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Inhibition by wortmannin of M-current in bullfrog sympathetic neurones

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1 The actions of wortmannin, an inhibitor of myosin light chain kinase (MLCK), on M-type potassium current of dissociated bullfrog sympathetic neurones have been examined.

2 The amplitude of M-current was measured by whole cell recordings from cells pretreated with wortmannin (0.01–10 μM) or the wortmannin vehicle, dimethylsulphoxide (0.0001–0.1 vol%), for 30 min. Internal (recording pipette) solutions having three different pCa values (6, 7 and 8) were used for the measurements.

3 Irrespective of the pCa, M-current was not detectable when the cells were pretreated with 10 μM wortmannin. Wortmannin, 3 μM, produced 85–95% inhibition of the M-current. Pretreatment with 10–30 nM wortmannin was without effect on M-current.

4 The M-current inhibition by wortmannin at concentrations of 0.1–1 μM depended on the pCa of the internal solution. Inhibition occurred only when the calcium-rich (pCa = 6) internal solution was used.

5 Pre-treatment of the cells with wortmannin (10 μM) did not affect rapidly-inactivating A-type or delayed rectifier-type potassium currents nor did it alter inwardly rectifying sodium-potassium current (I_H).

6 These observations show that M-current inhibition by wortmannin has two pharmacological profiles. One is calcium-dependent and occurs at lower concentrations (0.1–1 μM), and is attributed to inhibition of MLCK by wortmannin. At higher concentrations (3–10 μM), wortmannin has an additional, calcium-independent action, inhibiting the M-current by an unknown mechanism.

Keywords: Wortmannin; myosin light chain kinase; calmodulin; potassium current; autonomic neurones

Introduction

The M-current is a potassium current originally described in bullfrog sympathetic ganglion cells (Brown & Adams, 1980). The current is a non-inactivating outward current at potentials less negative than − 65 mV. Acetylcholine released from presynaptic nerve terminals inhibits M-current via muscarinic receptors, thereby increasing the excitability of the cell membrane. We have recently provided evidence suggesting that phosphorylation of myosin catalyzed by myosin light chain kinase (MLCK) acts to increase the amplitude of the M-current (Tokimasa & Akasu, 1991; Akasu *et al.*, 1993).

The goal of the present study was to obtain further evidence for the MLCK-dependence of the M-current by use of a microbial product, wortmannin, recently identified as an inhibitor of MLCK (Nakanishi *et al.*, 1992; Yano *et al.*, 1993). During the course of our experiments, wortmannin was reported to have the additional effect of inhibiting phosphatidylinositol 3-kinase with an IC_{50} value approximately 100 times lower than that for MLCK (Yano *et al.*, 1993). Hence, we have also examined whether the observed actions of wortmannin on M-current could result from its ability to inhibit phosphatidylinositol 3-kinase.

Three main approaches were adopted. First, cells were pretreated with wortmannin (0.1–10 μM) for 30 min and then subjected to whole-cell recordings of M-current. Second, internal (pipette) solutions having three different concentrations of free calcium ions were used for measuring the M-current amplitude. A calcium-rich (free calcium, 1 μM) solution was used to activate the calmodulin/MLCK-complex fully (Olwin *et al.*, 1984; Yazawa *et al.*, 1987). A calcium-deficient (free calcium, 10 nM) solution was used to test whether wortmannin has actions unrelated to calcium/calmodulin/MLCK-dependent processes. Another internal solution (free calcium, 100 nM) was used to compare the results in the present study with those reported for the actions of a peptide inhibitor, MLCK(783-804), on the M-current (Akasu *et al.*, 1993). For the third approach, wortmannin was applied directly to single cells while recording the M-current so that the acute actions of the drug could be observed.

These experiments have revealed two distinct effects of wortmannin on M-type potassium current. Some of our observations are consistent with our working hypothesis that wortmannin can inhibit the M-current by inhibiting MLCK. However, we will also provide evidence suggesting that wortmannin has a second, though quite selective inhibitory effect on the M-current, perhaps by exerting a direct blocking action at the M-channel.

Methods

Tissue culture

Dissociated bullfrog sympathetic neurones were incubated in a culture medium for 2–7 days as described previously (Tokimasa & Akasu, 1990a,b). The cells were resuspended in Ringer solution 2 h before electrophysiological experiments. This solution had the following composition (mM): NaCl 128,

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KCl 2.4, $CaCl_2$ 1.8, $MgCl_2$ 1.8 and HEPES 10 (pH adjusted with NaOH to 7.4).

Pretreatment of the cells with wortmannin

For pretreatment with wortmannin, the cells were resuspended in Ringer solution for 1.5 h then transferred to a Ringer solution containing wortmannin (0.01–10 μM) for 30 min. For the 24 h pretreatment, wortmannin (10 μM) was added to the culture medium 22 h before the cells were resuspended in Ringer solution. The cells were then resuspended for 2 h in a Ringer solution which contained wortmannin (10 μM). Following either of the above pretreatments, the cells were pipetted into the recording chamber then continuously superfused with Ringer solution containing no added wortmannin and 10–90 min later, electrophysiological experiments started. The superfusate always contained tetrodotoxin (3 μM) to block the inward sodium current.

