Essential Role of Myosin Light Chain Kinase in the Mechanism for MgATP-Dependent Priming of Exocytosis in Adrenal Chromaffin Cells

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Ca^{2+}-induced exocytosis in chromaffin cells now seems to consist of at least two distinct steps: MgATP-dependent Ca^{2+}-enhanced priming of the secretory apparatus, and Ca^{2+}-dependent MgATP-independent step that triggers exocytosis (Bittner and Holz, 1992). Recently we found that a specific inhibitor of myosin light chain kinase (MLCK), wortmannin, inhibits Ca^{2+}-induced catecholamine release from digitonin-permeabilized chromaffin cells, suggesting an implication of MLCK in the mechanisms of Ca^{2+}-induced exocytosis (Imaizumi et al., 1992b). To elucidate further the implication of MLCK in the mechanism of exocytosis, we studied the effects of wortmannin and a peptide inhibitor (SM-1) corresponding to the pseudosubstrate domain of MLCK on MgATP-dependent and MgATP-independent release in digitonin-permeabilized chromaffin cells. Ca^{2+}-induced exocytosis from the permeabilized cells in the presence of MgATP was inhibited by both SM-1 and wortmannin. Inhibitory effect of wortmannin on the rate of release induced by 10 μM Ca^{2+} in the presence of MgATP was much prominent in the later phase (1–10 min), although the initial rate was also decreased. SM-1 strongly inhibited ATP-dependent release without affecting Ca^{2+}-dependent ATP-independent release at all. In addition, priming effect of MgATP that underlies Ca^{2+}-dependent ATP-independent release was remarkably reduced by both wortmannin and SM-1. These results suggest that MLCK plays an essential role in ATP-dependent priming of Ca^{2+}-induced exocytosis in chromaffin cells.

[Key words: chromaffin cells, exocytosis, myosin light chain kinase, ATP-dependent priming, wortmannin, MLCK pseudosubstrate domain peptide]

Recent studies on mechanisms of regulated secretion in chromaffin cells have revealed possible implication of guanine nucleotides and guanine nucleotide–binding proteins (Knight and Baker, 1985; Bittner et al., 1986; Ohara-Imaizumi et al., 1992a; Vitale et al., 1993), various protein kinases (Morgan and Bur-

goyne, 1990; Terbush and Holz, 1990; Wilson, 1990; Ohara-Imaizumi et al., 1992b), cytoplasmic proteins (Sarafian et al., 1987; Ali et al., 1989; Morgan and Burgoyne, 1992; Nishizaki et al., 1992), and granule-associated proteins (Darchen et al., 1990; Perin et al., 1991; Elferink et al., 1993). While the progress by these studies does not allow us yet to fully understand the biochemical events responsible for regulated exocytosis, multiple processes in Ca^{2+}-dependent secretion have been suggested recently. Holz and collaborators suggested that MgATP primed secretion before Ca^{2+}-triggering process in digitonin-permeabilized cells, and that Ca^{2+}-evoked exocytosis from the primed pool was independent of ATP (Holz et al., 1989; Bittner and Holz, 1992). Hay and Martin (1992) dissected the regulated secretion, in mechanically disrupted PC12 cells, into MgATP-dependent priming and Ca^{2+}-dependent MgATP-independent triggering stages. In mast cells permeabilized by streptolysin-O, ATP was required to maintain a high affinity of the response system for Ca^{2+} and GTPγS, but was not required for the final stage of triggered exocytosis (Howell et al., 1989).

Recently we reported the inhibition by wortmannin of Ca^{2+}-induced catecholamine (CA) release from digitonin-permeabilized chromaffin cells as well as the release by ACh and high K$^+$ from the intact cells (Ohara-Imaizumi et al., 1992b). Wortmannin inhibits myosin light chain kinase (MLCK) specifically and potently (Nakanishi et al., 1992). Thus, we suggested an implication of MLCK in the mechanisms of Ca^{2+}-induced exocytosis. Possible involvement of the contractile proteins or their regulatory proteins in the mechanism of exocytosis in chromaffin cells has been proposed from earlier (Douglas and Rubin, 1964; Poisner and Trifaro, 1967; Trifaro, 1978). MLCK is present in adrenal medulla (Kanda et al., 1985), and exogenous MLCK or secretagogues have been shown to phosphorylate intracellular myosin light chain (MLC) in these cells (Lee et al., 1987; Gutierrez et al., 1989). Despite these findings, physiological studies have failed to provide any evidence that suggests the involvement of MLCK activation in the mechanism of exocytosis (Lee et al., 1987; Nakanishi et al., 1989) until our previous report (Ohara-Imaizumi, 1992b).

To clarify the implication of MLCK activation in the multiple processes for the regulated exocytosis, in this study we examined the effects of inhibition of MLCK activation on MgATP-dependent and MgATP-independent release of CA in digitonin-permeabilized chromaffin cells. We particularly used a potent inhibiting peptide, SM-1, that corresponds to the pseudosubstrate domain of MLCK required for autoinhibition (Kargacin

et al., 1990) and other inhibiting peptides in addition to wortmannin. We also studied the localization of myosin II for understanding the site of MLCK implication.

Materials and Methods

Primary cultures. Adrenal chromaffin cells were isolated from fresh bovine adrenal glands by collagenase digestion, and cells were purified by differential plating as previously described (Kumakura et al., 1986). The purified chromaffin cells were plated (3×10^5 cells/ml) on collagen-coated 16 mm diameter dishes (Nunc), and were maintained in monolayer culture in Dulbecco's modified Eagle's Medium (DMEM; GIBCO) supplemented with 10% fetal calf serum (Boehringer Mannheim) as was described previously (Ohara-Imaizumi et al., 1992a). All the experiments were performed with the cells in culture for 3–10 d.

Permeabilization of cells. The chromaffin cells in monolayer were permeabilized by incubating for 4 min at 37°C in a buffer (P-buffer) containing 140 mM Na-glutamate, 20 mM PIPES, 5 mM glucose, 5 mM EGTA, and 0.02 mM digitonin (Calbiochem) adjusted pH to 6.80 with NaOH (Ohara-Imaizumi, 1992a). This permeabilizing buffer was subsequently replaced with a buffer for a secretion experiment.

