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MYOSIN LIGHT-CHAIN KINASE INHIBITOR, 1-(5-CHLOR-NAPHTHALENE-1-SULFONYL)-1H-HEXAHYDRO-1,4-DIAZEPINE (ML-9), INHIBITS CATECHOLAMINE SECRETION FROM ADRENAL CHROMAFFIN CELLS BY INHIBITING Ca^{2+} UPTAKE INTO THE CELLS

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Abstract—For determination of whether myosin light-chain kinase (MLCK) is involved in the secretory mechanism of adrenal chromaffin cells, the effect of a preferential inhibitor of the enzyme, 1-(5-chlor-naphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-9), on catecholamine secretion from cultured bovine adrenal chromaffin cells was studied. ML-9 did not affect basal catecholamine secretion, but inhibited catecholamine secretion stimulated by acetylcholine, high K^+, veratridine or palytoxin. At similar concentrations to those inhibiting the secretion of catecholamine, ML-9 also inhibited increased $[^{45}Ca]^{2+}$ uptake by the cells induced by these stimulants. However, it did not inhibit catecholamine secretion induced by the Ca^{2+} ionophore A23187. Moreover, it did not affect catecholamine secretion from digitonin-permeabilized cells induced by a micromolar Ca^{2+} concentration in the presence of Mg ATP. These results indicate that ML-9 inhibits catecholamine secretion from adrenal chromaffin cells by inhibiting the transmembrane Ca^{2+} uptake mechanism, but not by inhibiting the intracellular Ca^{2+}-dependent mechanism. The possible role of MLCK in stimulus–secretion coupling in adrenal chromaffin cells is discussed.

Secretion of catecholamine from adrenal chromaffin cells is known to occur by Ca^{2+}-dependent exocytosis [1–6]. However, its intracellular mechanism is still largely unknown. It has been suggested that the mechanism of stimulus–secretion coupling may be similar to that of excitation–contraction coupling in muscle and that contractile proteins or their regulatory proteins may be involved in regulation of the secretory mechanism [1, 5, 7–9]. Recently, several proteins were found to be phosphorylated during secretory stimulation of adrenal chromaffin cells [10–14] or during secretion of catecholamine from permeabilized chromaffin cells induced by a micromolar Ca^{2+} concentration in the presence of Mg ATP [15–18], suggesting a possible role of protein phosphorylation in the secretory mechanism. Moreover, the Ca^{2+}-mediator protein calmodulin and protein kinase C have also been suggested to play roles in the secretion of catecholamine from chromaffin cells [3, 5, 6, 19, 20].

Myosin light-chain kinase (MLCK) is a Ca^{2+}-calmodulin dependent protein kinase that regulates the contraction or activation of muscle and non-muscle cells through phosphorylation of myosin light-chains [21]. A preferential inhibitor of MLCK, 1-(5-chlor-naphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-9) has also been used to elucidate the role of MLCK in these cells [22]. Recently, Nagatsu *et al.* [23] demonstrated that ML-9 inhibited the release of dopamine from rat pheochromocytoma PC 12 cells stimulated by high K^+ and suggested that the phosphorylation of myosin light-chains might stimulate release of dopamine from PC 12 cells.

In this study, we investigated the effect of ML-9 on catecholamine secretion from adrenal chromaffin cells to determine whether MLCK is involved in their secretory mechanism. Results showed that ML-9 inhibited catecholamine secretion stimulated by acetylcholine, high K^+, veratridine or palytoxin, by inhibiting Ca^{2+} uptake into the cells, but did not affect catecholamine secretion induced by the Ca^{2+} ionophore A23187, or catecholamine secretion from digitonin-permeabilized cells induced by a micromolar Ca^{2+} concentration in the presence of Mg ATP.

MATERIALS AND METHODS

Isolated bovine adrenal chromaffin cells were prepared by sequential digestion of adrenal medullary slices with collagenase [24] and were maintained as monolayers on 24-well cluster plates (Costar, Cambridge, MA) at a density of 5×10^5 cells/well for 3–4 days as described previously [25].