Whole-cell recordings

Membrane currents were recorded in the whole-cell configuration with methods described previously (Simmons *et al.*, 1990; Tokimasa & Akasu, 1990a,b,c). The tip resistance of glass pipettes was 0.8–1.2 MΩ when filled with the pipette (internal) solution. A sample-and-hold/voltage-clamp amplifier (Axoclamp 2A, Axon Instruments) was used in most experiments (sampling rate, 9–11 kHz). Another amplifier (Axopatch 1C, Axon Instruments) was also used in a limited number of experiments. In either case liquid junction potentials between internal and external solutions have been corrected. Axodata 1.14 (Axon Instruments) and an ITC-16 interface (Instrutech) were used for data acquisition. Axograph 2.0 (Axon Instruments) and Sigmaplot 4.14 (Jandel) were used for analyzing the data. A single-cell superfusion system was used for direct applications of wortmannin during whole cell recordings. Wortmannin was applied intracellularly by inclusion in the pipette solution as described previously (Simmons *et al.*, 1990; Akasu *et al.*, 1993).

Internal solutions

Five different pipette (internal) solutions were used for measuring the amplitude of M-current. A 'normal calcium, plus calmodulin' solution had the following composition (mM): KCl 120, $MgCl_2$ 2.5, $CaCl_2$ 0.32, BAPTA 1, Na_2ATP 1.15, Na_2GTP 1.5, HEPES 10 and calmodulin 0.001 (Akasu *et al.*, 1993). The pCa was estimated as 7.0 (Godt & Lindley, 1982). The concentration of $CaCl_2$ was increased to 0.826 mM (pCa = 6.0) for preparing a 'calcium-rich, plus calmodulin' solution. The activity of the calcium/calmodulin/MLCK-complex should be maximal with this internal solution (Olwin *et al.*, 1984; Yazawa *et al.*, 1987). A 'normal calcium' solution was the same as the 'normal calcium, plus calmodulin' solution, but with calmodulin omitted. A 'calcium-deficient' solution was the same as the 'normal calcium' solution, but with concentration of $CaCl_2$ reduced from 0.32 mM to 0.045 mM, giving a calculated pCa of 8.0. An 'ATP-free' solution contained 5'-adenylylimidodiphophate (ANP-PNP) in place of 1.15 mM ATP, but no added $CaCl_2$ or calmodulin. The pH of the internal solutions was adjusted with KOH to 6.8.

M-current

M-type relaxations on the current trace were detected by use of two different protocols (see Akasu *et al.*, 1993). First, the M-current was continuously deactivated by setting a holding potential at − 65 mV. The cells were subjected to 5 different step commands starting from − 35 mV with − 10 mV increments every 4 s. Total duration of the steps was 0.5 s. The peak amplitude of the deactivating tails was measured upon stepping back to − 65 mV. Second, M-current was continuously activated at a holding potential of − 35 mV then briefly (0.5 s) deactivated during 6 different step commands starting from − 45 mV with − 15 mV increments every 5–6 s. The peak amplitude of the deactivating M-current tail was measured upon steps to − 60 mV.

A-current

The amplitude of the A-current was measured using conventional digital subtraction techniques as described previously (Tokimasa *et al.*, 1991; Akasu *et al.*, 1993). A- and M-currents were activated upon steps from − 100 mV to − 30 mV and M-current alone was activated upon steps from − 45 mV to − 30 mV.

Delayed rectifier potassium current

This potassium current was recorded by superfusing the cells with a modified Ringer solution of the following composition (mM): choline chloride 124, KCl 2.4, $BaCl_2$ 1.8, $MgCl_2$ 1.8, $CdCl_2$ 0.1, CsCl 2, 4-aminopyridine 3, scopolamine 0.0001 and HEPES 10 (pH adjusted with CsOH to 7.4) (Tokimasa *et al.*, 1993). Scopolamine was used to prevent muscarinic receptors from being activated by choline. The peak amplitude of the delayed rectifier was measured upon steps from − 120 mV to + 50 mV (Akasu *et al.*, 1993; Tokimasa *et al.*, 1993).

H-current

The cells were superfused with a potassium-rich (22.4 mM) Ringer solution to increase the amplitude of this inwardly rectifying sodium-potassium current (H-current or I_H: Tokimasa & Akasu, 1990a; Tokimasa *et al.*, 1990). I_H was activated during hyperpolarizing step commands (15–60 mV, 3 s) from the holding potential of − 50 mV. The amplitude of I_H was measured at the end of 3 s-pulses to − 110 mV. In cells which had not been pretreated with wortmannin (10 μM) for 24 h, the superfusate contained $BaCl_2$ (1.8 mM) to eliminate M-current since otherwise both I_H activation and M-current deactivation occur simultaneously upon steps from − 50 mV (Tokimasa & Akasu, 1990a).

