the complete absence of extracellular Ca^{2+} (and other divalent cations) when the Ca^{2+} channel current is carried by Na^+, and when intracellular Ca^{2+} is heavily buffered to a fixed level by 20 mM BAPTA (Beech, 1993).

The possibility that transient and sustained components of Ca^{2+} channel suppression may be linked to phospholipid metabolism has led us to investigate the effects of wortmannin and D609, which are reported to inhibit phospholipases (Müller-Decker, 1989; Powis *et al*., 1991; Schütze *et al*., 1992; Gratas & Powis, 1993; Cross *et al*., 1995; Bonser *et al*., 1991). U73122, another agent reported to inhibit phospholipases (Powis *et al*., 1991), was not investigated because it directly blocks Ca^{2+} channels (Macrez-Leprêtre *et al*., 1996).

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Methods

Male guinea-pigs, weighing 350–450 g were killed by stunning and exanguination. Smooth muscle cells were isolated from the longitudinal layer of the small intestine using enzymatic and mechanical procedures described previously (Unno *et al*., 1995; McHugh & Beech, 1995). Prior to making recordings, cells were suspended in bath solution containing 0.5 mM Ca^{2+} and stored at 4°C until use on the same day. All recordings were made at room temperature (21–26°C).

Membrane currents were recorded from isolated smooth muscle cells in the conventional whole-cell configuration of the patch-clamp technique (voltage-clamp recordings) and using an SEZ-2300 (Nihon Kohden, Japan) or Axopatch 1D patch-clamp amplifier (Axon Instruments, Inc., U.S.A.). Current signals were filtered at 0.5 or 1 kHz (−3 dB, Bessel) and then digitized at 1 or 4 kHz using either a 1401-plus CED analogue-to-digital converter (Cambridge Electronic Design (CED) Ltd., U.K.) with a 486 IBM-compatible PC, or the MacLab 4 (AD Instruments, Australia) data acquisition system with a Macintosh IIVX or LC 475 computer (Apple, U.S.A.). Patch pipettes were made from borosilicate glass (Clark Electromedical Instruments: outside and inside diameters of 1 and 0.58 mm respectively) and had resistances of 2–6 MΩ after fire-polishing and when filled with pipette solution. The solution in the recording chamber was exchanged using a gravity-flow perfusion system with multiple input reservoirs. The bath volume was 100–150 μl and the flow rate through the bath was about 2–4 ml min^{-1}; solutions were fully exchanged in <1 min. No corrections were made for liquid junction potentials, which were <2 mV. Measurements of membrane capacitance and series resistance were made from the amplitude and single exponential decay time-constant of capacity current, which was sampled at 20 kHz and filtered at 10 kHz, and elicited by applying a 10-mV hyperpolarizing pulse.

When measuring Ca^{2+} channel current, depolarizing pulses were applied at a high frequency of 0.2 or 0.25 Hz in order to allow detection of the transient component of Ca^{2+} channel current inhibition. Although the pulses were brief, in some experiments there was run-down of current amplitude, especially if Ca^{2+} was used as the charge carrier. If run-down was detected, its time-course was described by an exponential curve so that the extent of run-down at the time of agonist-induced current inhibition could be estimated. Leakage current (non-Ca^{2+} channel current) was estimated in the presence of 100 μM Cd^{2+} which was applied at the end of each experiment.

Flash photolysis was performed with a XF-10 xenon flashlamp system equipped with a UG-11 filter and using a charge voltage of 100 V (Hi-Tech Scientific, Salisbury, U.K.). Flashes in the absence of caged-compound did not induce K^+-current.

All statistical comparisons were made using unpaired Students *t*-tests and differences were taken to be statistically significant if $P<0.05$. Results are expressed as means ± s.e.-mean. The value of 'n' indicates number of isolated cells. Data presentation and mathematical fitting of functions to data using a least-squares method were performed by the program Origin 4.1.

For the experiments relating to the study of wortmannin (Figures 1–3) and ML-7, bath solution contained (mM) NaCl, 126; KCl, 6; $CaCl_2$, 2; $MgCl_2$, 1.2; glucose, 14; HEPES 10.5 (pH 7.2). In experiments where Ba^{2+}-current was recorded the $CaCl_2$ was replaced with an equimolar concentration of $BaCl_2$. The patch pipette solution contained (mM): CsCl or 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 CsOH or KOH). For the experiments relating to the study of D609 (Figures 4 and 5) the pipette solution contained (in mM) CsCl, 130; $MgCl_2$, 2; $CaCl_2$, 0.05; HEPES, 10; EGTA, 0.2; Na_2ATP, 3 and NaGTP 0.1, and the bath solution contained (in mM) NaCl, 135; $MgCl_2$, 1.2; $CaCl_2$, 1.5; glucose, 8 and HEPES, 10. For K^+-current recording, KCl replaced CsCl in the pipette and 5 mM of the NaCl was replaced by 5 mM KCl in the bath solution. All solutions were titrated to pH 7.4 and filtered (pore size 0.2 μm; Gelman Sciences). Pipette solutions were frozen in aliquots at −18°C.