Cultured chromaffin cells were washed once with 1 ml of balanced salts solution [BSS: 135 mM NaCl, 5.6 mM KCl, 1.2 mM $MgSO_4$, 2.2 mM $CaCl_2$, 10 mM glucose and 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES)/NaOH, pH 7.4]. They were then pre-incubated at 37° for 10 min in 250 μl of BSS in the absence or presence of ML-9, and stimulated with secretagogues for 10 min. The

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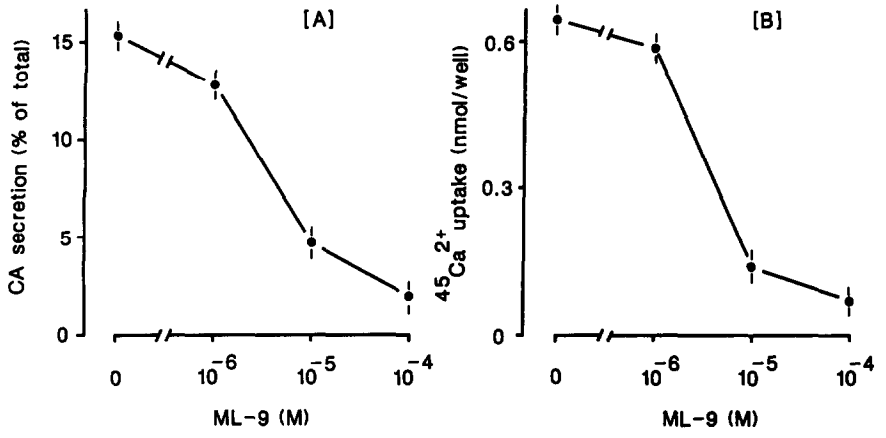

Fig. 1 (A) Inhibitory effects of ML-9 on catecholamine secretion from cultured bovine adrenal chromaffin cells stimulated by acetylcholine. Cells were incubated with or without various concentrations of ML-9 and stimulated with acetylcholine (3×10^{-4} M) as described in Materials and Methods. Catecholamine (CA) secretion is shown as a percentage of the total cellular catecholamine content. Basal CA secretion was approx. 2–3%. Acetylcholine-stimulated secretion was calculated by subtracting the basal values. Points and bars are means ± SE for four experiments. (B) Inhibitory effects of ML-9 on $[^{45}Ca]^{2+}$ uptake into cells stimulated by acetylcholine. Cells were incubated with $^{45}CaCl_2$ and stimulated with acetylcholine (3×10^{-4} M) as described in Materials and Methods. $[^{45}Ca]^{2+}$ uptake by the cells is shown in nmol/well. Points and bars show acetylcholine-stimulated $[^{45}Ca]^{2+}$-uptake into the cells in the presence of various concentrations of ML-9 as means ± SE for four experiments.

56 mM K^+ solution had the same composition as BSS except that NaCl was replaced by an equimolar amount of KCl. After incubation, the medium was withdrawn and the cells were lysed by adding 250 μl of 10% acetic acid followed by a freeze-thawing. Catecholamine in the medium and the cell lysate was assayed fluorometrically [26].

For measurement of $[^{45}Ca]^{2+}$ uptake by the cells, 3.0 μCi of $[^{45}Ca]^{2+}$ was added to the incubation medium. After incubation, the medium was discarded and the cells were washed four times with 1 ml of ice-cold Ca^{2+}-free BSS. Then intracellular $[^{45}Ca]^{2+}$ was extracted with 1% Triton X-100 and measured in a liquid scintillation counter.

For study of catecholamine secretion from digitonin-permeabilized cells [25, 27–29], the cultured cells were incubated at 37° for 10 min with 250 μl of digitonin-permeabilizing medium [20 μM digitonin in 140 mM monosodium glutamate, 20 mM 1,4-piperazinediethanesulfonic acid (PIPES)/NaOH (pH 6.8), 5 mM glucose, 5 mM $MgSO_4$, 5 mM ATP, 0.5 mM ascorbic acid, 5 mM EGTA and 0 mM or 5 mM $CaCl_2$] in the absence or presence of ML-9. The free Ca^{2+} concentration calculated by the method of Portzehl *et al.* [30], was approximately 1 nM in Ca^{2+}-free medium and 20 μM in Ca^{2+}-containing medium. After incubation, catecholamine in the medium and in the cells was measured as described above.

The following reagents were used: ML-9 (Seikagaku Kogyo, Tokyo, Japan), acetylcholine (Nakarai Chem., Kyoto, Japan), veratridine (Sigma Chemical Co., St Louis, MO), palytoxin (donated by Prof. Muramatsu, Dept. of Pharmacology, Fukui Univ. School of Medicine), digitonin (Calbiochem-Behring, Cleveland, OH), A23187 (Boehringer-Mannheim, F.R.G.), HEPES, PIPES and EGTA (Dohjin Chem., Kumamoto, Japan).