MLCK activity

MLCK from chicken gizzard and calmodulin from bovine brain were purified as described previously (Walsh *et al.*, 1983; Ikebe *et al.*, 1987). The 20 kD light chain of myosin (LC20) was purified by the method of Hathaway & Haeberle (1983). The activity of MLCK was monitored using LC20 at 0.4 mg ml^{-1} in 30 mM Tris/HCl (pH 7.5) which contained 0.1 mM [γ-^{32}P]-ATP (DuPont-New England Nuclear), 1 mM $MgCl_2$, 30 mM KCl, 0.1 mM $CaCl_2$, 1 μg ml^{-1} calmodulin and 0.5 μg ml^{-1} MLCK (Walsh *et al.*, 1983). The specific activity of MLCK was measured at least three different times between 0.5 and 1.5 min after the start of the reaction.

Drugs

Drugs used were the disodium salt of adenosine 5'-triphosphate (Sigma), 4-aminopyridine (Sigma), lithium salt of 5'-adenylylimidodiphophate (Sigma), calmodulin obtained from bovine testes (Biomedical Technologies), guanosine 5'-triphosphate sodium salt (Sigma), scopolamine hydrochloride (Sigma), tetrodotoxin (Wako Pure Chemicals) and wortmannin isolated from the fungal strain *Penicillium fumiculosum* (Sigma). Wortmannin was dissolved in dimethylsulphoxide (Sigma) then stored at − 35°C. This stock solution (10 mM) was diluted first with Ringer solution by a factor of 10 then diluted further with Ringer solution to achieve the final concentration of the drug in the superfusate between 0.01 and 3 μM. The stock solution (10 mM) was diluted directly

with normal or modified Ringer solution for solutions containing wortmannin (10 μM).

Experiments were carried out at room temperature (22–24°C) under ordinary fluorescent lighting. Statistics are expressed as mean ± s.e.mean for the cells tested.

Results

Effects of wortmannin on MLCK activity

Wortmannin used in the present study has been isolated from the fungal strain *Penicillium fumiculosum* (see Methods). The drug (0.01–10 μM) was incubated with a reaction mixture lacking ATP for 3, 10 or 30 min. The phosphorylation reaction was initiated by adding [γ-^{32}P]-ATP. As shown in Figure 1, wortmannin is an effective inhibitor of MLCK. The ability of wortmannin to inhibit MLCK activity depended on both concentrations of the drug (0.1–10 μM) and the pre-incubation time (3–30 min). The IC_{50} for the wortmannin effect on MLCK was 0.09 μM with a 30 min pre-incubation. Higher IC_{50} values were obtained for the 10 min pre-incubation (0.2 μM) and the 3 min pre-incubation (0.9 μM). The presence of a higher concentration of calmodulin (50 μg ml^{-1}) in the reaction mixture did not significantly alter the actions of wortmannin on MLCK activity (data not shown). These results are consistent with recent observations with wortmannin obtained from *Talaromyces wortmannin* (Nakanishi *et al.*, 1992).

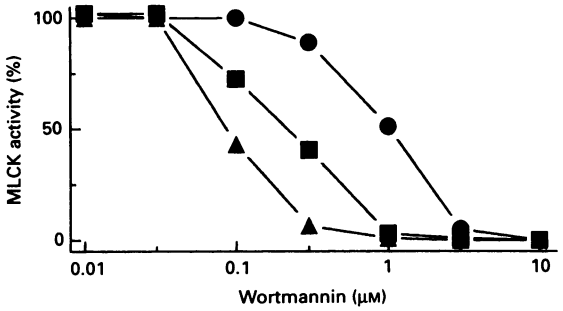

Figure 1 Dose-response curves for the actions of wortmannin on myosin light chain kinase (MLCK)-activity. Ordinate scale denotes the specific activity of MLCK with respect to its control value measured in the absence of wortmannin. Pre-incubation times were 3 min (●); 10 min (■) and 30 min (▲).

The effects of Ca^{2+} and calmodulin on M-current

M-current was recorded from cells which had not been pre-treated with wortmannin. Recordings were made first with the 'normal calcium, plus calmodulin' internal solution (pCa = 7, 1.15 μM ATP and 1 μM calmodulin present). Figure 2a shows an example of recordings obtained from one of 35 cells tested. As has been demonstrated previously (Adams *et al.*, 1982; Akasu *et al.*, 1993), the relaxation during the voltage step command, and that occurring after the termination of the command represent the activating ('on') M-current at − 35 mV followed by its deactivating tail current at − 65 mV, respectively. Consistent with previous observations that the M-current is not activated at potentials