Secretion experiments. The release of CA from the digitonin-permeabilized cells was measured as previously described (Ohara-Imaizumi, et al., 1992a). At the end of permeabilization, P-buffer was replaced with a buffer (S-buffer) containing (in mM) 140 Na-glutamate, 20 piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 5 glucose, 5 MgSO$_4$, 5 ATP, and 5 EGTA (without or with various amount of CaCl$_2$), with pH adjusted to 6.80 with NaOH. The cells were incubated for an additional 6 min and the medium was collected for measurement of Ca^{2+}-evoked CA release. Calcium concentrations in Ca^{2+}-EGTA buffers were calculated according to Nanninga and Kempen (1971). For the measurement of MgATP-independent release, the cells were incubated for 1–2 min in S-buffer containing 100 μM Ca^{2+} without MgSO$_4$ and ATP. For MgATP-dependent release, the concentration of free Ca^{2+} in S-buffer was adjusted to 10 μM, and the cells were incubated for 6 min according to Bittner and Holz (1992) in the presence of 5 mM MgSO$_4$ and 5 mM ATP. The release of endogenous CA from intact chromaffin cells in monolayer culture was measured as previously described (Ohara-Imaizumi et al., 1992a). Briefly, the cells were stimulated in Locke's solution for 3 min following the preincubation for 30 min at 30°C. Released CA and cellular CA were extracted with 0.4 M perchloric acid and assayed by HPLC (Kumakura et al., 1986).

Priming effect of ATP. To examine the priming effect of ATP, priming incubation was done simultaneously with permeabilization for 4 min in the permeabilizing buffer (P-buffer) containing 5 mM MgSO$_4$, with or without 5 mM ATP. The buffer was subsequently replaced with S-buffer without ATP, and MgATP-independent release was measured.

Wortmannin and MLCK-inhibiting peptides. Wortmannin was prepared as previously described (Nakanishi et al., 1992). SM-1 was synthesized and purified according to Wysolmerski and Lagunoff (1991). SM-1 has the following sequence: Ala-Lys-Lys-Leu-Ser-Lys-Asp-Arg-Met-Lys-Lys-Tyr-Met-Ala-Arg-Arg- Lys-Trp-Gln-Lys-Thr-Gly (MLCK, 480–501). Myosin kinase-inhibiting peptide (MKIP; Lys-Lys-Arg-Ala-Ala-Arg-Ala-Thr-Ser-NH$_2$; analog of MLC$_{20}$, 11-19) and N-terminal acetylated MKIP (MKIPAc) were prepared according to Hunt et al. (1989). A pseudosubstrate peptide for MLCK (K-MLC 11-23), and for protein kinase C (PKC), [Ser25]PKC(19-31), were purchased from Peninsula Laboratories (Belmont, CA).

To examine the effects of wortmannin, 30 min preincubation was done in the presence of the drug prior to the digitonin permeabilization. The peptide inhibitors were added in the permeabilizing P-buffer and also in S-buffer for some experiments (see figure captions).

Monitoring of intracellular calcium. For the monitoring of intracellular calcium, the cells cultured on a coverslips coated with Cell-Tak (Biopolymers, Farmington, CT) were loaded with 2 μM fura-2/AM in DMEM for 1 hr at 37°C using a Sykus-Moore culture chamber, and rinsed three times with Locke's solution. Fura-2–loaded cells were incubated for another 30 min in the presence or absence of wortmannin prior to Ca^{2+} monitoring. The cells were superfused (2 ml/min) with Locke's solution at 30°C for fluorescence measurement. Digital imaging of fura-2 fluorescence was carried out with an inverted microscope (TMD-2, Nikon, Tokyo, Japan) and a digital image processor (Argus-100/Ca, Hamamatsu Photonics, Hamamatsu, Japan). The concentration of intracellular free Ca^{2+} ([Ca^{2+}]$_i$) was estimated from the fluo-

rescence ratio (340 nm:380 nm) using the equation described by Grynkiewicz et al. (1985).

Preparation of antibody and immunoblotting. Myosin heavy chain was purified from chicken gizzard smooth muscle according to the method previously described (Nakamura and Nonomura, 1984). Preparation and characteristics of polyclonal anti-myosin II IgG will be described in details elsewhere (Ogiwara, unpublished observations). This antibody specifically and strongly reacted with mammalian nonmuscle myosin II heavy chain as well as smooth muscle one (see Results and Fig. 10A).

For immunoblotting, cultured chromaffin cells were collected after rinsed with PBS, and homogenized by a small Grass-homogenizer in the solution containing 50 mM KCl, 25 mM Tris-HCl (pH 7.6), 50 μM DFP, 50 μM NaN$_3$, and 1 mM MgCl$_2$. The homogenate was centrifuged in an Eppendorf tube at 3500 × g for 10 min. The supernatant was centrifuged at 16,000 × g for 90 min, and the precipitate solved in SDS solution was used as microsome-rich fraction. Proteins resolved by SDS-PAGE were transferred to nitrocellulose sheets (Schleicher and Schuell, Inc.; 0.25 μm), and after staining with Ponceau S and photographed, the samples were reacted with anti myosin II IgG (diluted 1:500) and HRP-conjugated anti-rabbit IgG according to the conventional method, and stained with 4-chloro-naphthyl phosphate.

Immunofluorescent microscopy. Chromaffin cells cultured on coverslips were fixed with 0.8% paraformaldehyde in PBS for 1 min and, after washing with PBS, with 100% acetone for 5 min at room temperature. The cells were treated with 1% Triton X-100 in PBS for 5 min at room temperature, rinsed in PBS, and then incubated with the blocking solution containing 2.5% bovine serum albumin and 2% chicken serum for 30 min. Subsequently, the cells were incubated with anti myosin II IgG (diluted 1:400) for 3 hr at 37°C. After rinsing in 400 mM MgCl$_2$ and 20 mM Tris-HCl (pH 7.6) and PBS, the cells were incubated for 1 hr at 37°C with FITC-conjugated anti rabbit IgG in the blocking solution. The samples were mounted in 5% DABCO in PBS/glycerol (1:1) and visualized under Nikon FEX-A fluorescent microscopy.

Results

Inhibition of CA release by wortmannin

Preincubation of adrenal chromaffin cells with wortmannin produced a marked decrease in CA release evoked by ACh from the intact cells (Fig. 1). The inhibitory effect of wortmannin on ACh-evoked release was dose dependent, and a significant inhibition was observed at concentrations above 0.3 μM. The apparent IC$_{50}$ value for the inhibition was approximately 2 μM that was consistent with IC$_{50}$ for the inhibition of high-K$^+$-evoked and Ca^{2+}-induced release (Ohara-Imaizumi et al., 1992b). Wortmannin at 10 μM suppressed ACh-evoked release almost completely as was observed for high-K$^+$-evoked and Ca^{2+}-induced release in our previous report (Ohara-Imaizumi et al., 1992b). Wortmannin did not affect either the basal release or the noradrenaline:adrenaline ratio in the released CA (data not shown). The release in the absence of Ca^{2+} in the digitonin-permeabilized cells was not affected by wortmannin (4.4 ± 0.2% and 4.1 ± 0.2% in 10 μM wortmannin-untreated and -treated cells, respectively). Wortmannin did not affect the cellular content of CA at all (data not shown). These results together with our previous findings suggest that the inhibitory action of wortmannin on the evoked CA release from intact cells and on Ca^{2+}-induced release from the permeabilized cells is mediated by the same mechanism.

