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Differential effects of wortmannin on the release of substance P and amino acids from the isolated spinal cord of the neonatal rat

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1 Effects of wortmannin, an inhibitor of myosin light chain kinase, on the release of substance P and amino acids, GABA and glutamate, were investigated in the isolated spinal cord preparation of the neonatal rat.

2 Wortmannin at 0.5–10 μM depressed the release of substance P evoked by high-K^+ (90 mM) medium from the spinal cord (IC_{50} = 1.1 μM). Wortmannin also depressed the high-K^+ (70 mM)-evoked release of substance P from cultured dorsal root ganglion neurons of neonatal rats. In contrast, the high-K^+ (90 mM)-evoked release of GABA and glutamate from the spinal cord was not affected by wortmannin (0.1–10 μM).

3 Upon stimulation of a dorsal root, a monosynaptic reflex and a subsequent slow ventral root depolarization were evoked in the ipsilateral ventral root of the same segment in the isolated spinal cord preparation. The magnitude of the slow ventral root depolarization was depressed gradually to about 70% of the control during the course of 30 min under wortmannin (1 μM). In contrast, the monosynaptic reflex was unaffected by wortmannin.

4 Immunofluorescent staining revealed that immunoreactivities of substance P and myosin II were colocalized at presynaptic terminals in the dorsal horn of the neonatal rat spinal cord.

5 The present results suggest that myosin phosphorylation by myosin light chain kinase may play a crucial role in the release of substance P, but not in the release of GABA and glutamate in the neonatal rat spinal cord. This may reflect a difference in the exocytic mechanisms of substance P-containing large dense core vesicles and amino acid-containing small clear vesicles.

Keywords: Amino acids; myosin light chain kinase; neurotransmitter release; spinal cord; substance P; wortmannin

Introduction

Clarification of the mechanisms of neurotransmitter release from nerve terminals is crucial for understanding synaptic transmission in the central nervous system (Bennett & Scheller, 1993; Trifaró & Vitale, 1993; Burns & Augustine, 1995; Südhof, 1995). Recent studies by Nonomura and colleagues showed that wortmannin, an inhibitor of myosin light chain kinase (MLCK), exerts inhibitory effects on various exocytic events: histamine secretion from rat basophilic leukaemia (RBL-2H3) cells (Kitani *et al*., 1992; Ozawa *et al*., 1996), catecholamine secretion from bovine adrenal chromaffin cells (Ohara-Imaizumi *et al*., 1992; Kumakura *et al*., 1994), insulin secretion from pancreatic β cell line MIN6 (Hagiwara *et al*., 1995), and the release of human immunodeficiency virus type 1 from host cells (Sasaki *et al*., 1995). These results suggest that phosphorylation of myosin II is involved in these exocytic events.

Phosphorylation of myosin II may also be involved in neurotransmitter release from presynaptic nerve terminals, since wortmannin inhibited synaptic transmission in the superior cervical ganglion of the rat (Mochida *et al*., 1994). However, whether myosin phosphorylation is involved in synaptic transmission in the central nervous system is not known. Furthermore, the role of myosin phosphorylation in the release process may be different among neurotransmitters. Peptide neurotransmitters such as substance P (SP) are stored in large dense-core vesicles and are thought to be released by slower exocytic mechanisms than amino acid neurotransmitters such as glutamate and GABA stored in small vesicles (Jessell & Kandel, 1993). In addition, small vesicles are clustered at active zones of presynaptic terminals, whereas large dense-core vesicles are not. It is therefore conceivable that mobilization, docking and fusion mechanisms are different between small and large synaptic vesicles (Jessell & Kandel, 1993).

The isolated spinal cord preparation enables us to examine effects of drugs on synaptic responses electrophysiologically and also on neurotransmitter release biochemically under controlled conditions (Suzuki & Otsuka, 1993). Characteristics of the releases of SP and glutamate and those of synaptic responses mediated by these transmitters have been studied in this preparation (Akagi *et al*., 1985; Jahr & Yoshioka, 1986; Sakuma *et al*., 1991; Suzuki *et al*., 1994; Maehara *et al*., 1995). In the present study we, therefore, investigated biochemically and electrophysiologically the possible involvement of myosin phosphorylation in the release of SP in the isolated spinal cord preparation of the neonatal rat, using wortmannin as an inhibitor of MLCK. We further compared the effects of wortmannin on the release of SP with those on the releases of amino acid neurotransmitters, GABA and glutamate, to assess the difference in exocytic mechanisms between SP-containing large dense core vesicles and amino acid-containing small clear vesicles.