For the pretreatment of cells with wortmannin the cell suspension in bath solution containing 0.5 mM Ca^{2+} was divided into two volumes. Wortmannin was added to one volume and allowed to act in a dark room at 25°C for 60 min and then the cell suspension was centrifuged at 600 r.p.m. for 2 min. The cells were re-suspended in wortmannin-free solution before being placed on coverslips. Control cells experienced the same procedure but in the absence of wortmannin. Control and test conditions were not compared strictly alternately and so the total numbers of test and control recordings were not necessarily equal.

Carbachol chloride (CCh) and wortmannin (WT) were from Wako (Osaka City, Osaka, Japan). 1-(5-iodonapthalene-1-sulonyl)-1H-hexahydro-1, 4-diazepine hydrochloride (ML-7) was from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.), and myosin kinase inhibiting peptide (KKRAARATS-NH_2) was from Peninsula Laboratories (Belmont, CA, U.S.A.). EGTA (ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid), HEPES (N-2-hydroxy-ethylpiperazine-N′-2-ethanesulphonic acid), D609 (tricyclodecan-9-ylxanthogenate), diC_8 and histamine were purchased from Sigma. Pertussis toxin and D-*myo*-inositol 1,4,5-triphosphate, $P^{4(5)}$-1-(2-nitrophenyl)-ethyl ester (NPE-caged IP_3) were from Calbiochem. (Heat-inactivated pertussis toxin was produced by boiling pertussis toxin for 15 min). Stock solutions of WT and ML-7 were in 100% DMSO and the final concentration of DMSO was <0.1%. All other drugs were dissolved in distilled water, or directly in bath or pipette solution. Salts were purchased from either BDH, Sigma or Aldrich.

Results

Extracellular application of carbachol or histamine induced a rapid inhibition of Ca^{2+} channel current. Over the next 10–20 s, in the continued presence of agonist, there was recovery of current amplitude, but recovery was partial and, in some recordings, the development of a second component of inhibition was clearly evident. Thus, the agonist effects were characterized by having transient and sustained components (Figures 1 and 4). After wash-out of either agonist, Ca^{2+} channel current amplitude recovered slowly to the control level (Figure 1; Beech, 1993; Unno *et al*., 1995).

Application of carbachol not only inhibited Ca^{2+} channel current but also induced monovalent cationic current (Figure 1; Benham *et al*., 1985). Histamine also elicited cationic current in some cells but this current was much smaller than with carbachol (Figure 4; Komori *et al*., 1992). Cationic current was not expected to contaminate study of Ca^{2+} channel current because it reversed near 0 mV and Ca^{2+} channels were activated by depolarization to 0 mV.

Similarity between the pathways which mediate transient and sustained components of Ca^{2+} channel suppression has previously been suggested by the observation that pertussis

toxin pretreatment has no effect on transient or sustained effects induced by carbachol (Unno *et al.*, 1995). To check whether this was also true for the action of histamine, ileal smooth muscle strips were preincubated for 24 h at 37°C with 0.2 μg ml^{-1} pertussis toxin (test cells) or heat-inactivated pertussis toxin (time-matched control cells) prior to isolation of single smooth muscle cells and recording. In time-matched control cells, 10 μM histamine induced transient and sustained suppressions of Ca^{2+}-current which averaged 64.0$\pm$2.3% and 45.5$\pm$5% reductions in current amplitude respectively (n=4 for each). In test cells, 10 μM histamine induced transient and sustained suppressions of Ca^{2+}-current which averaged 60.4$\pm$2.5% and 44.8$\pm$1.7% reductions in current amplitude (n=4 for each). Pertussis toxin had inhibited G_i GTP-binding proteins in these cells because 10 μM carbachol did not induce cationic current in the four test cells, whereas it did in all of the four time-matched control cells. Therefore, both components of agonist action on Ca^{2+} channels are resistant to pertussis toxin and the pathways are distinct from that mediating activation of cationic channels which is pertussis toxin-sensitive.