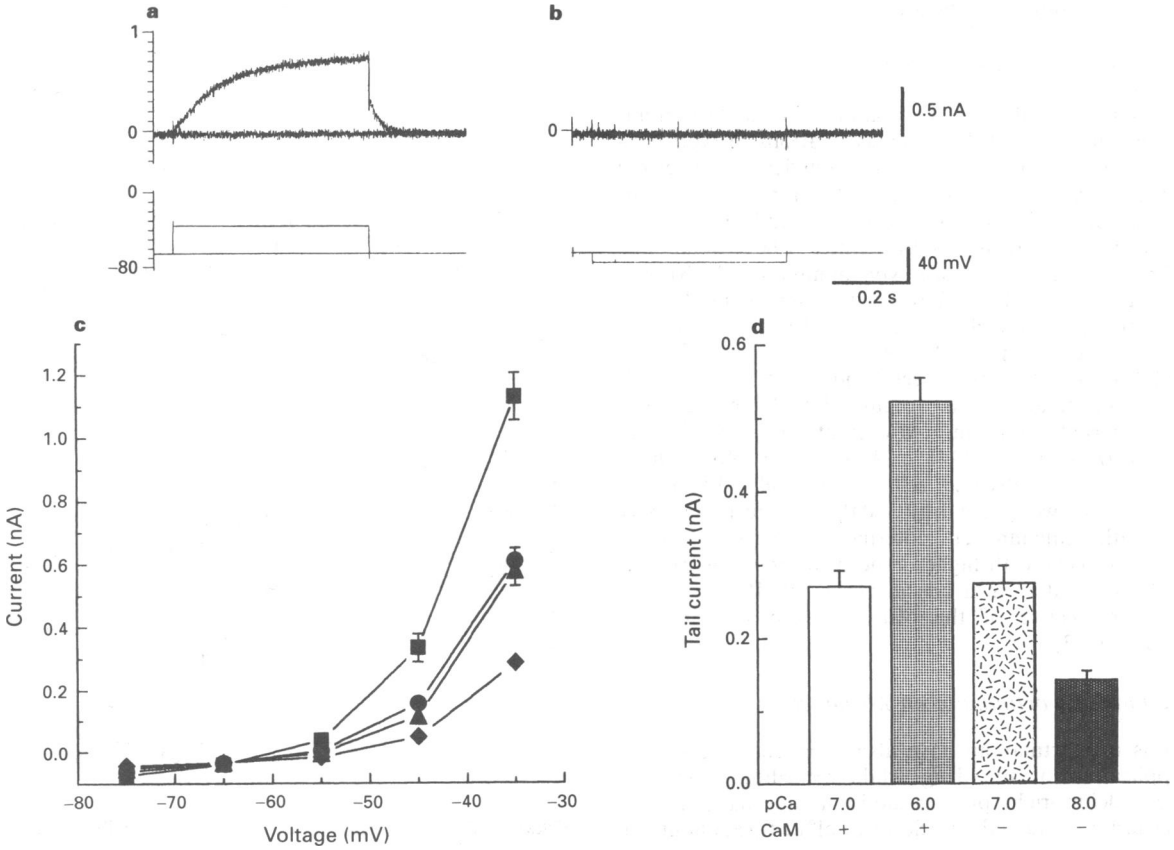

Figure 2 M-current in control cells. (a) Two superimposed current traces (top) are holding current at − 65 mV and M-type relaxations in response to a depolarizing step command (30 mV, 0.5 s) from the holding potential of − 65 mV. Ohmic current in response to a hyperpolarizing step command (10 mV, 0.5 s) is superimposed on the holding current in (b). (c)-(d) Results are based on pooled data. Four *I–V* curves in (c) were obtained using internal solutions termed the 'normal calcium (pCa = 7), plus calmodulin' solution (●, $n = 35$), the 'calcium-rich (pCa = 6), plus calmodulin' solution (■, $n = 21$), the 'normal calcium (pCa = 7)' solution (▲, $n = 8$) and the 'calcium-deficient (pCa = 8)' solution (◆, $n = 22$). (d) Amplitude histogram for the M-current tail upon stepping back from − 35 to − 65 mV. From left to right are the results with these four different internal solutions. CaM + on the abscissa scale denotes plus calmodulin in the internal solution.

negative to − 65 mV (Adams *et al.*, 1982), the current response during the hyperpolarizing step was essentially ohmic and of very small amplitude (Figure 2b). Depolarizing step commands to − 55 mV as well as to − 45 mV (pulse duration was 0.5 s for both commands) were also used to observe M-type relaxations. The amplitude of the 'on' current was measured at the end of step commands (between − 35 mV and − 75 mV in 10 mV-increments) then plotted as a function of the membrane potential at which it was measured, which gave current-voltage ($I-V$) curve between − 75 and − 35 mV. Results obtained from 35 cells are summarized in Figure 2c. The averaged peak amplitude of the tail current was 271 ± 22 pA ($n = 35$) upon stepping back from − 35 mV to the holding potential of − 65 mV (Figure 2d). In all cells, whole-cell recordings started about 3 min after the patch break and results were obtained during the next 2–5 min.