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Methods

Perfusion experiments with isolated spinal cords

Perfusion experiments for the release of SP and amino acids were carried out following procedures described previously (Suzuki *et al*., 1994; Maehara *et al*., 1995). A 1–4 day-old Wistar rat was deeply anaesthetized with ether and the spinal cord below the upper thoracic segment was removed with the vertebral column and placed in a dissecting dish filled with artificial cerebrospinal fluid (CSF) saturated with a gas mixture of 95% O_2 and 5% CO_2. After ventral laminectomy, the whole thoraco-lumbar spinal cord was isolated and hemisected sagittally into the right and left halves. One hemisected spinal cord was fixed on a thin glass plate and placed in a perfusion chamber of 1 ml volume and perfused with artificial CSF at a flow rate of 0.7 ml min^{-1}. The composition of normal artificial CSF was as follows (in mM): NaCl, 138.6; KCl, 3.35; $CaCl_2$, 1.26; $MgCl_2$, 1.15; $NaHCO_3$, 21.0; NaH_2PO_4, 0.58; glucose, 10.0. High-K^+ medium (90 mM) was prepared by substituting equimolar KCl for NaCl. In the experiments examining the release of SP from the spinal cord, dithiothreitol (5 μM) and a mixture of peptidase inhibitors consisting of actinonin 10 μM, captopril 10 μM and thiorphan 1 μM were added to the perfusion medium to inhibit degradation of SP (Suzuki *et al*., 1994). Perfusion medium was saturated with a gas mixture of 95% O_2 and 5% CO_2. The temperature in the chamber was maintained at 27°C. After a hemisected spinal cord was washed with normal artificial CSF for 2 h, perfusate was collected through a glass wool filter for every 3 min into a tube (referred to as 3-min fraction).

To examine effects of wortmannin on the release of SP, we used a pair of hemisected spinal cords derived from the same rat, which were simultaneously perfused in two independent chambers, one with normal artificial CSF without wortmannin and the other with artificial CSF containing wortmannin for 30 min before starting the collection of perfusates. Two successive 3-min fractions were collected as pre-controls under normal-K^+ medium without or with wortmannin. Perfusion medium was then changed to the high-K^+ medium without or with wortmannin and two 3-min fractions were collected. Subsequently perfusion medium was returned to normal-K^+ medium without or with wortmannin and one 3-min fraction was collected. Perfusion with 90 mM K^+ medium constantly evoked the release of SP. The collected samples were frozen and lyophilized. Each lyophilized sample was dissolved in 1 or 2 ml of a solution and the amount of SP was measured by enzyme immunoassay (EIA) according to the manufacturer's instruction (Cayman Chemical Co.). The detection limit of the assay was about 0.4 fmol $well^{-1}$. The cross-reactivities with neurokinin A and neurokinin B were 2.7 and 0.04%, respectively (see manufacturer's product description). The net evoked SP release was calculated as sum of the fourth and fifth fractions after subtracting the basal outflow value from each fraction.

To examine the effect of wortmannin on the release of amino acids, glutamate and GABA, each hemisected spinal cord received stimuli with high-K^+ (90 mM) two times with an interval of 1 h. This concentration of K^+ was chosen because the stimulus with 90 mM K^+ was needed for the constant release of glutamate. Procedures for collecting perfusate samples were similar to those used to measure the release of SP. In control experiments, a hemisected spinal cord was perfused with artificial CSF for 1 h, and two successive 3-min fractions were collected as pre-controls; perfusion medium was then changed to the high-K^+ solution and two 3-min fractions were collected; subsequently perfusion medium was returned to normal-K^+ artificial CSF and one 3-min fraction was collected as post-control. The same collection was once again carried out after 1 h of perfusion with normal artificial CSF. In test experiments, the first collection was carried out without wortmannin in the same way as in control experiments, but the second collection was started after perfusion with normal artificial CSF for 30 min and further with artificial CSF containing wortmannin at various concentrations for another 30 min and was carried out with similar procedures under wortmannin at the same concentration. To each collected sample 250 pmol of D, L-β-aminoisobutyric acid was added as an internal standard. After the samples were frozen and lyophilized, the amounts of amino acids were measured using high-performance liquid chromatography (HPLC) with a pre-column derivatization technique by *o*-phthalaldehyde/2-mercaptoethanol (Lindroth & Mopper, 1979). Each lyophilized sample was dissolved in 200 μl of water and 40 μl of the solution was mixed with an equal volume of *o*-phthalaldehyde (2.7 mg ml^{-1})/2-mercaptoethanol (2.5 μl ml^{-1}) in 0.1 M boric acid (pH 9.36), and 40 μl of the mixed solution was then injected into an HPLC system using an autosampler (CMA/200, Carnegie Medicine). The HPLC apparatus consisted of a reverse-phase column (MF6026, BAS) and a Nihon Bunko 800 system equipped with a fluorescence detector (Nihon Bunko) with an excitation wavelength of 330 nm and an emission wavelength of 440 nm. The mobile phase buffer consisted of 9% acetonitrile and 3% tetrahydrofurane in 0.1 M sodium acetate (pH 5.9). The detection limits for glutamate and GABA were about 10 fmol and 100 fmol, respectively. These levels were much lower than the amounts of the amino acids in each sample in basal conditions (about 13 pmol for glutamate and 0.8 pmol for GABA in each sample that was injected to HPLC). The net evoked release of glutamate or GABA was calculated as sum of the third, fourth and fifth fractions after subtracting the basal outflow value (mean of the first and second fractions) from each fraction. The net evoked release in the second collection was expressed as percentage of that in the first collection in the same preparation.

The content of protein was measured by the method of Lowry *et al*. (1951). The average wet weight and protein content of the hemisected spinal cord were 17.8 mg and 0.082 mg protein mg^{-1} wet weight, respectively.