RESULTS

Effect of ML-9 on catecholamine secretion and $[^{45}Ca]^{2+}$ uptake by cells stimulated by acetylcholine

Figure 1A shows the effects of different concentrations of ML-9 on catecholamine secretion from cultured bovine adrenal chromaffin cells stimulated by acetylcholine. ML-9 alone did not affect basal catecholamine secretion, but inhibited catecholamine secretion stimulated by acetylcholine in a concentration-dependent fashion; the inhibition was observed at concentrations of more than 10^{-6} M and the IC_{50} value was approx. 3×10^{-6} M. In cells stimulated by acetylcholine, Ca^{2+} uptake is essential for the initiation of catecholamine secretion, so we examined the effect of ML-9 on $[^{45}Ca]^{2+}$ uptake by the cells. As shown in Fig. 1B, ML-9 inhibited acetylcholine-stimulated $[^{45}Ca]^{2+}$ uptake and the concentration–response curve was similar to that for inhibition of catecholamine secretion. Therefore, the inhibition of catecholamine secretion by ML-9 seemed to result from inhibition of $[^{45}Ca]^{2+}$ uptake into the cells.

Effect of ML-9 on catecholamine secretion and $[^{45}Ca]^{2+}$ uptake by cells stimulated by other secretagogues

Table 1 shows the effects of ML-9 on catecholamine secretion and $[^{45}Ca]^{2+}$ uptake by cells stimulated by high K^+, veratridine or palytoxin, which are known to activate the voltage-dependent Ca^{2+} channel, leading to the secretion of catecholamine. ML-9 was found to inhibit both catecholamine secretion and $[^{45}Ca]^{2+}$ uptake by cells treated with these stimulants. These inhibitory effects of ML-9 were observed at concentrations of more than 10^{-6} M and were maximal at 10^{-4} M. These results indicated that ML-9 inhibited catecholamine secretion by inhibiting not only acetyl-

Table 1. Effects of ML-9 on catecholamine secretion and $[^{45}Ca]^{2+}$ uptake stimulated by various secretagogues

	ML-9 (10^{-4} M)	CA secretion (% of total)	$[^{45}Ca]^{2+}$ uptake (nmol/well)
High K^+	(−)	16.3 ± 0.5	0.90 ± 0.05
(56 mM)	(+)	7.0 ± 0.1	0.50 ± 0.05
Veratridine	(−)	10.0 ± 0.4	0.37 ± 0.04
(2×10^{-5} M)	(+)	5.4 ± 0.3	0.15 ± 0.05
Palytoxin	(−)	40.7 ± 0.5	4.26 ± 0.02
(3×10^{-8} M)	(+)	13.8 ± 0.1	0.92 ± 0.04
A23187	(−)	3.0 ± 0.1	—
(10^{-5} M)	(+)	3.4 ± 0.4	—

Cultured bovine adrenal chromaffin cells were incubated with or without ML-9 (10^{-4} M) and then stimulated with various secretagogues as described in Materials and Methods. Catecholamine (CA) secretion is shown as a percentage of the total cellular catecholamine content. Basal CA secretion was approx. 2–3%. Stimulated secretion was calculated by subtracting the basal values. $[^{45}Ca]^{2+}$ taken up into the cells is shown in nmol/well. Values are means ± SE for four experiments.

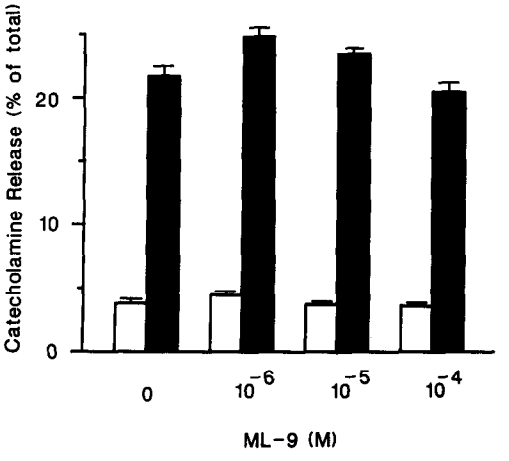

Fig. 2. Effect of ML-9 on catecholamine secretion from digitonin-permeabilized adrenal chromaffin cells. Cells were incubated in digitonin-permeabilizing medium with or without ML-9 (10^{-6}, 10^{-5}, 10^{-4} M) in the absence (open columns) or presence (shaded columns) of Ca^{2+} as described in Materials and Methods. Catecholamine secretion is expressed as a percentage of the total catecholamine content. Columns and bars are means ± SE for four experiments.

choline receptor-mediated, but also voltage-dependent Ca^{2+} uptake into the cells. On the other hand, ML-9 did not inhibit catecholamine secretion induced by the Ca^{2+} ionophore A23187 (Table 1), suggesting that it did not interfere with the intracellular secretory mechanism initiated by increase in cytoplasmic free Ca^{2+} concentration.