Time dependency of the inhibitory action of wortmannin

Since the inhibition of purified MLCK by wortmannin *in vitro* is dependent on the preincubation time that is required for the interaction of the drug with the enzyme (Nakanishi et al., 1992), we examined the effect of preincubation time on susceptibility of Ca^{2+}-induced release from the permeabilized cells. When the cells were preincubated in the presence of 10 μM wortmannin for a different time prior to the permeabilization, clear inhibition
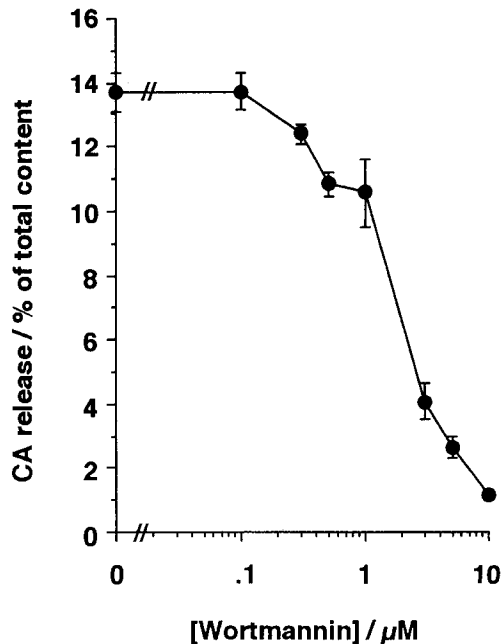
Figure 1. Dose–response curve for wortmannin inhibition of ACh-evoked from intact chromaffin cells. CA release from the intact cells was measured by stimulating the cells with 10^{-4} M ACh for 3 min following the preincubation for 30 min at 30°C in the presence or absence of increasing concentrations of wortmannin. The amounts of released CA are expressed as the percentage of total cellular content. Spontaneously released CA was measured for each group, and the mean value for each group was subtracted from each evoked release. Data are the mean ± SEM of six determinations from individual wells.

of Ca^{2+}-induced release was observed only after the preincubation for more than 15 min, and preincubation for 30 min was required for nearly maximal inhibition (Fig. 2). This time dependency was similar to that observed for the interaction of wortmannin with MLCK *in vitro* (Nakanishi et al., 1992).

Wortmannin does not inhibit Ca^{2+} influx

In human platelet, our group found an effective blockade by wortmannin of a new type of Ca^{2+} channel that acted in the delayed phase of the response to thrombin stimulation (Hashimoto et al., 1992) Thus, it was necessary to check the effect of wortmannin on Ca^{2+} entry in the present study.

Stimulation of fura-2–loaded intact cells with carbamylcholine (CCh; 2×10^{-4} M) by adding 100 μl of CCh into superfusing chamber produced a transient increase of $[Ca^{2+}]_i$ to 482 ± 43 nM (n = 6). The same concentration of CCh produced $[Ca^{2+}]$ increase to 539 ± 71 nM (n = 6) in wortmannin-treated cells. These concentrations of $[Ca^{2+}]_i$ are comparable to those required for the diphosphorylation of MLC by MLCK *in vitro* (Itoh et al., 1992). Representative fura-2 signals are shown in Figure 3. These results suggest that wortmannin acted on the downstream $[Ca^{2+}]_i$ increase or on the prerequisite process for Ca^{2+}-triggering events in CA release in intact cells.

Effects of SM-1 and other peptide inhibitors

To clarify that the inhibitory action of wortmannin is attributable to its inhibition of MLCK, we examined the effects of various synthetic peptides that are known to inhibit MLCK: SM-1, K-MLC 11-23, MKIP, and its inactive derivatives MKI-PAc. The effects of pseudosubstrate inhibitory peptides for PKC
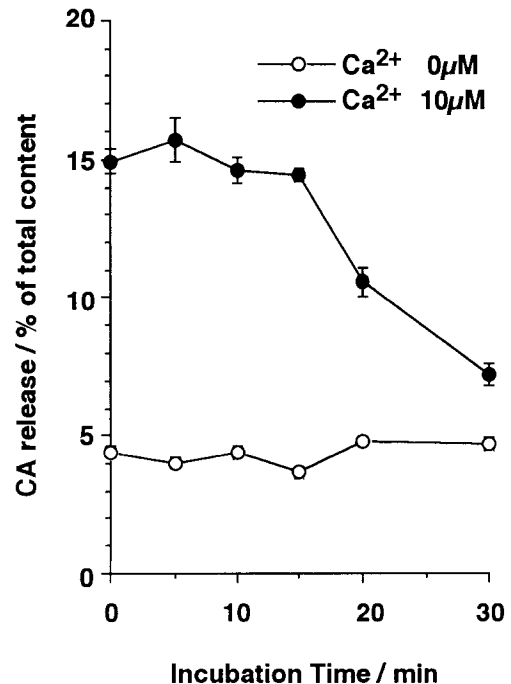

Incubation Time / min

Figure 2. Requirement of preincubation time for wortmannin inhibition of Ca^{2+}-evoked CA release from the permeabilized cells. The cells were preincubated with 10 μM wortmannin for the indicated times at 30°C prior to permeabilization. Permeabilization was done with 10 μM digitonin for 10 min in this particular experiment. The permeabilized cells were stimulated with 10 μM Ca^{2+} for 10 min to measure CA release. The amounts of released CA are expressed as the percentage of total cellular content. Data are the mean ± SEM of eight determinations from individual wells. Note that wortmannin did not affect the basal release in the absence of Ca^{2+}.

were also examined. As was shown in Figure 4, SM-1 inhibited Ca^{2+}-induced release from the permeabilized cells by approximately 25% at 100 μM, 50% at 370 μM, and 90% at 500 μM. K-MLC 11-23 caused approximately 20% inhibition at 500 μM. MKIP was much weaker than SM-1 in inhibiting Ca^{2+}-induced release. The inhibition by 500 μM MKIP was about 15%, although it was statistically insignificant, and such an inhibition was not observed with the inactive analog MKIPAc. In contrast, [Ser25]PKC(19-31) at 100 μM did not affect the Ca^{2+}-induced release from the permeabilized cells at all in good agreement with a previous report (Terbush and Holz, 1990). None of these peptides significantly affected CA release in the absence of Ca^{2+}. It has been shown that MKIP is much less potent than SM-1 in inhibiting Ca^{2+}- and ATP-induced retraction of permeabilized endothelial cells (Wysolmerski and Lagunoff, 1991). The weak inhibitory action of MKIP seems to be due to the fact that this peptide is less potent in inhibiting MLCK in the permeabilized chromaffin cells as well.