Culture of dorsal root ganglion neurons and release of SP

Cultured dorsal root ganglion (DRG) neurons were prepared as described by Perney *et al*. (1986) with slight modifications. Dorsal root ganglia were dissected from 0–2 day-old Wistar rats, incubated for 1 h at 37°C with 0.1% collagenase A in Leibovitz's L-15 medium, and then mechanically dissociated through a Pasteur pipette. Cells from 15–20 ganglia were plated on 35-mm culture dishes (Primaria, Falcon) and cultured for 2 weeks. Cells were fed every 3–4 days with Ham's nutrient mixture F-12 supplemented with 10% heat-inactivated horse serum, 50 ng ml^{-1} of 2.5 S nerve growth factor, 44 mM glucose, 2 mM L-glutamine, 1% minimum essential medium (MEM) vitamin mixture (100×), and penicillin/streptomycin (100 units ml^{-1} and 100 μg ml^{-1}, respectively). To kill dividing non-neuronal cells, 5 μM cytosine arabinoside was added to the medium for the first 3 days of culture.

Experimental procedures to study the release of SP from cultured neurons were similar to those described by Mudge *et al*. (1979). DRG neurons were washed with phosphate-

buffered saline (PBS) and placed in normal-K^+ solution. The normal-K^+ solution contained 5.4 mM K^+, HEPES-buffered Eagle's MEM (pH 7.5) and 0.2% bovine serum albumin. High-K^+ solution (70 mM) was prepared by substituting equimolar KCl for NaCl. The K^+ concentration of 70 mM to evoke the release of SP was chosen according to Mudge *et al*. (1979). In control experiments, the neurons were incubated in 2 ml of normal-K^+ solution without wortmannin for 30 min before collection. Then the neurons were incubated for 5 min in 1 ml of normal-K^+ solution (pre-control) and thereafter incubated for 5 min in 1 ml of the high-K^+ solution followed by incubation for 5 min in 1 ml of normal-K^+ solution (post-control). To examine the effect of wortmannin, the neurons were incubated in 2 ml of the normal-K^+ solution containing 1 or 10 μM wortmannin for 30 min before starting collection. The procedures of test experiments were the same as those for control experiments except wortmannin at 1 or 10 μM was added to the medium throughout the experiments. After each experiment, the neurons were extracted with 2 ml of 2 M acetic acid. Both the extract of DRG neurons and the incubation samples were lyophilized and the SP contents were measured with the same procedures as those for the release experiments with spinal cord preparations. Mean value of the total SP content was 0.98 ± 0.09 pmol dish^{-1}. We expressed the amount of SP in each incubation sample as a percentage of the total SP content that was calculated by summing the amounts of SP in the incubation samples and that in the cell extract (Mudge *et al*., 1979).

Electrophysiological experiments

A spinal cord below upper thoracic segment was isolated from a 1–3 day-old rat, hemisected, placed in a recording chamber of 0.2 ml volume and perfused with artificial CSF saturated with a gas mixture of 95% O_2 and 5% CO_2 at a rate of 2 ml min^{-1} (Suzuki & Otsuka, 1993). The composition of artificial CSF was the same as that used for perfusion experiments. The temperature in the chamber was kept at 27°C. A lumbar (L3-5) dorsal root was electrically stimulated every 2 min with a single square pulse of 100 μs and supramaximal intensity (30–40 V) applied through a suction electrode, and monosynaptic reflex and subsequent slow depolarizing response (slow ventral root potential, slow VRP) were recorded extracellularly from the ipsilateral lumbar ventral root of the same segment with a tightly fitting suction electrode. Under these conditions, stable responses could be recorded for several hours. The magnitude of the slow VRP was expressed as the area encompassed by the base line and the curve of VRP (mV·s) and the amplitude of the monosynaptic reflex was expressed as the peak height from the base line (mV).

Immunofluorescent experiments

A lumbar portion of the spinal cord of a 2–3 day-old rat was fixed with 1% paraformaldehyde in PBS for 5 min and washed with PBS at room temperature. The cord was infused with sucrose overnight at 4°C and then embedded into Cryomolt and frozen in liquid nitrogen. The frozen sample was cut into 5–10 μm-thick slices, placed on poly-L-lysine-coated glass slides, dried, and treated with 0.5% Triton X-100 in PBS for 5 min at room temperature. After incubation for 30 min at room temperature with PBS containing 2.5% bovine serum albumin and 2% chick serum (blocking solution), the slices were incubated with a mixture of polyclonal anti-myosin II rabbit IgG (diluted 1 : 300–500; Nonomura, 1993) and monoclonal anti-synaptophysin mouse IgG (diluted 1 : 100), or with a mixture of polyclonal anti-myosin II rabbit IgG (diluted 1 : 300–500) and monoclonal anti-SP rat IgG (diluted 1 : 100), in the blocking solution for 3 h at 36°C. After excess antibodies were washed out twice with 0.4 M $MgCl_2$ in 20 mM Tris-HCl (pH 7.6)-buffered saline and then three times with PBS for 5 min each, the slices were incubated with a mixture of biotinylated anti-mouse or anti-rat IgG (diluted 1 : 300–500) and rhodamine-labelled anti-rabbit IgG (diluted 1 : 50–100) for 1 h at 36°C in blocking solution. After rinsing with PBS, the slices were incubated with fluorescein isothiocyanate (FITC)-labelled streptavidin (diluted 1 : 500) and rhodamine-labelled anti-rabbit IgG (diluted 1 : 50–100) for 30 min. After being washed with PBS sufficiently, the samples were embedded in 50% glycerol in PBS containing 2.5% diaminobenzidine carbon oxide, and were examined with a Nikon Microphoto-FXA fluorescent microscope. To avoid overlapping of a stray beam, a cut filter was inserted in the fluorescent microscope. The monoclonal antibodies to synaptophysin and SP used were from commercial source. The anti-synaptophysin antibody was reported to react with presynaptic vesicles in spinal neurons of rat (see manufacturer's product description). The anti-SP antibody was also reported to react specifically with SP in the rat spinal cord (Cuello *et al*., 1979), but not with other peptides such as Leu- or Met-enkephalin (see manufacturer's product description). Characterization of the anti-myosin II rabbit antibody was described elsewhere (Kumakura *et al*., 1994; Mochida *et al*., 1994). Immunoblotting of homogenates from various kinds of tissues including nervous system showed that the antibody binds to a single band of 200 KDa, which corresponds to the molecular weight of mysoin II heavy chain.