Essentially the same experiments as with the 'normal calcium, plus calmodulin' internal solution were repeated on 55 other cells using four other internal solutions (see Methods). Results are summarized in Figure 2c for the $I-V$ curve and Figure 2d for the tail current amplitude. The averaged peak amplitude of the tail current was 523 ± 32 pA ($n = 21$) with the 'calcium-rich, plus calmodulin' solution, 275 ± 24 pA ($n = 8$) with the 'normal calcium' solution and 143 ± 12 pA ($n = 22$) with 'calcium-deficient' solution upon stepping back from − 35 mV to the holding potential of − 65 mV. An unpaired t test shows that these values are significantly different from each other at the 5% level. In all 4 cells tested, the peak amplitude of the tail current was almost zero pA when measured with the 'ATP-free' internal solution, consistent with previous observations that M-current undergoes a rapid run-down when the internal solution does not contain a hydrolyzable form of ATP (Pfaffinger, 1988; Tokimasa & Akasu, 1991c; Simmons & Schneider, 1993).

M-current in cells pretreated with wortmannin

Figure 3 shows the effect of wortmannin on the M-current. Pretreatment of cells with lower concentrations of wortmannin (0.01–0.03 μM) for 30 min did not significantly affect the amplitude of the M-current. In 'calcium-rich, plus calmodulin' internal solution, the peak amplitude of the tail current in cells pretreated with vehicle (0.03 vol%) was 285 ± 35 pA ($n = 12$) and with wortmannin (0.03 μM) was 596 ± 53 pA ($n = 23$); this difference was significant. Vehicle alone had no significant effect. In 'normal calcium' internal solution the peak amplitude of the tail current was 226 ± 25 pA ($n = 5$) for vehicle cells and 202 ± 33 pA ($n = 8$) for wortmannin-treated cells (Figure 3c). With 'calcium-deficient' internal solution, M-current amplitude were 147 ± 19 pA ($n = 10$) and 146 ± 24 pA ($n = 6$) for vehicle cells and wortmannin-treated cells, respectively (Figure 3). Thus, the effect of wortmannin (0.3 μM) on M-current is only manifest with calcium-rich internal solutions. Finally, pretreatment of cells with higher concentrations of wortmannin (3–10 μM) resulted in a considerable (90–100%) loss of M-current irrespective of the pCa values of the internal solution (Figure 3).

Acute actions of wortmannin on M-current

The actions of wortmannin have also been observed during direct application of the drug while recording M-current using the 'calcium-rich, plus calmodulin' internal solution. The superfusate contained vehicle (0.1 vol%) throughout the experiments. Wortmannin was added to the superfusate approximately 180 s after the patch break and continued for another 600 s. Results obtained from these experiments are summarized in Figure 4 in which the currents were normalized to the maximum M-current for that particular cell. The average amplitude of the tail current at − 60 mV was 450 ± 60 pA when measured 2 min after the patch break ($n = 33$). In the absence of wortmannin, the M-current exhibited a slight run down with time. The run-down of M-current was much more rapid in the presence of wortmannin. When cells were superfused with 10 μM wortmannin, the M-current gradually disappeared over a 16 min recording period. At a concentration of 0.3 μM, wortmannin inhibited the M-current by an average of 60% over 16 min (Figure 4).

Wortmannin was also applied directly to the cell interior using the intracellular dialysis technique, via the patch pipette. (See Methods). Somewhat surprisingly, wortmannin (10 μM) which eliminates the M-current when applied extracellularly had no effect on M-current when applied intracellularly (Figure 4). Two-way analysis of variance showed that

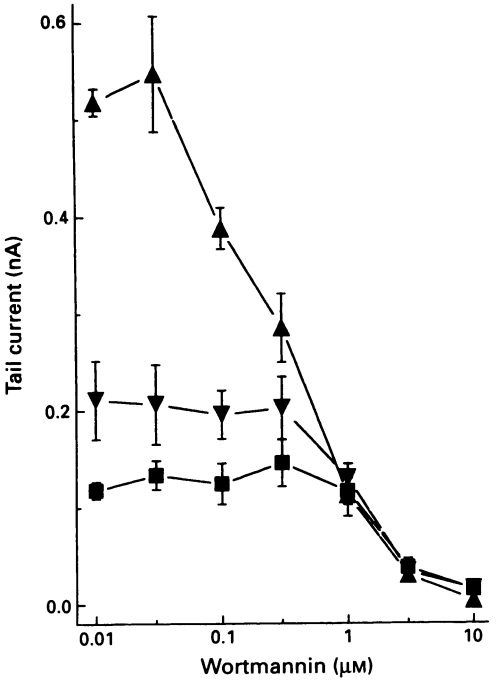

Figure 3 M-current inhibition by wortmannin. Results are based on pooled data ($n = 4–16$ cells at each concentration) obtained from cells pretreated with wortmannin (0.01–10 μM) for 30 min. Dose-response curves for the actions of wortmannin on the M-current tail at − 65 mV in 'calcium-rich, plus calmodulin' internal solution (▲), 'normal calcium' internal solution (▼) and 'calcium-deficient' internal solution (■).