These results, especially effective inhibition by SM-1 of Ca^{2+}-induced release from the permeabilized cells, strongly suggest that the inhibitory action of wortmannin resulted from the inhibition of MLCK activation.

Effects of wortmannin on time course of Ca^{2+}-induced release

In the time course of Ca^{2+}-induced release from permeabilized chromaffin cells, it has been shown that the initial rate of release during first 1–2 min is not altered by the absence and presence of MgATP while MgATP is required for the release to continue
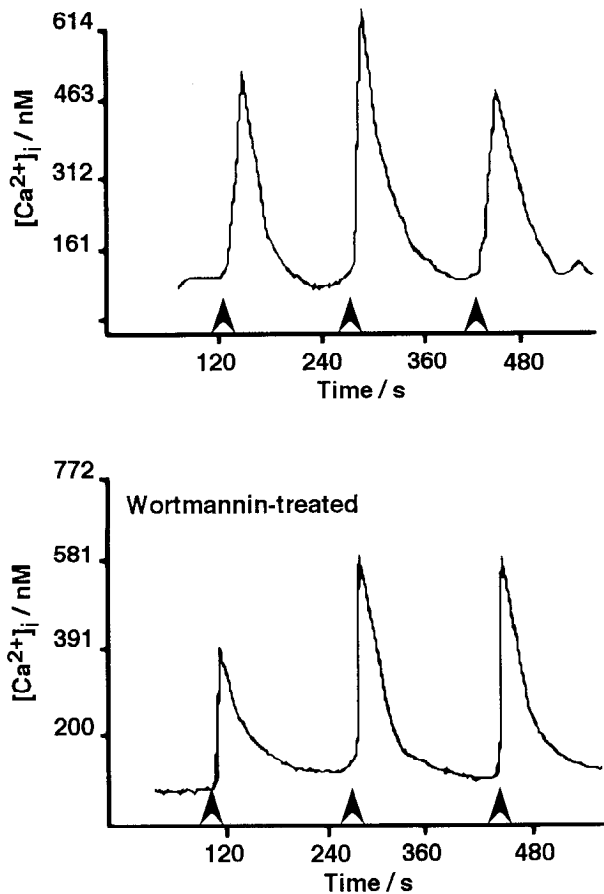
Figure 3. Effect of wortmannin on CCh-evoked increase of intracellular Ca^{2+} *([Ca^{2+}]$_i$)*. Ca^{2+} signals of a single cell were detected with a video-imaging system using fura-2 in control cells *(top)* and wortmannin-treated cells *(bottom)*. The cells were preincubated with or without 10 μM wortmannin for 30 min after loading of fura-2. Repetitive stimulation with 2×10^{-4} M CCh was given in the superfusing chamber at the points indicated by *arrows*. Responses in *A* and *B* are the representative fura-2 signals. Mean values of the increased [Ca^{2+}]$_i$ in control and wortmannin-treated cells were 482 ± 43 nM (*n* = 6) and 539 ± 71 nM (*n* = 6), respectively.

Figure 4. Effects of SM-1, K-MLC 11-23, MKIP, and MKIP-Ac on Ca^{2+}-evoked CA release from the permeabilized cells. The cells were permeabilized with 20 μM digitonin for 4 min, and then stimulated with 10 μM Ca^{2+} for additional 6 min at 30°C. Peptide inhibitors were present during whole procedure at the indicated concentration. The amounts of released CA are expressed as the percentage of total cellular content. The amounts of released CA are expressed as the percentage of total cellular content. Amount of CA released in the absence of Ca^{2+} was measured for each group, and the mean value for each group was subtracted from each evoked release. Data are the mean ± SEM of at least six determinations from individual wells. None of the peptides affected CA release in the absence of Ca^{2+}.

thereafter (Holz et al., 1989). Therefore, we studied the effects of three different concentrations (1, 3, and 10 μM) of wortmannin on the time course of CA release evoked by 10 μM Ca^{2+} from the permeabilized cells. As shown in Figure 5, the time course of Ca^{2+}-induced release in control cells apparently consisted of kinetically distinct three components: (1) the initial phase for the first 1 min, (2) the second phase from 1 to 5 min, (3) the third phase after 5 min. Wortmannin reduced the release in the initial phase to about one-third of control, without any dose dependency in both the extent and secretion rate. In contrast, effects of wortmannin on the later phase were much prominent. In the cells treated with 1 μM wortmannin, the release in the second and third phase persisted up to 10 min though the secretion rate was reduced. Wormannin at 3 μM caused cessation of the release at 2 min without further decrease in the rate of this shortened second phase. Wortmannin at 10 μM almost completely abolished both the second and third phase; thus, the release nearly ceased at the end of the initial phase. Consequently, the duration and final extent of the release were decreased by wortmannin with clear dose dependency that is comparable to that we reported previously (Ohara-Imaizumi et al.,
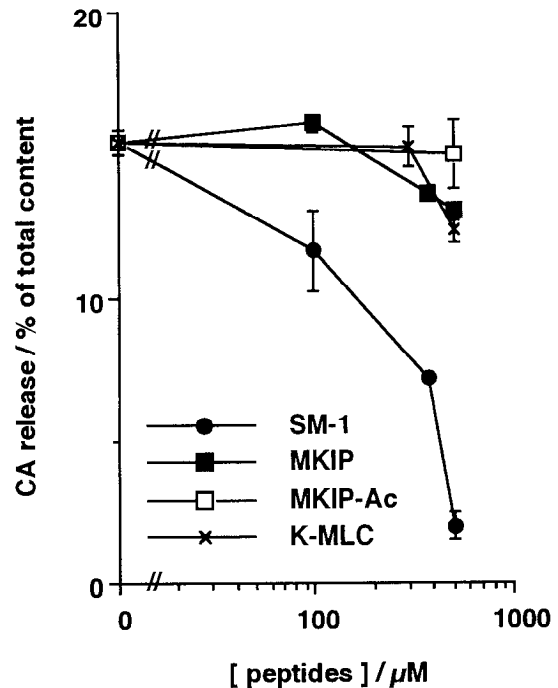
1992). These results clearly suggest a requirement of MLCK activation for the continuous release that is sustained by MgATP.