Materials

Actinonin, captopril, SP and thiorphan were purchased from Peptide Institute, Osaka, Japan; SP EIA kit from Cayman Chemical Co., Ann Arbor, MI, U.S.A.; monoclonal anti-synaptophysin mouse IgG was purchased from Boehringer-Mannheim GmbH, Mannheim, Germany; 2.5 S nerve growth factor was from Life Technologies, Gaithersburg, MD, U.S.A.; monoclonal anti-SP rat IgG was from Chemicon International, Temecula, CA, U.S.A.; wortmannin was from Sigma, St. Louis, MO, U.S.A.

Statistics

Statistical significance was assessed using Steel's non-parametric multiple comparison or Wilcoxon's signed rank test. $P < 0.05$ was regarded as significant.

Results

Release of SP from isolated spinal cords

Since repeated application of high-K^+ medium caused a decline in the evoked release of SP in preliminary experiments, effects of wortmannin were examined by comparing the net release in the presence of wortmannin with that in the absence of wortmannin using a pair of hemisected spinal cords derived from the same rat. Results of experiments with 5 μM wortmannin are shown in Figure 1A. Because of a delay in replacement of the medium in the chamber (Sakuma *et al*., 1991), the peak of the evoked release usually occurred in the fourth fraction, i.e. during the period 3–6 min after the start

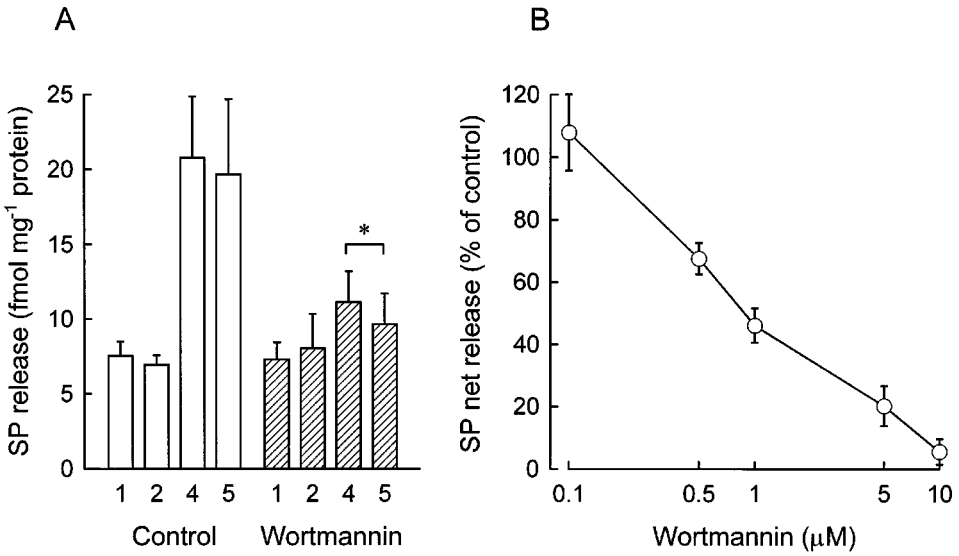

Figure 1 Effects of wortmannin on the release of SP evoked by high-K^+ (90 mM) from isolated spinal cords of neonatal rats. (A) Effect of 5 μM wortmannin on pairs of hemisected spinal cords. Each column and bar represent mean$\pm$s.e.mean ($n=4$). *$P<0.05$, compared with the control evoked release by Wilcoxon's signed rank test. (B) Dose-dependent effect of wortmannin on the net release of SP evoked by high-K^+ as described in Methods. Each point and bar represent mean$\pm$s.e.mean ($n=3-6$).