Pretreatment of cells with wortmannin did not significantly reduce the amplitude of Ca^{2+} channel current: the mean peak Ba^{2+}-currents elicited by depolarization to 0 mV were -6.36 ± 0.88 pA.pF^{-1} (n=11) and -4.59 ± 0.31 pA.pF^{-1} (n=12) in untreated and 10 μM-wortmannin treated cells respectively. Wortmannin did, however, inhibit transient and sustained components of Ba^{2+} current modulation induced by 10 μM carbachol (Figure 1). Transient and sustained components of carbachol action on Ba^{2+} current were inhibited similarly (IC_{50} values were 1.1 μM and 0.6 μM respectively; Figure 2). Cationic current was still induced in all cells after preincubation with wortmannin (Figure 1) but the character of the current was different because the initial transient cationic

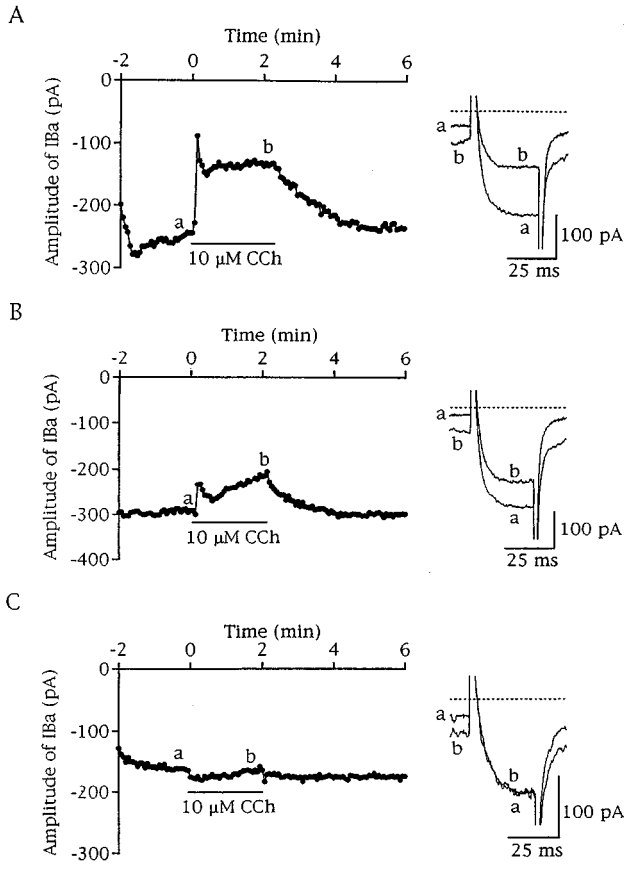

Figure 1 Transient and sustained components of carbachol-induced suppression of Ca^{2+} channel current are inhibited by wortmannin (WT). Ba^{2+}-current was elicited by 30-ms depolarizing pulses to 0 mV from a holding potential of -60 mV and at 0.25 Hz. On the left of (A), (B) and (C) are time-series plots for peak Ba^{2+}-current amplitude w here time zero indicates when the 10 μM carbachol application was initiated. (A) is for a control cell (no WT), (B) is for a 1 μM WT-treated cell, and (C) is for a 10 μM WT-treated cell. Examples of actual current records for before (a) and at the end of carbachol's application (b) are shown on the right. The downward deflection of holding current (a *c.f.* b) reflects the induction of cationic current.