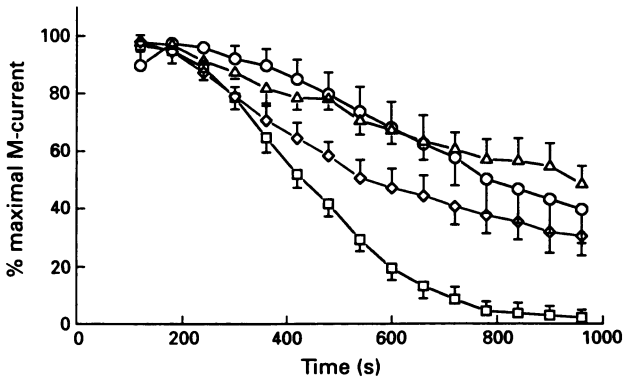

Figure 4 Effects of acute extracellular or intracellular application of wortmannin on M-current. The amplitude of the tail current (at − 60 mV, see Methods) were normalized so that the current recorded 120 s after patch break (abscissae) was set to 100%. Wortmannin was added to either the superfusate or the internal solution approximately 180 s after the patch break. The application was discontinued after another 600 s. Control cells (○, $n = 6$); 10 μM wortmannin in the internal solution (△, $n = 11$); 0.3 μM wortmannin added to the superfusate (◆, $n = 9$) and 10 μM wortmannin added to the superfusate (□, $n = 7$). Symbols show mean ± s.e.mean.

there is a statistically significant effect of both time ($P<0.001$) and wortmannin concentration ($P<0.003$). The effect of 10 μM wortmannin applied extracellularly was significantly different from the effect of either zero or 10 μM wortmannin applied intracellularly.

A-current and delayed rectifier current

The amplitude of A-type and delayed rectifier-type potassium currents was measured from cells pretreated with wortmannin (10 μM) or vehicle (0.1 vol%) alone, for 30 min (see Methods). The 'calcium-rich, plus calmodulin' internal solution was used.

The peak amplitude of A-current at −30 mV was 713 ± 81 pA ($n = 11$) and 617 ± 63 pA ($n = 6$), for control and wortmannin-treated cells, respectively (Figure 5a–c). The peak amplitude of delayed rectifier at +50 mV was 13.9 ± 2.2 nA ($n = 9$) and 15.9 ± 2.6 nA ($n = 7$) for the control and the wortmannin-treated cells, respectively (Figure 5d and 5e). Thus, wortmannin (10 μM) was without effects on these potassium currents, even when calcium-rich internal solutions are used.

Cyclic AMP-dependent H-current

Bullfrog sympathetic neurones also possess a cyclic AMP-dependent cationic inward rectifier current termed I_H

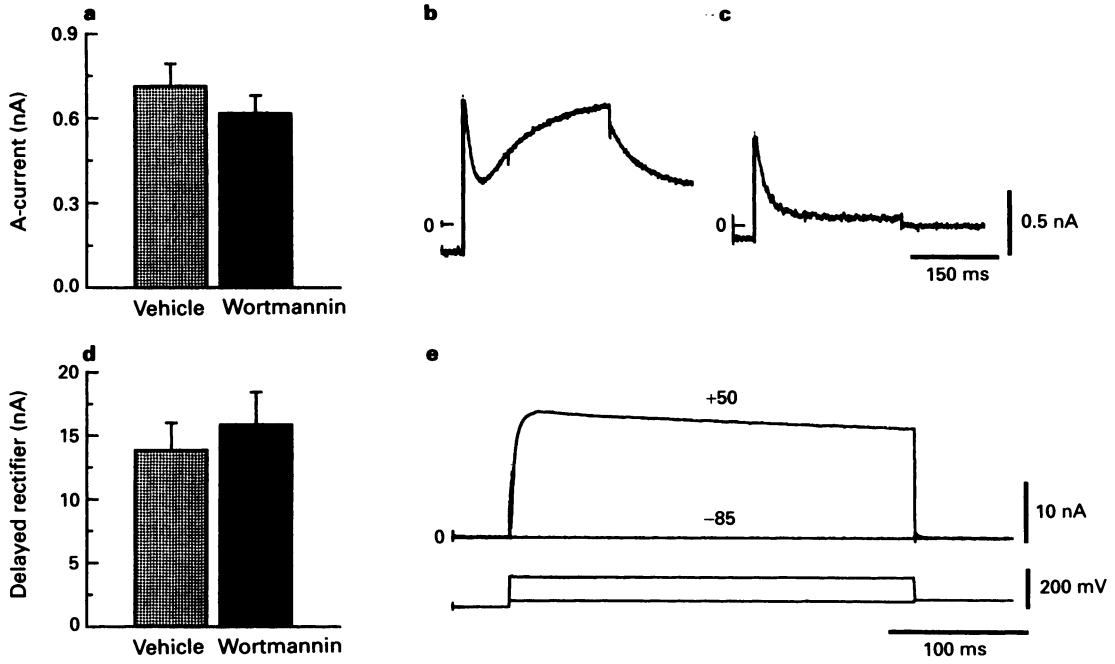

Figure 5 Lack of effect of wortmannin on A-current and delayed rectifier current. Pooled results are shown in (a) and (d). (a) The amplitude of A-current at −30 mV. Sample recordings are shown in (b) for a vehicle-treated cell and in (c) for a wortmannin-treated cell. Note the absence of M-current in the wortmannin-treated cell. (d) The peak amplitude of delayed recitifier at +50 mV. A sample recording for a wortmannin-treated cell is shown in (e). Ohmic current at −85 mV is also shown in this record. Above: current; below: voltage.