Effects of wortmannin and SM-1 on ATP-dependent and ATP-independent release

To obtain further evidence for requirement of MLCK for ATP-dependent release, we examined the effects of wortmannin and SM-1 on ATP-independent and ATP-dependent release from the permeabilized cells. When the cells were pretreated with 10 μM wortmannin, both ATP-independent release and ATP-dependent release were decreased approximately by 78% and by 90%, respectively (Fig. 6). In contrast to wortmannin, treatment of the cells with 370 μM SM-1 for 4 min prior to Ca^{2+} challenge caused 55% inhibition of ATP-dependent release without any inhibition of ATP-independent release. Another inhibiting peptide, MKIP, also decreased ATP-dependent release but slightly (Fig. 7). The inhibition by SM-1 and MKIP at this concentration were close to that observed in Figure 4.

The effects of wortmannin and of SM-1 on ATP-independent release were different. One possibility seems to be that a decrement in the population of the primed granules might be caused during the longer preincubation time (30 min) with wortmannin but not within 4 min of preincubation with SM-1, since ATP-dependent priming has been shown to be readily reversible (Holz et al., 1989; Hay and Martin, 1992). However, due to the experimental limitation with digitonin-permeabilized cells, we
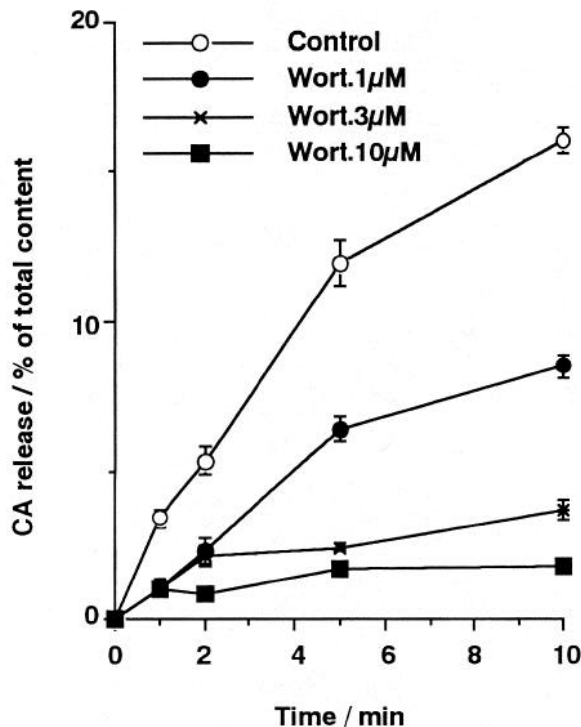
Figure 5. Effects of wortmannin on the time course of Ca²⁺-evoked CA release from the permeabilized cells. The cells were preincubated with or without different concentration of wortmannin for 30 min prior to permeabilization. The permeabilized cells were stimulated with 10 μM Ca²⁺ in the presence of MgATP for the indicated times. The amounts of released CA are expressed as the percentage of total cellular content. Each value was calculated as was described in Figure 4. Data are the mean ± SEM of at least six determinations from individual wells.
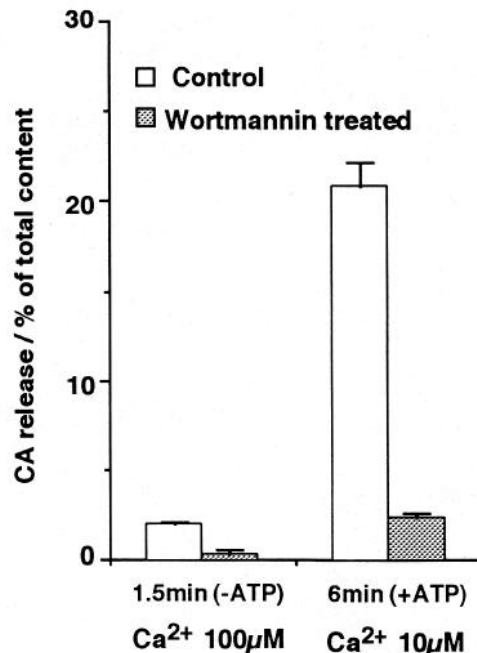

Figure 6. Effects of wortmannin on ATP-independent and ATP-dependent CA release from the permeabilized cells. The cells were preincubated at 30°C with or without 10 μM wortmannin for 30 min prior to permeabilization in the absence of MgATP. Then, ATP-independent ($-ATP$) and ATP-dependent ($+ATP$) release was measured by stimulating with Ca²⁺ at the indicated concentration. The amounts of released CA are expressed as the percentage of total cellular content. Each value was calculated as was described in Figure 4. Data are the mean ± SEM of eight determinations from individual wells.

could not examine the effect of longer treatment with SM-1. The secretory activity is known to run down rapidly after digitonin permeabilization. The response to 100 μM Ca²⁺ at 5 min after 4 min of digitonin permeabilization decreased to 17% of the response without an interval, and the response was almost lost after 15 min interval (data not shown). The inhibition of ATP-independent release by wortmannin should underlie its 70% inhibition of the initial release in the time course (Fig. 5) that was not dose dependent. This may imply alternatively that the inhibition of ATP-independent release by wortmannin is due to a mechanism other than MLCK inhibition. Whatever the mechanism of the inhibition of ATP-independent release by wortmannin that remained uncertain, these results together with the results in time course (Fig. 5) indicate a requirement of MLCK for the continuous release sustained by MgATP.