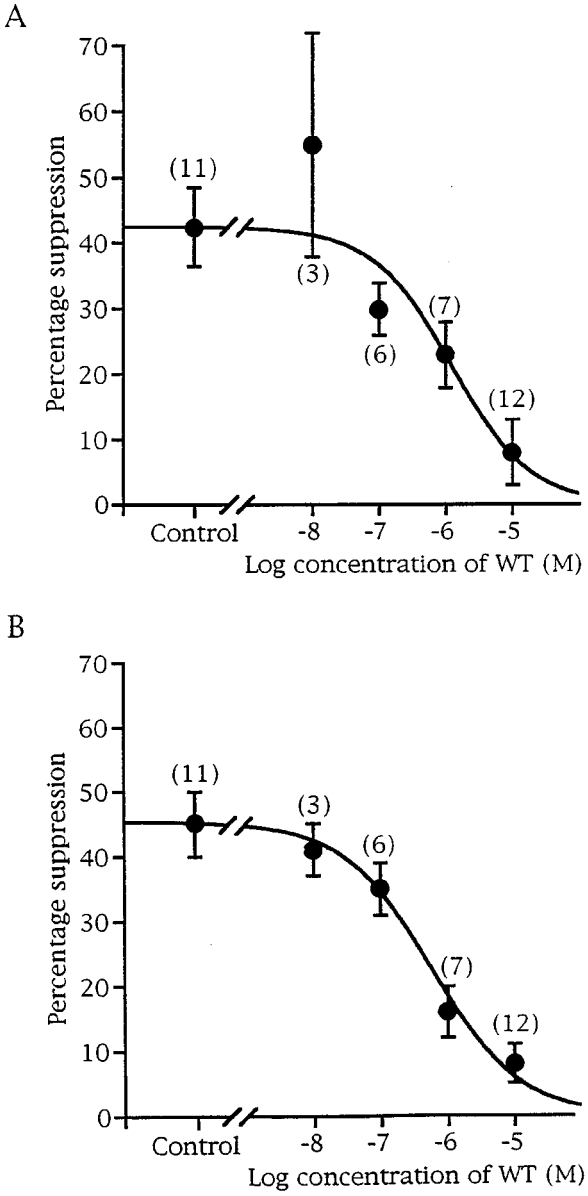

Figure 2 Concentration-response curves for inhibition of carbachol action on Ca^{2+} channels by wortmannin (WT). Mean percentage suppression of Ba^{2+}-current by carbachol (10 μM) is plotted as closed circles against the concentration of WT for transient (A) and sustained (B) components of carbachol's action. The sustained component was measured 2 min after application of carbachol had been initiated. In this figure and in Figure 3, vertical bars and parenthesized numbers indicate s.e.mean and the number of cells used for measurements respectively. The smooth curves are the Hill equation: $P_{cont}/\{1+([WT]/IC_{50})^s$, where P_{cont}, [WT], IC_{50} and s are mean percentage suppression in WT-untreated cells (control), WT concentration, the concentration required for WT to produce 50% inhibition, and the Hill coefficient respectively. (A) IC_{50} 1.1 μM, s=0.7. (B) IC_{50} 0.6 μM, s=0.8.

current was inhibited (IC_{50} 1.6 μM) whereas the sustained phase was unaffected (Figure 3A). The initial phase of cationic current is associated with IP_3-induced Ca^{2+}-release from sarcoplasmic reticulum. Further evidence that wortmannin inhibited this pathway for Ca^{2+}-release came from experiments with Ca^{2+}-dependent K^+-current which can be used as an indicator of submembrane intracellular Ca^{2+} levels. The induction of this K^+-current was inhibited by wortmannin with an IC_{50} of 1.7 μM (Figure 3B). The effect was probably not explained by direct inhibition of BK_{Ca} channels by wortmannin because 10 mM caffeine induced an outward K^+-current at 0 mV of 2.3 ± 0.5 nA ($n=4$) in cells treated with 10 μM wortmannin, which is similar to that observed in control cells (Komori *et al*., 1992).

Wortmannin inhibits myosin light chain kinase (MLCK) (Nakanishi *et al*., 1994) at the concentrations which were effective against carbachol-induced Ca^{2+} channel suppression. We, therefore, investigated whether MLCK might be involved by using ML-7 and MLCK inhibiting peptide which are reported to inhibit MLCK (Pearson *et al*., 1986; Saitoh *et al*., 1987). Inclusion of 100 μM ML-7 or 100 μM $MLCK_i$ peptide in the recording pipette solution did not significantly affect Ca^{2+} channel suppression: the percentage transient and sustained Ba^{2+}-current suppressions induced by 10 μM carbachol were $62.8 \pm 15.0\%$ and $44.8 \pm 7.0\%$ ($n=4$ for each) in the presence of ML-7 and $48.0 \pm 6.4\%$ and $46.4 \pm 10.5\%$ ($n=5$ for each) in the presence of $MLCK_i$ peptide. In control recordings the transient and sustained values were $42.5 \pm 6.0\%$ and $45.0 \pm 5.0\%$ ($n=11$ for each).

D609 inhibits phosphatidylinositol-specific (PI) or phosphatidylcholine-specific (PC) PLC and PLD (Müller-Decker, 1989; Powis *et al*., 1991; Schütze *et al*., 1992; Gratas & Powis, 1993). We used D609 intracellularly, without including it in the bath solution. This was achieved by making recordings alternately from separate cells with and then without D609 in the patch pipette. Histamine was bath-applied 10 min after breaking the cell-attached patch (i.e. after starting whole-cell recording). Using the method of Pusch & Neher (1988) and our series resistance (9 MΩ) and cell-capacitance (39 pF) measurements, it was estimated that the average intracellular concentration of D609 after 10 min of recording was about 70% of that included in the patch pipette (i.e. 126 μM when the patch pipette concentration of D609 was 180 μM). There was partial inhibition of both transient and sustained components of histamine action on Ca^{2+}-current when 180 μM D609 was in the pipette, and abolition of both components when 1.8 mM D609 was in the pipette (Figures 4 and 5). Fitting of the data with a one site binding isotherm indicated that IC_{50} values for D609 inhibition of transient and sustained components were roughly 130 μM (Figure 5A and B). Carbachol is more efficacious than histamine and so it induced additional Ca^{2+}-current suppression when current had already been suppressed in the presence of 10 μM histamine. The additional Ca^{2+}-current suppression induced by carbachol in the presence of histamine was measured with reference to the Ca^{2+}-current amplitude immediately before the application of carbachol (i.e. in the presence of histamine). This effect of carbachol was measured with and without 1.8 mM D609 in the patch pipette and was found to be reduced from $70.3 \pm 5.7\%$ to $23.9 \pm 3.5\%$ by D609 (six cells for each group).