of perfusion with the high-K^+ medium. In artificial CSF containing the mixture of peptidase inhibitors but not wortmannin, the basal release of SP was 7.2 ± 0.8 fmol mg^{-1} protein 3 min^{-1} ($n=4$). Bath-application of high-K^+ medium (90 mM) for 6 min to the spinal cord resulted in an increase in the release of SP, which became to 20.8 ± 4.1 fmol mg^{-1} protein 3 min^{-1} during the period of 3–6 min after starting the application of high-K^+ medium ($n=4$). Wortmannin decreased the amount of the maximal release but did not alter the time course (Figure 1A). In the presence of 5 μM wortmannin, the release of SP evoked by high-K^+ medium was inhibited to 11.1 ± 2.1 fmol mg^{-1} protein 3 min^{-1} ($P<0.05$, $n=4$), while the basal release was not significantly affected (7.6 ± 1.6 fmol mg^{-1} protein 3 min^{-1}, $n=4$). The net evoked release of SP was depressed in a dose-dependent manner under wortmannin (Figure 1B). The IC_{50} was estimated at 1.1 μM.

Release of SP from cultured DRG cells

The effects of wortmannin on the release of SP were also examined in DRG cells in culture. The basal release of SP in normal-K^+ solution during 5 min was 1.1 ± 0.2% of the total SP content. High-K^+ medium (70 mM) caused an increase in the release of SP to 8.7 ± 0.6% 5 min^{-1} in the absence of wortmannin ($n=7$, Figure 2). Wortmannin markedly inhibited the high-K^+-evoked release of SP at 1 and 10 μM. The basal release was not significantly affected by wortmannin (Figure 2).

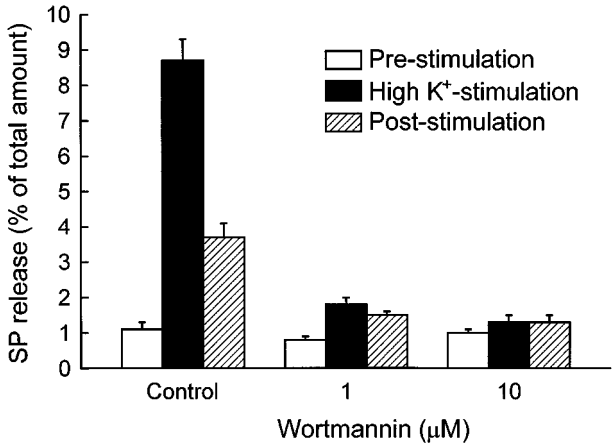

Figure 2 Effects of wortmannin on the release of SP evoked by high-K^+ (70 mM) from cultured dorsal root ganglion (DRG) neurons. In control experiments, the neurons were first incubated in normal-K^+ solution without wortmannin for 30 min, and then again in normal-K^+ solution for 5 min (the SP content shown by open column); subsequently the neurons were incubated in the high-K^+ solution for 5 min (closed column) followed by incubation in normal-K^+ solution for 5 min (hatched column). In test experiments, the neurons were treated in the same way except that the incubation solutions contained wortmannin at 1 or 10 μM throughout the experiments. Ordinate: the amount of SP release expressed as percentage of the total amount of SP that was calculated by summing the amounts of SP in the incubation solutions and that in the cell extract. The mean value of the total amount of SP was 0.98 ± 0.09 pmol dish^{-1}. Abscissa: concentration of wortmannin. Each column and bar represent mean$\pm$s.e.mean ($n=3-7$).

Release of amino acids from isolated spinal cords

Effects of wortmannin on high-K^+-evoked release of glutamate and GABA from the spinal cord were investigated for comparison with those on the release of SP. The basal releases of glutamate and GABA in the second collection were not affected by wortmannin at 0.1–10 μM (data not shown). The net evoked release of glutamate or GABA in the second collection after an interval of 1 h was expressed as percentage of that in the first collection with the same preparation. In control experiments, the net evoked release of glutamate or GABA in the second collection was slightly lower than that in the first collection but the difference was not significant (Figure 3). The net evoked release of glutamate and GABA in the second collection was not significantly depressed by wortmannin at 0.1–10 μM (Figure 3).

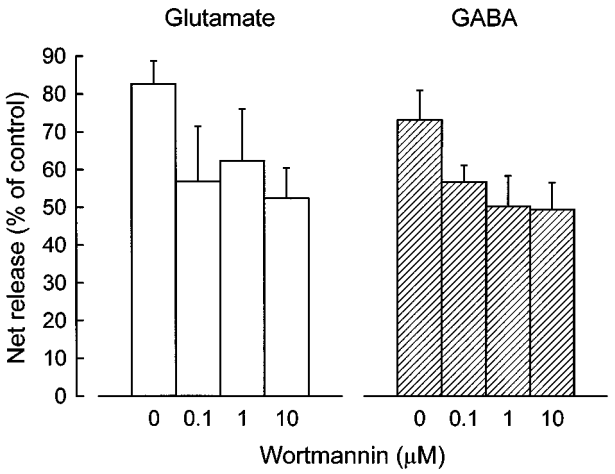

Figure 3 Effects of wortmannin on the release of glutamate and GABA evoked by high-K^+ (90 mM) from isolated spinal cords. Ordinate: the net evoked release of glutamate (open columns) and GABA (hatched columns) in the second collection expressed as percentage of that in the first collection. The amount of the net evoked release was calculated as described in Methods. In control experiments, the mean values of the net evoked release of glutamate and GABA in the first collection were 93.3 ± 60.1 and 60.0 ± 4.1 pmol mg^{-1} protein and those in the second collection were 68.1 ± 37.9 and 41.3 ± 9.1 pmol mg^{-1} protein ($n=3$). Abscissa: concentration of wortmannin. Each column and bar represent mean $\pm$ s.e.mean ($n=3-6$).