Wortmannin, SM-1, and K-MLC 11-23 inhibit priming action of ATP

Since ATP-dependent release from the permeabilized cells has been suggested to be attributable to the action of ATP to prime the secretory machinery (Bittner and Holz, 1992), we examined the effect of wortmannin and MLCK-inhibiting peptides on priming action of ATP. As shown in Figure 8, priming with ATP for 4 min remarkably increased both the initial rate and final extent of ATP-independent release. Pretreatment of the cells with wortmannin caused a great decrease in the priming effects of ATP, in addition to a decrease in ATP-independent release in the unprimed cells (Fig. 8). SM-1 at 100 μM caused

Figure 7. Effects of SM-1 and MKIP on ATP-independent and ATP-dependent CA release from the permeabilized cells. SM-1 or MKIP was added at 370 μM during 4 min of permeabilization in the absence of MgATP. ATP-independent ($-ATP$) and ATP-dependent ($+ATP$) release were measured by stimulating with Ca²⁺ at the indicated concentration. The amount of released CA is expressed as the percentage of total cellular content. Each value was calculated as was described in Figure 4. Data are the mean ± SEM of eight determinations from individual wells.
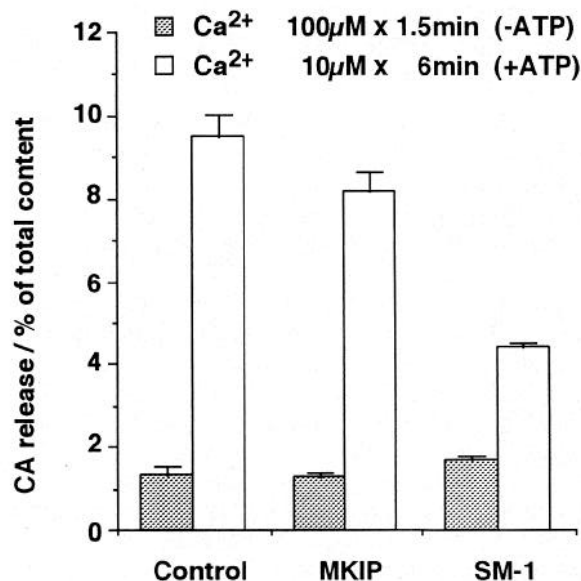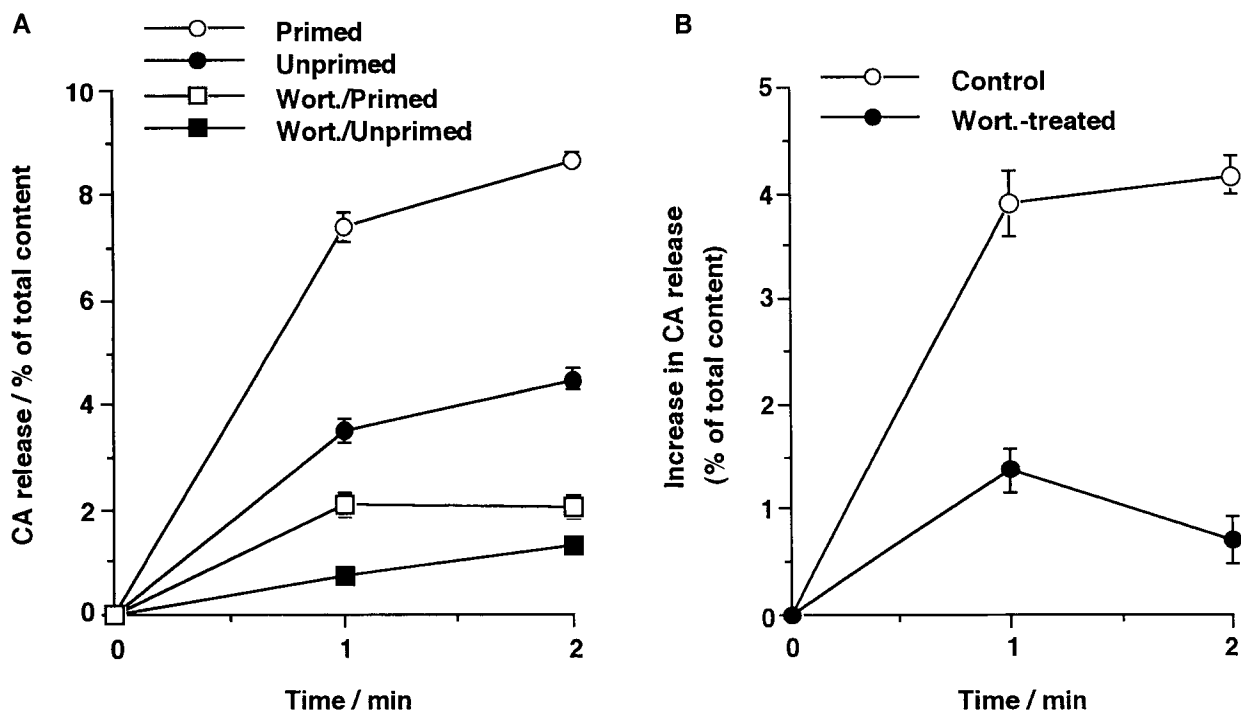
Figure 8. Effect of wortmannin (*Wort.*) on ATP-dependent priming of CA release in the permeabilized cells. The cells were preincubated with or without 10 μM wortmannin for 30 min prior to permeabilization. Priming incubation with 5 mM MgATP was done simultaneously with 4 min of permeabilization. Stimulation with 100 μM Ca^{2+} was done for the indicated times in the absence of ATP. Amount of CA released in the absence of Ca^{2+} was subtracted as described in Figure 4. The amount of released CA is expressed as the percentage of total cellular content. Data are the mean ± SEM of eight determinations from individual wells.

36% inhibition of the priming effect of ATP, and K-MLC 11-23 at 500 μM caused 30% inhibition (Fig. 9). The inhibition by SM-1 at this lower concentration was more pronounced than that observed in Figure 4. These results suggest that activation of MLCK is an essential step for the priming of secretion by MgATP.

Immunoblotting and immunofluorescent localization of myosin

The myosin light chain kinases are known to possess an absolute specificity for myosin light chains (for review, see Kennelly and Krebs, 1991). Therefore, we studied the cellular distribution of antigen site for myosin antibodies in order to understand the site of MLCK implication. The anti-myosin II IgG used in this experiments specifically recognized a single band in immunoblotting of the microsome-rich fraction. The position slightly lower than that of chicken gizzard myosin heavy chain (200 kDa) was very reasonable as nonmuscle myosin II heavy chain (Fig. 10A). Strong immunofluorescence was observed mainly at the subplasmalemmal region (Fig. 10B) as both intense rings and patched distribution. The weak immunofluorescence observed in the cytoplasmic area could be interpreted as either the subplasmalemmal distribution of myosin beneath the area or the cytoplasmic distribution as has been reported (Gutierrez et al., 1989). These immunoblotting and immunofluorescent distributions of myosin may suggest that myosin II is the most probable substrate, situated at the site of secretion, for MLCK involved in ATP-dependent priming mechanisms. Hence, phosphorylation of myosin II in the subplasmalemmal region by MLCK activation may be a part or prerequisite of the priming process.