In order to test whether D609 might have inhibited agonist-induced Ca^{2+} channel suppression because it inhibited IP_3-mediated Ca^{2+}-release from sarcoplasmic reticulum we circumvented receptor-coupling to IP_3 levels and released free IP_3 in cells by flash-photolysing NPE-caged IP_3, which was included in the patch pipette at a concentration of 100 μM. The resulting rise in intracellular Ca^{2+} levels was detected by recording Ca^{2+}-dependent K^+-current. The peak amplitude and half-decay time of K^+-current at 0 mV which was induced by flash-photolysis of NPE-caged IP_3 were 749 ± 305 pA ($n=8$) and 176 ± 65 ms ($n=7$) and 1222 ± 318 pA ($n=4$) and 324 ± 156 ms ($n=4$) for control and 1.8 mM D609 groups

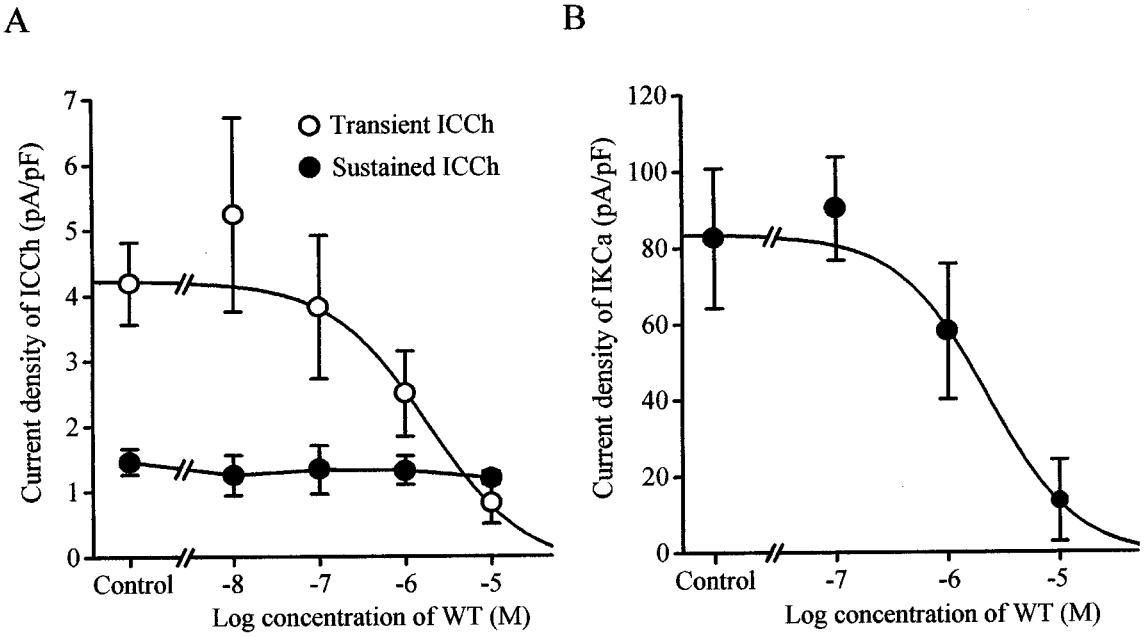

Figure 3 Effect of wortmannin (WT) on carbachol-induced cationic current and Ca^{2+}-dependent K^+-current. (A) Mean amplitude of cationic current induced by 10 μM carbachol is plotted against WT concentration for the transient (open circles) and sustained (closed circles) components of the cationic current measured at -60 mV. The sustained component was measured 2 min after carbachol application was initiated. The data points were obtained from the same cells described in Figure 2. The smooth curve for the open circles is the fitted Hill equation (IC_{50} 1.6 μM, s = 1.0). (B) Mean amplitude at 0 mV of peak Ca^{2+}-dependent K^+-current induced by 10 μM carbachol is plotted against WT concentration. The smooth curve is the fitted Hill equation (IC_{50} 1.7 μM, s = 1.1).