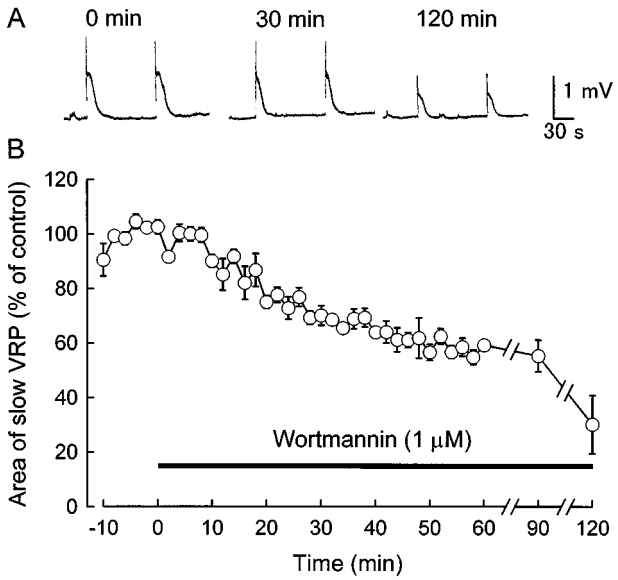

Figure 4 Effects of wortmannin (1 μM) on slow ventral root potentials (VRPs) evoked by dorsal root stimulation in isolated spinal cord preparations of neonatal rats. (A) Sample records: immediately before (control responses), 30 min, and 120 min after addition of wortmannin (1 μM). (B) Time course of the effect of wortmannin on slow VRPs. Areas of the responses in mV·s were measured and expressed as percentage of the average value of five control responses in normal artificial CSF immediately before application of wortmannin. Wortmannin was applied during the period indicated by the horizontal bar. Each point and bar represent mean $\pm$ s.e.mean ($n=4$).

Electrophysiological experiments

When a lumbar (L3–5) dorsal root was stimulated every 2 min with a single square pulse of 100 μs duration at 30–40 V in an isolated spinal cord preparation, a monosynaptic reflex was elicited in the ipsilateral ventral root of the same segment and was followed by a slow VRP lasting about 30 s. Previous studies showed that the slow VRP was depressed by various tachykinin NK_1 receptor antagonists (Akagi *et al.*, 1985; Guo *et al.*, 1993), suggesting the involvement of SP and neurokinin A in the slow VRP. When monosynaptic reflexes and slow VRPs became stable after about 1 h of perfusion with normal artificial CSF, wortmannin at 1 μM was added to the perfusion medium. The magnitude of the slow VRP was gradually decreased to about 70% of the control at 30 min after addition of wortmannin ($P<0.05$, Figure 4). In contrast, the amplitude of monosynaptic reflex was unchanged for 120 min after addition of 1 μM wortmannin (Figure 5).

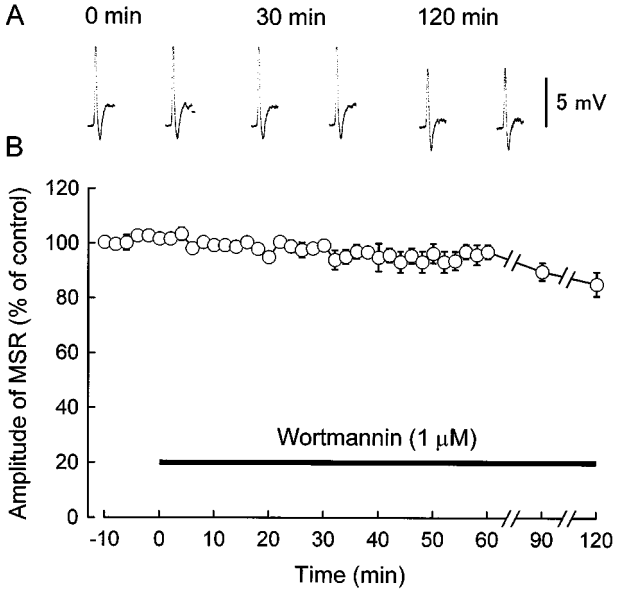

Figure 5 Effects of wortmannin (1 μM) on monosynaptic reflexes (MSRs) evoked by dorsal root stimulation in isolated spinal cord preparations. Experimental procedures were similar to those in Figure 4. (A) Sample records: immediately before (control responses), 30 min, and 120 min after addition of wortmannin (1 μM). In each panel, two successive MSRs are shown. (B) Time course of the effect of wortmannin. Peak amplitude of the reflex was measured and expressed as percentage of the average value of five control responses in normal artificial CSF immediately before application of wortmannin. Each point and bar represent mean $\pm$ s.e.mean ($n=4$).