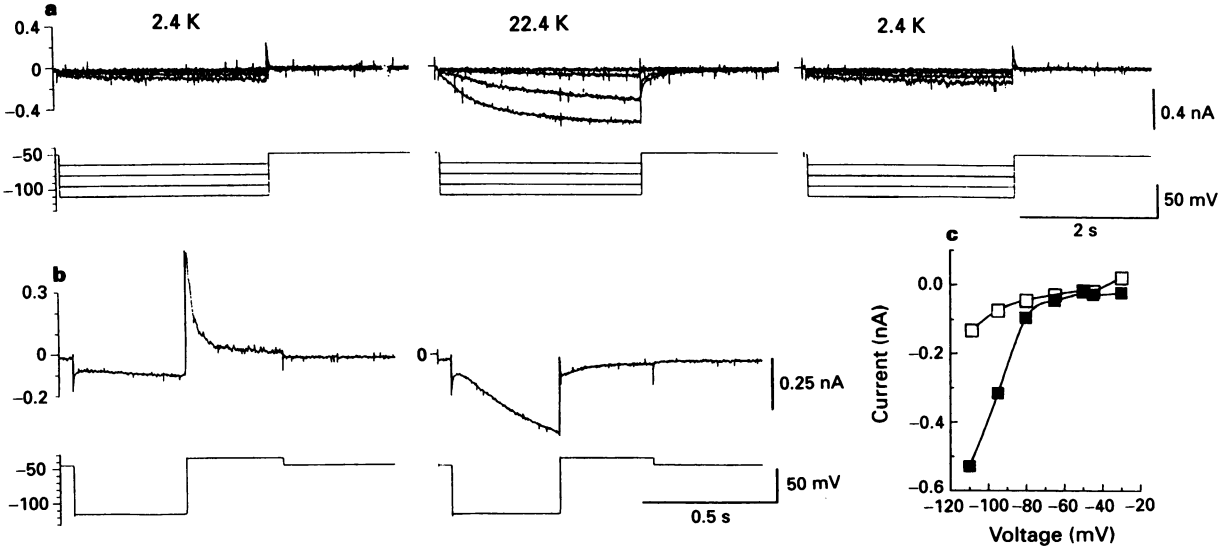

Figure 6 H-current of a cell pretreated with wortmannin (10 μM) for 24 h. (a) From left to right are: H-current with the concentration of KCl in the superfusate at 2.4, 22.4 and 2.4 mM. Above: current; below: voltage. Holding potential was −50 mV. (b) A mixture of A-current and H-current. Left: 2.4 mM KCl; right: 22.4 mM KCl. Above: current; below: voltage. (c) Current-voltage curves in 2.4 mM KCl (□) and 22.4 mM KCl (■). The amplitude of membrane current was measured at the end of 3 s-step pulses such as that shown in (a).

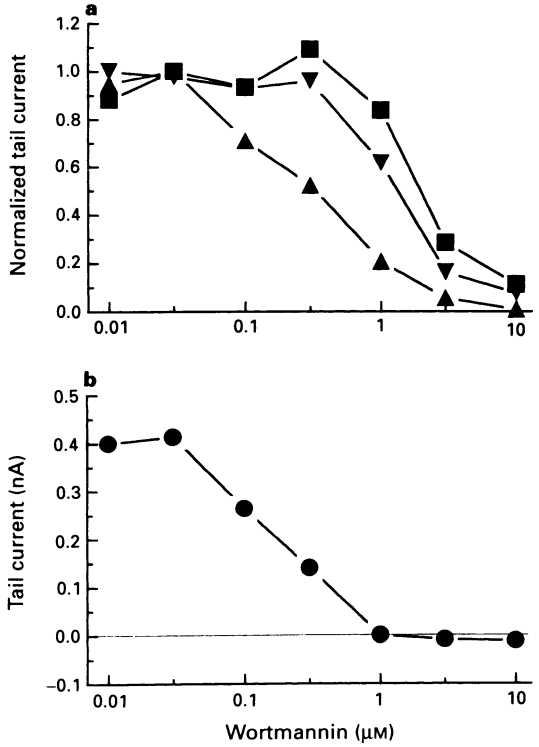

Figure 7 Plot of wortmannin effects with different intracellular calcium concentrations. Data points are based on those in Figure 3. (a) The dose-response curves for the three different internal solutions shown in Figure 3a have been normalised according to the maximum current amplitude: (▲) 'calcium-rich, plus calmodulin' solution; (▼) 'normal calcium' solution and (■) 'calcium-deficient' solution. (b) The amplitudes of the tail current measured with the 'calcium-deficient' internal solution (■ in Figure 3) was subtracted from that measured with the 'calcium-rich, plus calmodulin' internal solution (▲ in Figure 3).