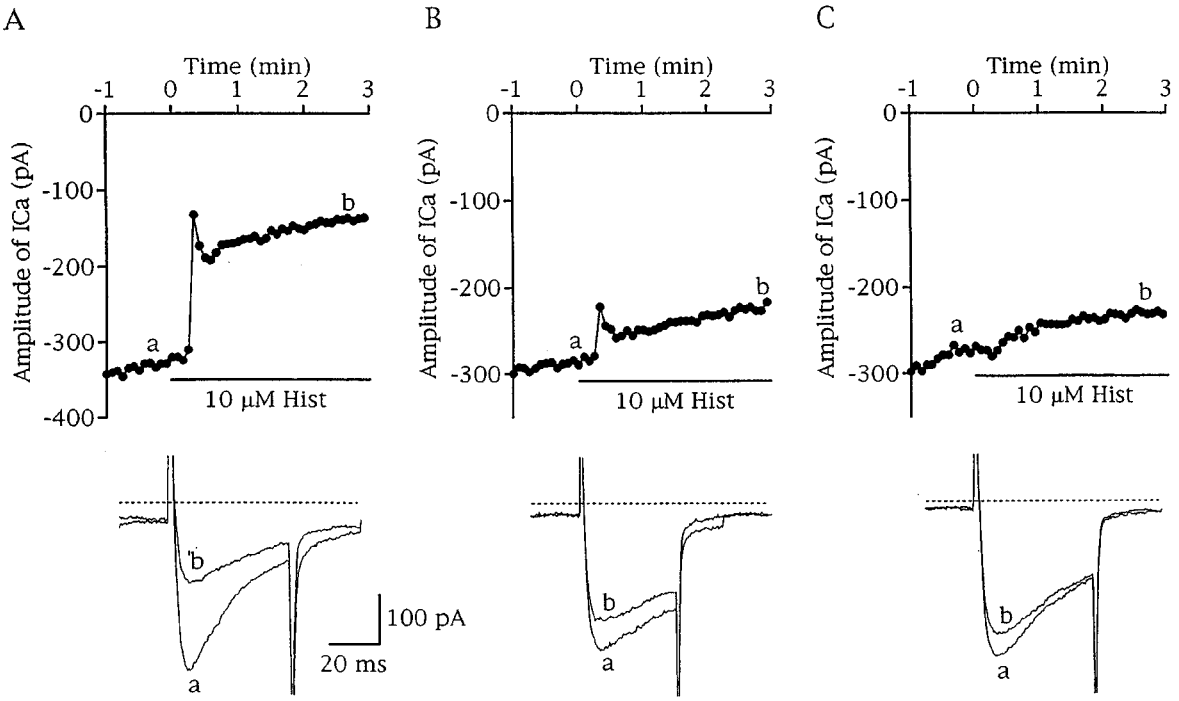

Figure 4 Effect of intracellular D609 on transient and sustained components of histamine-induced suppression of Ca^{2+} channel current. Ca^{2+}-current was evoked by 40- or 50-ms depolarizing pulses to 0 mV from the holding potential of −60 mV and at 0.2 Hz. The time-series plots are for peak Ca^{2+}-current amplitude (closed circles). (A) no D609 (control recording), (B) 180 μM D609 in the patch pipette and (C) 1.8 mM D609 in the patch pipette. Histamine (Hist, 10 μM) was bath-applied 10-min after breaking the cell-attached patch and as indicated by horizontal bars. Examples of actual current records are shown below the time-series plots for before (a) and at the end of each period of histamine application (b).

respectively (Figure 5C and D). Therefore, D609 did not inhibit IP_3-induced Ca^{2+}-release, did not chelate intracellular Ca^{2+}, or inhibit Ca^{2+}-dependent K^+ channels. D609 (1.8 mM) in the patch pipette also did not block Ca^{2+} channels or cationic channels. After 10 min of whole-cell recording, the peak amplitude of Ca^{2+}-current elicited by depolarization to 0 mV was -8.68 ± 1.61 pA.pF^{-1} ($n=6$) and -6.7 ± 0.98 pA.pF^{-1} ($n=6$) in control and D609 recordings respectively (the values are not significantly different). Cationic current was measured by application of 10 μM carbachol in the presence of histamine, which had already released Ca^{2+} from sarcoplasmic reticulum. Measured at −60 mV, and immediately before the depolarizing steps which elicited Ca^{2+}-current, the amplitude of cationic current was -7.26 ± 2.46 pA.pF^{-1} ($n=6$) and -4.83 ± 1.91 pA.pF^{-1} ($n=6$) in control and D609 recordings respectively (the values are not significantly different).

Discussion

This study makes progress towards elucidating the mechanism by which spasmogenic agonists induce sustained suppression of voltage-dependent Ca^{2+} channel current in smooth muscle cells of the small intestine. It has been observed that two chemically unrelated agents – wortmannin and D609 – inhibit sustained Ca^{2+} channel suppression at concentrations that are the same as those which inhibit transient agonist-induced Ca^{2+} channel suppression and other effects in these cells which are known to occur *via* receptor-coupling to PLC and the consequent production of IP_3 and Ca^{2+}-release from sarcoplasmic reticulum. The data suggest that there is a common, or at least similar, molecular mechanism for sustained and transient agonist effects. This mechanism may be activity of a PLC/D isoform.

The evidence is good that the transient suppression of Ca^{2+} channel current, which is induced by carbachol and histamine, occurs *via* the IP_3-pathway (Beech, 1997). We have found that D609 does not prevent IP_3-induced Ca^{2+}-release and does not prevent rises in intracellular Ca^{2+} levels. There are no reports that D609 inhibits G-proteins, or the coupling of receptors to G-proteins, but there are reports that D609 will inhibit phosphatidylinositol-specific (PI) or phosphatidylcholine-specific (PC) PLC and PLD at concentrations which are similar to those used in our experiments (Müller-Decker, 1989; Powis *et al.*, 1991; Schütze *et al.*, 1992; Gratas & Powis, 1993). On this basis it seems reasonable to assume that D609 inhibited transient Ca^{2+}-current suppression because it inhibited PI-PLC. We know from previous experiments that the sustained agonist effect will occur in the absence of the transient effect (Beech, 1993) and so inhibition of the sustained effect by D609 shows that a D609-sensitive process is required for the sustained as well as the transient effect. The similar sensitivity of both components to D609 suggests that PI-PLC is also required for sustained Ca^{2+} channel suppression. However, PC-PLC and PLD are also inhibited by D609 and so cannot be excluded. The previously reported observation that neomycin, an inhibitor of phosphatidylinositolbisphosphate availability for PI-PLC, prevented transient but not sustained Ca^{2+} channel suppression (Unno et al., 1995) lends support to the hypothesis that it could be PC-PLC or PLD which is involved in the sustained effect.