Effect of ML-9 on catecholamine secretion from digitonin-permeabilized cells induced by micromolar Ca^{2+}

To avoid the influence of the effect of ML-9 on transmembrane Ca^{2+} uptake, we next examined the effect of ML-9 on catecholamine secretion from digitonin-treated permeabilized cells induced by a micromolar Ca^{2+} concentration. As shown in Fig. 2, ML-9 (10^{-6}–10^{-4} M) did not significantly affect the basal catecholamine secretion or Ca^{2+} (20 μM)-induced catecholamine secretion from the permeabilized chromaffin cells. These results support the above suggestion that ML-9 did not interfere with the Ca^{2+}-dependent intracellular secretory mechanism.

DISCUSSION

In this study, we examined the effect of ML-9, a preferential inhibitor of myosin light-chain kinase (MLCK) [22], on catecholamine secretion from cultured bovine adrenal chromaffin cells to determine whether MLCK, that is, phosphorylation of myosin light-chain, is involved in regulation of the Ca^{2+}-dependent secretory mechanism.

We found that ML-9 inhibited catecholamine secretion stimulated by acetylcholine in a concentration-dependent manner (10^{-6}–10^{-4} M). ML-9 also inhibited $[^{45}Ca]^{2+}$ uptake into the cells stimulated by acetylcholine. The effective concentration range of ML-9 for inhibition of $[^{45}Ca]^{2+}$ uptake into the cells was similar to that for its inhibition of catecholamine secretion from the cells. These results suggested that ML-9 inhibited catecholamine secretion stimulated by acetylcholine by inhibiting Ca^{2+} uptake into the cells. ML-9 was also found to inhibit catecholamine secretion stimulated by high K^+, veratridine or palytoxin which are known to activate the voltage-dependent Ca^{2+} channel and initiated the secretion of catecholamine. ML-9 inhibited $[^{45}Ca]^{2+}$ uptake into cells stimulated by these secretagogues at similar concentrations to those inhibiting the secretion of catecholamine. These results indicated that ML-9 also inhibited catecholamine secretion stimulated by high K^+, veratridine or palytoxin by inhibiting Ca^{2+} uptake into the cells. Therefore, ML-9 inhibited the functions of both acetylcholine receptor-mediated Ca^{2+} channels and voltage-dependent Ca^{2+} channels and thereby inhibited catecholamine secretion.

Next, to study the direct effect of ML-9 on the intracellular Ca^{2+}-dependent mechanism in the

absence of its inhibitory effect on transmembrane Ca^{2+} uptake, we examined the effect of ML-9 on catecholamine secretion induced by the Ca^{2+} ionophore, A23187, and on catecholamine secretion from digitonin-permeabilized cells induced by a micromolar Ca^{2+} concentration in the presence of Mg ATP [25, 27–29]. We found that ML-9 did not inhibit Ca^{2+} ionophore-induced catecholamine secretion. Moreover, it did not affect either the basal secretion or the Ca^{2+}-induced secretion from digitonin-permeabilized cells. These results indicate that ML-9 did not interfere with the intracellular secretory mechanism, which is initiated by an increase in cytoplasmic free Ca^{2+} concentration.

It is still uncertain whether ML-9 inhibits Ca^{2+} uptake into the cells by inhibiting MLCK, or by some mechanism that is unrelated to MLCK. However, provided that at the concentrations of ML-9 used in this study its effect is specific for MLCK, our results strongly suggest that MLCK, i.e., phosphorylation of myosin light-chains, is involved in the secretory mechanism by regulating transmembrane Ca^{2+} uptake rather than by regulating the intracellular Ca^{2+}-dependent mechanism.

Several proteins are reported to be phosphorylated during secretory stimulation of adrenal chromaffin cells [10–14] or during secretion of catecholamine from permeabilized chromaffin cells induced by a micromolar Ca^{2+} concentration in the presence of Mg ATP [15–18], suggesting a possible role of protein phosphorylation in stimulus–secretion coupling. However, the phosphorylation of myosin light-chains (20 kDa) has not yet been demonstrated [17], although in rat pheochromocytoma PC12 cells, myosin light-chains (20 kDa) were found to be phosphorylated in the presence of a soluble fraction of the cells and [^{32}P]-ATP, and this phosphorylation was shown to be inhibited by the presence of ML-9 [23].

Recently, introduction of DNase 1 or heavy meromycin into adrenal chromaffin cells with the aid of liposomes was observed to cause depolarization of the plasma membrane and increase in Ca^{2+} uptake into the cells, followed by secretion of catecholamine [31, 32]. These results, together with the finding that myosin is associated with the plasma membrane in bovine adrenal medulla [6, 8, 9, 33, 34], suggest that an actomyosin-like protein may be involved in regulation of transmembrane ion transport and the secretory response of chromaffin cells.

Further studies are required to obtain more direct evidence for the role of MLCK or phosphorylation of the myosin light-chain in stimulus–secretion coupling of adrenal chromaffin cells.

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