Discussion

Is MLCK involved in the mechanism of exocytosis?

Our results suggest that MLCK is involved in the mechanisms of Ca^{2+}-induced exocytosis in chromaffin cells as follows. Wortmannin inhibited ACh-evoked release from the intact cells with IC_{50} of approximately 2 μM (Fig. 1), which is consistent with IC_{50} for Ca^{2+}-induced release in permeabilized cells (Ohara-Imaizumi et al., 1992b). The preincubation time required for clear inhibition by wortmannin (Fig. 2) was very similar to that required for the interaction of this drug with MLCK *in vitro* (Nakanishi et al., 1992). Wortmannin inhibits MLCK activity acting irreversibly at or near the catalytic site of the enzyme, whereas it had no effect on cAMP-dependent protein kinase, cGMP-dependent protein kinase, Ca^{2+}/calmodulin-dependent protein kinase II, and little effect on PKC (Nakanishi et al., 1992). Although we found very recently that wortmannin also inhibits phosphatidylinositol 3-kinase (Yano et al., 1993), its IC_{50} (2–3 nM) is extremely lower than that for the inhibition of MLCK (Nakanishi et al., 1992) and of CA release observed here. Hence, the inhibition of CA release by wortmannin observed here seems to be attributable to its inhibitory action on MLCK.

Among MLCK-inhibiting peptides, SM-1 at 500 μM produced almost complete inhibition of Ca^{2+}-induced release from the permeabilized cells. Another MLCK-inhibiting peptide, K-MLC 11-23, caused a significant inhibition, while MKIP was much weaker (Fig. 4). SM-1 is known to be extremely potent (Foster et al., 1990) and highly specific inhibitor for MLCK (Kargacin et al., 1990). The primary structure of nonmuscle MLCK and that of smooth muscle MLCK are identical except for N-ter-
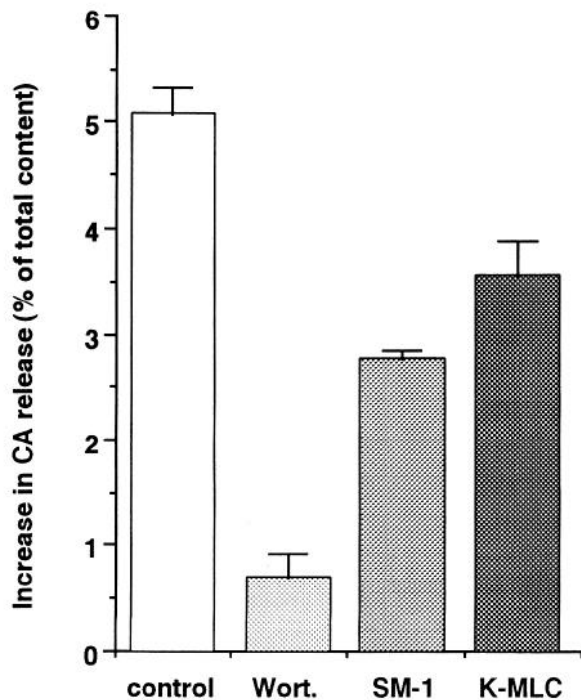
Figure 9. Effects of SM-1 and K-MLC 11-23 on ATP-dependent priming of CA release in the permeabilized cells. Priming incubation with 5 mM MgATP was done simultaneously with 4 min of permeabilization in the presence or absence of 100 μM SM-1 or of 500 μM K-MLC 11-23 (*K-MLC*). MgATP-independent release for 1.5 min was then measured in the absence of the inhibiting peptide. Value is the extent of increase by priming. Mean value of the release in unprimed cells was subtracted from individual value of the release in primed cells. The peptides affected neither the basal release nor the release from the unprimed cells. Data are the mean $\pm$ SEM of eight determinations from individual wells. Inhibition by wortmannin (*Wort.*) detected at 2 min in Figure 8 was also shown.

high as 100 μM was required for clear inhibition even with an intracellular injection (Akasu et al., 1993). Excer presence of endogenous substrate and possible leakage of protein phosphatase from cytosol in our experimental condition might explain the requirement of this high concentration. MKIP was much less potent than SM-1 as was reported for Ca^{2+}- and ATP-induced retraction of permeabilized endothelial cells (Wysolmerski and Lagunoff, 1991).

Anti-calmodulin antibody has been shown to inhibit high-K^+-evoked and ACh-evoked CA release when introduced into chromaffin cells (Kenigsberg and Trifaro, 1985). This seems to be consistent with the present findings, since MLCK is a Ca^{2+}/calmodulin-activated enzyme.

How is MLCK involved?

Ca^{2+}-induced release of CA from the permeabilized chromaffin cells or from the semiintact PC12 cells has been resolved into at least two distinct stages: MgATP-dependent priming of secretion, and Ca^{2+}-triggered ATP-independent release from the primed pool (Bittner and Holz, 1992; Hay and Martin, 1992). In Ca^{2+}-induced release from digitonin-permeabilized chromaffin cells, the initial release for 1–2 min reflects ATP-independent release from the primed pool, and the release thereafter is sustained by ATP-dependent priming (Holz et al., 1989; Bittner and Holz, 1992). The former of these two components can be distinguished as the release evoked by 100 μM Ca^{2+} in the absence of ATP that terminates by 1–2 min, thus termed ATP-independent release. The latter one is distinguished as the release evoked by 10 μM Ca^{2+} in the presence of MgATP that continues for 10–12 min, thus termed ATP-dependent release.

In addition, the priming effect of ATP can be observed as such that preincubation of permeabilized cells with MgATP enhances Ca^{2+}-dependent release during a subsequent incubation in the absence of MgATP either in chromaffin cells or in PC12 cells (Holz et al., 1989; Hay and Martin, 1992). Similar multiple processes of Ca^{2+}-dependent release in chromaffin cells were suggested very recently from kinetic analysis of the membrane capacitance and intracellular Ca^{2+} concentration measured simultaneously (Neher and Zucker, 1993).