Intracellular D609 appeared to be selective for the signal transduction mechanism coupling receptors to Ca^{2+} channels because it did not inhibit Ca^{2+} channels or cationic channels directly, and it did not inhibit IP_3-induced Ca^{2+}-release. The

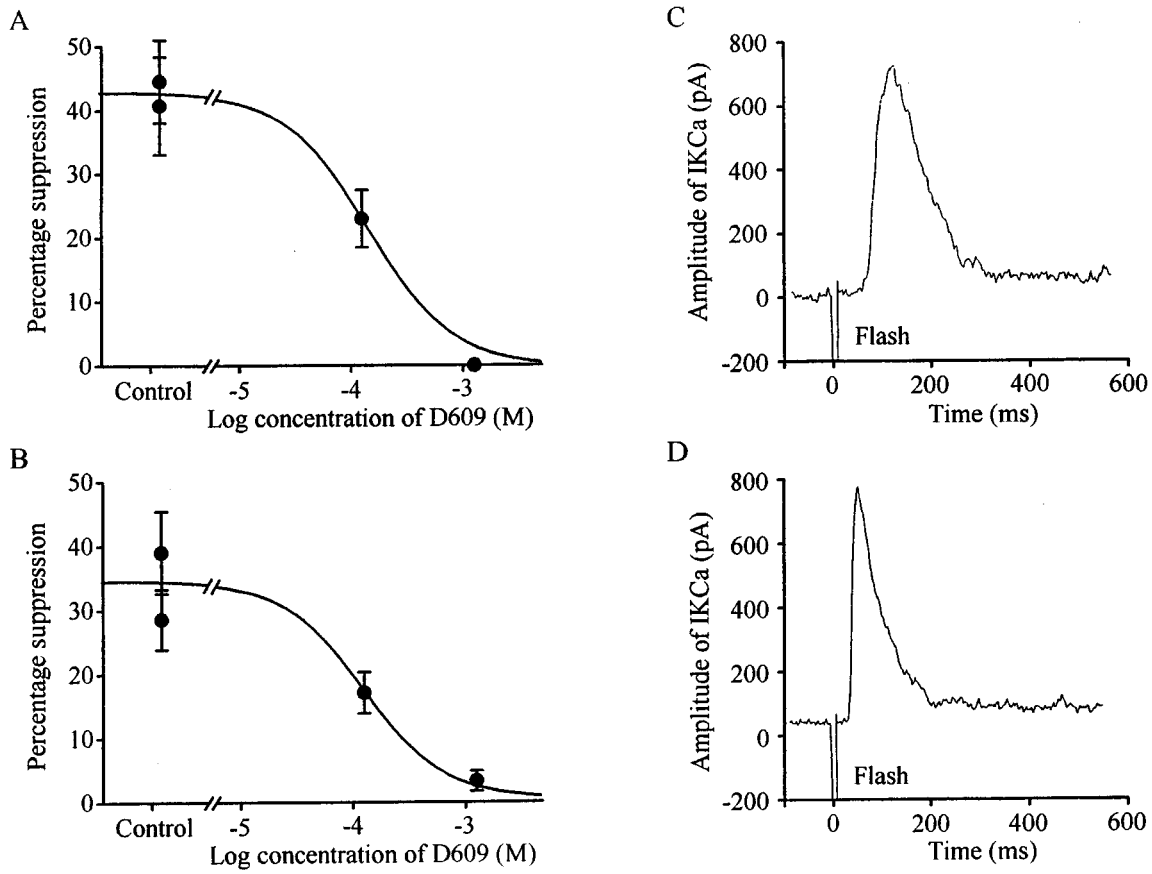

Figure 5 Concentration-dependence and selectivity of D609. (A) and (B) are plots of the percentage suppression of Ca^{2+}-current induced by 10 μM histamine against the intracellular D609 concentration estimated as described in the text. Closed circles are mean $\pm$ s.e.mean values. There are separate zero D609 values for comparison with the low and high concentrations of D609. The smooth curves are fitted one-site Hill equations which give IC_{50} values of 129 μM for (A) and (B). (A) and (B) are for the transient and sustained components of histamine-induced suppression respectively. (C) and (D) are current measurements at a holding potential of 0 mV and using the KCl-containing bath and pipette solutions. In both cases there was 100 μM NPE-caged-IP_3 in the patch pipette and a flash of light was applied at time = zero where a brief downward artefact is evident. The slower upward deflection is a transient K^+-current. In (D) there was 1.8 mM D609 in the patch pipette. (C) is a time-matched control recording.

reported inhibitory effect of extracellular D609 on caffeine-induced Ca^{2+}-release in vascular smooth muscle (Macrez-Leprêtre *et al.*, 1996) indicates that D609 may have an additional action on the Ca^{2+}-induced Ca^{2+}-release channel of the sarcoplasmic reticulum.