Immunofluorescence studies

Localization of myosin II and SP in the dorsal horn region of the neonatal rat spinal cord was examined. As previously reported (Cuello *et al.*, 1979), SP immunoreactivity was concentrated in the superficial layers of the dorsal horn. Myosin II immunoreactivity was also observed as clear fluorescent spots in the dorsal horn (arrowheads in Figure 6A,B). Double staining showed that the majority of SP-immunoreactive spots also displayed the immunoreactivity of myosin II (Figure 6B,C) in this region, although the extent of distribution of myosin immunoreactivity was broader than that of SP immunoreactivity. In some sections examined, SP showed stronger fluorescent signals than myosin II (arrowheads in Figure 6D,E). When adjacent sections were stained with secondary antibodies only, no fluorescent images were observed. To examine whether or not myosin II is present at presynaptic terminals, double staining of myosin II with synaptophysin, a marker protein of presynaptic terminals, was carried out. Myosin II immunoreactivity was found to be colocalized with synaptophysin immunoreactivity in the

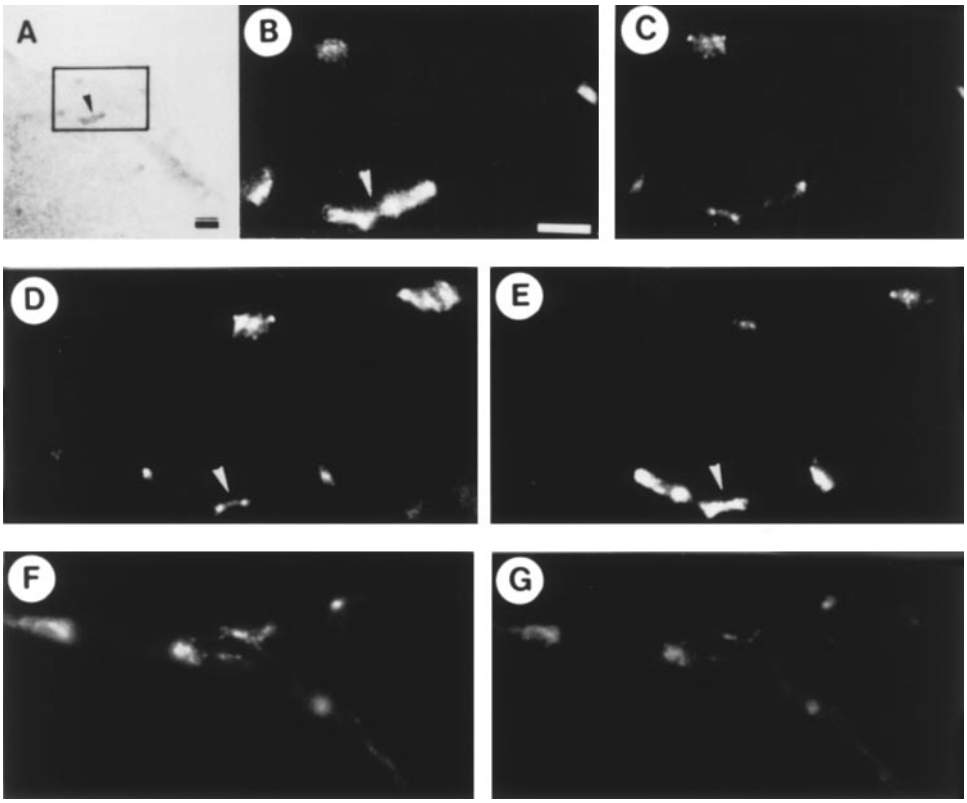

Figure 6 Localization of myosin II, substance P (SP) and synaptophysin in dorsal horn region demonstrated by fluorescent immunohistochemistry. (A – C) Immunofluorescent images of myosin II and SP in the dorsal horn region of a neonatal rat spinal cord. A, negative image of the dorsal horn region showing myosin II labelled by anti-myosin II rabbit IgG and rhodamine-conjugated anti-rabbit IgG under low magnification. Scale bar, 20 μm. B and C show the framed area in A under higher magnification. (B) Fluorescent image of myosin II. Arrowheads in A and B indicate the identical spot. Scale bar, 5 μm. (C) Fluorescent image of SP labelled by anti-SP rat IgG, biotinylated anti-rat IgG and FITC-conjugated streptavidin in the same area as that of B. (D and E) fluorescent images of myosin II (D) and SP (E) in the adjacent area to that shown in A, B and C. Note that immunoreactivity of SP is more intense than that of myosin II in some parts of this field (e.g. arrowheads). (F and G) Fluorescent images of myosin II (F) and synaptophysin (G) labelled by anti-synaptophysin mouse IgG, biotinylated anti-mouse IgG and FITC-conjugated streptavidin. The magnifications in C to G are the same as that in B.

superficial layer of the dorsal horn (Figure 6F,G), suggesting that myosin exists at presynaptic terminals. But not all immunoreactive spots of synaptophysin were overlapped with those of myosin II.