(Tokimasa & Akasu, 1990a,c; Tokimasa *et al.*, 1990). In order to test any potential effects of wortmannin on cyclic AMP-dependent protein kinase, I_H was measured from cells pretreated with wortmannin (10 μM) ($n = 9$) or vehicle (0.1 vol%) ($n = 6$) for 24 h. The 'calcium-rich, plus calmodulin' internal solution was used. I_H averaged 574 ± 49 pA ($n = 9$) when measured at the end of 3 s-pulses to − 110 mV, in cells pretreated with wortmannin (Figure 6a). This was not significantly different ($P < 0.05$, unpaired t test) from I_H in cells pretreated with vehicle alone (510 ± 78 pA, $n = 6$) (see Tokimasa & Akasu, 1990a). Figure 6b also shows that A-current can be recorded even after the 24 h pretreatment of the cells with wortmannin (see Figure 5c).

Discussion

We have demonstrated that the M-current is larger when measured with a calcium-rich (free calcium at 1 μM) internal solution, than when measured with solutions containing free calcium ions between 10 and 100 nM. We have also demonstrated that wortmannin (0.1–1 μM) inhibits this calcium-activated portion of the M-current. These observations can be interpreted as further supporting our argument that a calcium-dependent process positively modulates M-current (Akasu *et al.*, 1993) and that this modulation is inhibited by relatively lower concentrations of wortmannin, presumably as a result of MLCK inhibition.

At higher concentrations (3–10 μM), however, wortmannin inhibits M-current irrespective of the pCa values for the internal solutions (Figure 3). We have considered two possibilities for the calcium-independent effect. First, calcium may modulate the sensitivity of MLCK to wortmannin in such a manner that MLCK is more sensitive to wortmannin when tested with calcium-rich internal solutions than when tested with calcium-deficient solutions. If this were the case, we would expect the wortmannin dose-response curve shown in Figure 3a to shift to the right as the intracellular concentration of calcium ions is decreased. To compare the wortmannin dose-response curves for three different concentrations of free calcium ions, the data points in Figure 3a have been re-plotted in Figure 7a so that the ordinate scale now represents the relative amplitude of the M-current. The estimated IC_{50} values of wortmannin are 0.33 μM, 1.1 μM and 2.8 μM for concentrations of free calcium in the internal solution at 1 μM, 100 nM, 100 nM and 10 nM, respectively. The main problem with this interpretation is that the slope of the curve with 1 μM of free calcium ions differs from that for 10 and 100 nM. This difference suggests that different mechanisms may be involved in mediating the actions of wortmannin at different free calcium concentrations.

The other possibility is that wortmannin has a direct M-channel blocking activity at the higher concentrations. In this case we would conclude that the wortmannin-induced inhibition of the M-current detected with the internal solution containing lower (10–100 nM) concentrations of free calcium ions is due to direct channel block, and that wortmannin at concentrations below 1 μM does not have this channel blocking activity. This implies that the dose-response curve indicated by the upward triangles (free calcium at 1 μM) in Figure 3a is a sum of two dose-response curves, one for calcium-dependent and the other for calcium-independent actions of wortmannin. The calcium-independent actions of wortmannin should be represented by the dose-response curve indicated by the square symbols in Figure 3a. By taking the dose-response curve for the concentration of free calcium ions at 1 μM and subtracting the calcium-dependent actions, a dose-response curve for the calcium-independent actions of wortmannin should be obtained. This subtracted curve is plotted in Figure 7b.

Possible mechanisms underlying the inability of intracellularly applied wortmannin to block M-current remain to be clarified. For example there is no evidence suggesting that MLCK has transmembrane structure, such as that occurring for adenylate cyclase (see Hartzell & Fischmeister, 1987; Hartzell & Budnitz, 1992).

Nonomura and his co-workers have reported that, in addition to affecting MLCK, wortmannin (10–30 nM) inhibits phosphatidylinositol 3-kinase (PI3-kinase) to less than 20% of its control activity, while leaving MLCK activity almost unaffected (Nakanishi *et al.*, 1992; Yano *et al.*, 1993). We have shown that M-current is insensitive to these concentrations of wortmannin (Figure 4a).

With regard to the actions of wortmannin on protein kinases other than MLCK and PI3-kinase, Nakanishi *et al.* (1992) have reported that wortmannin inhibits calcium-activated, phospholipid-dependent protein kinase (C-kinase; Nishizuka, 1984) only slightly without significantly affecting cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase and calmodulin-dependent protein kinase II. It is unlikely that wortmannin inhibited M-current by inhibiting C-kinase in the present study since activators of C-kinase have been shown to decrease the amplitude of M-current (Brown & Adams, 1987). The inability of wortmannin to inhibit cyclic AMP-dependent protein kinase agrees well with our observations that wortmannin does not alter the cyclic AMP-dependent cationic inward rectifier or I_H (Figure 6).

In summary, the data are consistent with inhibition of MLCK by wortmannin, thereby resulting in the removal of a tonic effect of MLCK on the M-current when the intracellular concentrations of calcium ions is very high.

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