The time course of Ca^{2+}-induced release from the permeabilized cells in this study consisted of kinetically distinct three components. Wortmannin differently affected these three stages, and the effects of wortmannin were more pronounced on the
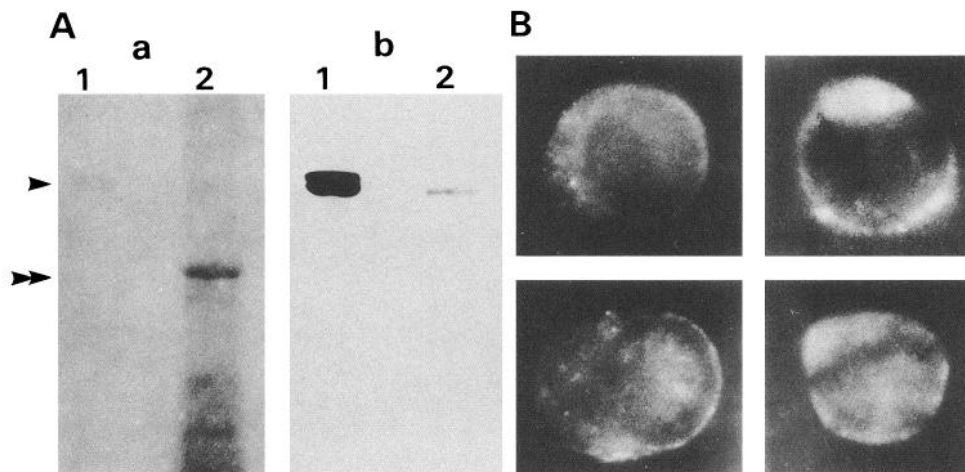
minal region (Shoemaker et al., 1990). Therefore, the inhibition of CA release by SM-1 in the present results can be interpreted as a result from specific inhibition of MLCK. Significant inhibition by K-MLC 11-23 supports this interpretation. The IC_{50} of SM-1 for this inhibition seems considerably high. However, SM-1 was recently shown to inhibit voltage-dependent potassium currents in sympathetic neurons, and a concentration as

Figure 10. *A*, Immunoblotting results by anti-myosin II IgG: *a*, protein staining by Ponceau staining; *b*, immunostaining; *1*, chicken gizzard myosin; *2*, membrane rich fraction from chromaffin cells. *Arrowhead* indicates the position of 200 kDa molecular weight of myosin II heavy chain. *Double arrowhead* indicates the position of 100 kDa. The band at 100 kDa in protein staining shows perhaps the Ca-ATPase in membrane fraction. *B*, Distribution of myosin in chromaffin cells detected by Immunofluorescent method.

release during the second and third phases than on the initial release. Wortmannin reduced dose dependently both the rate and duration of the release in the second and third phases (Fig. 5). Consistently, ATP-dependent release was inhibited by SM-1, while ATP-independent release was not affected at all (Fig. 7). The priming by MgATP that underlies ATP-dependent release was inhibited remarkably by wortmannin, and significantly by either SM-1 or K-MLC 11-23 (Figs. 8, 9). All of these results clearly suggest that MLCK has an essential role in ATP-dependent priming pathway.

A question arises, then, whether phosphorylation of MLC_{20} mediates the putative role of MLCK or not in the regulated exocytosis. The catalytic and calmodulin regulatory segments of nonmuscle MLCK are virtually identical to that of smooth muscle MLCK (Shoemaker et al., 1990). The sequences around the phosphorylation sites of smooth muscle and nonmuscle myosin II of higher eukaryotes are highly conserved (for review, see Tan et al., 1992). These findings imply that also nonmuscle MLCK may possess an absolute specificity for MLC_{20}. Phosphorylation of MLC_{20} by activated MLCK regulates the actin–myosin interaction in contraction of smooth muscle (for review, see Adelstein and Eisenberg, 1980). The phosphorylated adrenal medullary myosin has been shown to enhance the actin-activated Mg^{2+}-ATPase activity (Kanda et al., 1985). However, an implication of actin–myosin interaction in the putative role of MLCK for ATP-dependent priming remains uncertain for two reasons. First, the role of actin as a barrier to the movement of secretory vesicles to exocytotic sites has been suggested (Aunis and Bader, 1988; Vitale et al., 1991; reviewed by Trifaro et al., 1992). If actin–myosin interaction is involved in ATP-dependent priming, therefore, the actin should be distinct from filamentous actin that acts as a barrier. One may speculate the interaction of myosin with oligomeric actin molecules. In this regard, electron microscopic studies by quick freezing and deep etching have demonstrated that exocytosis occurred more frequently in the areas with few actin network (Nakata and Hirokawa, 1992). Second, MLCK was suggested very recently to modulate voltage-dependent potassium current (Akasu et al., 1993). This implies that phosphorylated MLC_{20} might interact with unknown molecules in the vicinity of the channel molecule or the channel it self. Such molecules might be membrane-bound proteins, and priming of secretion might be affected by an interaction of myosin with such molecules.

The present results do not exclude partial responsibility of phosphatidylinositol (PI) kinase or PI transfer protein for ATP-dependent priming of exocytosis (Holz et al., 1989; Hay and Martin, 1993). However, an essential role of PI 3-kinase at least is unlikely considering IC_{50} of wortmannin for inhibition of the enzyme (Yano et al., 1993). IC_{50} of wortmannin in the present results (1 μM) is more consistent with the inhibition of MLCK. It is likely that priming process is regulated through multiple reactions. From this point of view, MLCK or phosphorylation of MLC_{20} may be one of the essential components for priming.

The putative role of MLCK in the mechanism of exocytosis suggested here might be quite common and fundamental in different neurosecretory cells, since we now know that wortmannin inhibits secretory function in the various types of cells such as rat basophilic leukemia cells, human basophils (Kitani et al., 1992), PC12 cells (Inoue and Kumakura, 1992), and cultured rat sympathetic ganglion (Mochida et al., 1992; Muramoto et al., 1993). In cultured rat sympathetic ganglion, particularly, inhibition of neurotransmitter release at presynaptic terminal

by intracellular injection of SM-1 and anti-myosin antibodies was clearly demonstrated by detecting EPSP in response to presynaptic stimulation. In that study, the initial burst of the transmitter release seems to be resistant to wortmannin while the sustained release is effectively blocked by the inhibitor. Thus, MLCK seems to be essential for priming of exocytosis also in sympathetic nerve terminals (S. Mochida, H. Kobayashi, Y. Matsuda, Y. Yuda, K. Muramoto, and Y. Nonomura, unpublished observation).

In conclusion, our results strongly suggest an essential role of MLCK for priming of exocytosis. Most probably phosphorylation of MLC_{20} by MLCK activation plays an essential role in ATP-dependent priming of exocytotic machinery. It has to be determined whether phosphorylation of MLC_{20} is essential as the intrinsic mechanism of priming or as the microenvironment to maintain priming. Reevaluation of the role of MLCK in Ca^{2+}-dependent exocytosis is desirable in terms of ATP-dependent priming mechanism.

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