Discussion

The present study demonstrated that wortmannin inhibited the high-K^+-evoked release of SP from the isolated spinal cord of the neonatal rat at the concentration range of 1 – 10 μM (IC_{50} value of about 1 μM). Besides primary afferent fibres, SP is known to be contained in some population of interneurons and descending fibres in the spinal cord (Otsuka & Yoshioka, 1993). The relative contributions of these three SP pools in the release from the spinal cord is not clear. However, wortmannin at the similar concentration range also inhibited the SP release from cultured DRG neurons, demonstrating its inhibitory action on primary afferent neurons. In electrophysiological experiments, the slow VRP was depressed to 70% of the control 30 min after the application of wortmannin at 1 μM (Figure 4). There is evidence that SP released from primary afferent C fibres produces slow excitatory postsynaptic potentials in dorsal horn neurons resulting in the generation of the slow VRP (Otsuka & Yoshioka, 1993). The present observations, therefore, suggest that wortmannin depresses the slow VRP by inhibiting the release of SP from primary afferent nerve terminals.

In contrast to SP, the release of glutamate and GABA from the spinal cord was not affected by wortmannin at 0.1 – 10 μM. Furthermore, the monosynaptic reflex, which is mediated by glutamate (Jahr & Yoshioka, 1986), was unaffected by wortmannin. These results clearly show a difference in the susceptibilities to wortmannin of the release of SP and amino acids. SP is contained in large dense-core vesicles, whereas amino acid neurotransmitters like glutamate and GABA are stored in small synaptic vesicles. Mobilization, docking and fusion mechanisms seem different between small and large synaptic vesicles: for example, small vesicles are clustered at active zones of presynaptic terminals, whereas large dense-core vesicles are not (Jessell & Kandel, 1993; Kelly, 1993). The present study suggests that wortmannin may inhibit the SP release by suppressing the function of a component that is essential in the exocytosis of SP-containing vesicles, but not that of amino acid-containing vesicles.

Wortmannin was reported to inhibit effectively MLCK (Nakanishi *et al*., 1992) and later it was found to inhibit strongly phosphatidyl inositol 3 (PI3)-kinase. *In vitro*, the value of IC_{50} for inhibition of MLCK is 0.17 μM (Nakanishi *et al*., 1992), whereas that for inhibition of PI3-kinase is 3 nM (Yano *et al*., 1993). In accordance, differential effects of wortmannin were observed by application of the drug in different concentration ranges in two kinds of cell lines. Firstly, wortmannin potentiated glucose-induced insulin secretion in MIN6 cells through the inhibition of PI3-kinase at low nM concentration and inhibited the insulin secretion through the

inhibition of MLCK at a 100 fold higher concentration (Hagiwara *et al*., 1995). Secondly, in RBL-2H3 cells, IgE receptor-mediated histamine secretion was inhibited by wortmannin through inhibition of PI3-kinase with IC_{50} of 0.8 nM whereas Ca^{2+}-ionophore-mediated histamine secretion was inhibited by the drug through inhibition of MLCK with IC_{50} of 0.1 μM (Ozawa *et al*., 1996). In the present study wortmannin inhibited the release of SP with IC_{50} value of about 1 μM (Figure 1B). Therefore, it seems likely that the depressant effect of wortmannin on the release of SP was *via* inhibition of MLCK. It might be argued that micromolar concentrations of wortmannin inhibited PI3-kinase, but not MLCK, in the isolated spinal cord because of the difficulty of penetration of wortmannin to the site of action in the spinal cord. The depressant action of wortmannin on the SP release from cultured DRG neurons, in which wortmannin is expected to penetrate more easily to the site of action, was slightly more potent than that from the spinal cord. However, the effective concentration range of wortmannin for inhibiting the release of SP from cultured DRG neurons was higher than that inhibiting PI3-kinase. Furthermore, wortmannin showed similar IC_{50} values (approximately 1 μM) for inhibition of the release of catecholamines in both intact and permealized chromaffin cells (Ohara-Imaizumi *et al*., 1992; Kumakura *et al*., 1994), suggesting that cell membranes do not hamper the penetration of wortmannin to the interior of neurons. Taken together, the effect of wortmannin in the present study suggests that phosphorylation of myosin II plays an important role in the release of SP.

In consonance with this notion, the present immunofluorescence study showed colocalization of SP, myosin II, and a presynaptic protein, synaptophysin, suggesting that SP and myosin II co-exist in the presynaptic terminals. Myosin II has also been reported to exist in growth cones of rat DRG neurons (Miller *et al*., 1992) and in presynaptic axon terminals of sympathetic ganglion cells (Mochida *et al*., 1994). Actin has also been identified near synaptic vesicles (Burns & Augustine, 1995). The function of actin was thought to capture the vesicles and thereby prevent them from fusing to the presynaptic plasma membrane (Bähler & Greengard, 1987). As shown in muscle cells, where actin-myosin interaction is essential for contraction, myosin may possibly interact with actin to work cooperatively at nerve terminals for releasing SP and possibly other peptides.

In conclusion, wortmannin at 0.5–10 μM depressed high-K^+-evoked release of SP but not of amino acids from the isolated spinal cord of the neonatal rat. These results suggest that myosin phosphorylation by MLCK is crucial for the release of SP, but not for the release of GABA and glutamate. The differential effects of wortmannin may be due to a difference in the exocytic mechanisms between large dense core vesicles containing neuropeptides and small clear vesicles containing amino acids.

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