Wortmannin inhibits phosphoinositide 3-kinase and phospholipase A_2 at nanomolar concentrations (Ui *et al.*, 1995; Cross *et al.*, 1995). However, 10 nM wortmannin had no effect on agonist-action on Ca^{2+} channels and 100 nM wortmannin induced only small, statistically insignificant, inhibition. Therefore, it is necessary to look for an alternative target which could explain the effects of 1–10 μM wortmannin in our experiments (see also: Unno *et al.*, 1996 regarding phospholipase A_2). Possibilities include MLCK, PI-PLC and PLD, which are all inhibited by wortmannin at low micromolar concentrations (Bonser *et al.*, 1991; Nakanishi *et al.*, 1994). MLCK is probably not involved because ML-7 and $MLCK_i$ peptide were ineffective against Ca^{2+} channel current suppression. Our data suggest that PI-PLC was inhibited by wortmannin because transient Ca^{2+} channel suppression was prevented. Therefore, phosphoinositide 3-kinase, phospholipase A_2 and MLCK are unlikely targets to explain the effects of wortmannin in our experiments. Isoforms of PLC/D are the only other reported targets of wortmannin and thus our wortmannin data support the involvement of phospholipase, but not phospholipase A_2, in transient and sustained Ca^{2+} channel suppressions.

The above discussion leads us to consider the hypothesis that the sustained component of Ca^{2+} channel suppression occurs *via* a pathway involving PLC-mediated production of diacylglycerol and the activation of a protein kinase C (PKC). Although PKC commonly stimulates L-type Ca^{2+} channels there are also reports describing inhibition (Beech, 1997). However, we have not observed inhibition of Ca^{2+} channel current in response to phorbol esters (Unno *et al.*, 1995) or 10 μM ($n=2$) or 50 μM ($n=2$) of the diacylglycerol analogue diC_8 (data not shown). DiC_8 (10 μM) did, however, inhibit by 64.7 $\pm$ 6.8% ($n=3$) K^+-current induced by 10 μM levcromakalim (not shown). Furthermore, preincubation for at least 20 min with 1 μM staurosporine or 100 μM H-7 had no effect on transient or sustained carbachol-induced suppression of Ca^{2+} channel current (Unno *et al.*, 1995). (Staurosporine and H-7 inhibit a broad range of serine/threonine protein kinases, including most isozymes of PKC.) There was no independent test that PKCs were inhibited, but the data do not support a role for PKC. Our data are not positive in this regard but it is premature to rule out a role for diacylglycerol and PKC in sustained Ca^{2+} channel suppression. DiC_8 is a membrane permeant diacylglycerol which does activate PKCs but which is not the same as the

longer-chain diacylglycerols formed by the cellular action of PLC (Pessin *et al*., 1990). Perhaps these diacylglycerols have an action on Ca^{2+} channels which is independent of PKC. Alternatively, the Ca^{2+} channels may need to be primed in order to be inhibited by PKC. Priming may only happen in the presence of agonist and *via* a parallel but distinct signal transduction pathway.

An alternative to a diacylglycerol/PKC hypothesis might seem to be that it is a maintained elevation of IP_3 levels which is able to suppress Ca^{2+} channel current. This is unlikely because the sustained effect is not dependent on Ca^{2+}-release from sarcoplasmic reticulum and heparin prevents the effect of flash-photolysis of caged-IP_3 (Komori & Bolton, 1991) but not sustained agonist-induced Ca^{2+} channel suppression (Beech, 1993). If the sustained effect does not involve diacylglycerol or PKC it must occur *via* a separate pathway which is also sensitive to wortmannin and D609. An untested possibility is that phosphatidic acid or phosphocholine is involved in the sustained component of Ca^{2+} channel suppression.

The study has demonstrated that wortmannin and D609 are inhibitors of the receptor-coupling to Ca^{2+} channels in smooth muscle cells from guinea-pig small intestine. The data indicate a strong pharmacological similarity between transient and sustained components of Ca^{2+} channel suppression, supporting the hypothesis that there is a link between the two effects. There appears to be a common, or at least pharmacologically similar, element in the signal transduction pathways mediating the two effects. Our working hypothesis is that the element is a PLC or PLD.

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