Enhancement by L-Methionine of Contractile Responses to Acetylcholine and High KCl in Uterine Segment

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Abstract—The contractile responses of isolated uterine segments from 17β-estradiol-3-benzoate-treated ovariectomized rats to acetylcholine (ACh) and high KCl in Ca-depleted modified Locke-Ringer solution on addition of CaCl₂ were used as indicators of Ca²⁺ influxes through ACh receptor- and voltage-operated Ca²⁺ channels, respectively. L-Methionine (L-Met) significantly enhanced these responses. The enhancement depended on the time of treatment with L-Met and concentration of L-Met. 3-Deazaadenosine (3-DAA) plus homocysteine thiolactone (HCTL), which inhibit S-adenosylmethionine-dependent methylation, caused dose-dependent inhibition of these contractile responses to ACh and high KCl. These inhibitory effects of 3-DAA plus HCTL were significantly attenuated in the presence of L-Met. Protein carboxymethyltransferase and phospholipid methyltransferase activities were detected in the isolated uterine segment under conditions similar to those in which the contractile responses were observed. 3-DAA plus HCTL inhibited these enzyme activities. These findings suggest that S-adenosylmethionine-dependent methylations of protein and/or phospholipid in isolated uterine segment are involved in the contractile responses to ACh and high KCl in Ca-depleted modified Locke-Ringer solution on addition of CaCl₂.

Biological transmethylations of carboxyl groups in proteins and phosphatidylethanolamine (PE), with S-adenosyl-L-methionine (SAM) as a methyl donor, are involved in a number of important physiological processes in various types of living cells (1-3). For example, the methylation of side chains such as carboxyl groups in proteins of cell membranes can alter the surface charge and affect the structure and function of the cell membranes (4). Moreover, methylation of PE in cell membranes can increase membrane fluidity (5) and affect coupling of the beta adrenergic receptor and adenylate cyclase in rat reticulocyte ghosts (6). Ishizaka et al. (7) also observed that increase of phospholipid methylation in rat mast cells induced by divalent antireceptor antibodies results in influx of Ca²⁺ and subsequent release of histamine. From these observations, it seems likely that methylation of carboxyl groups in proteins and/or of PE of excitable cell membranes affect the process of Ca²⁺ influx (opening of Ca²⁺ channels) through the membranes.

Recently, we reported that the contractile response of isolated uterine smooth muscle to acetylcholine (ACh), serotonin and high KCl in Ca-depleted modified Locke-Ringer (Ca-depleted Ringer) solution on addition of CaCl₂ resulted from increased influx of Ca ions into the uterine cells from the medium, and we suggested that this system was useful for studies on the biological mechanism(s) of voltage- and receptor-operated membrane Ca²⁺ channels (8).

In this work, we investigate the effects of L-methionine and/or blockers of SAM-dependent transmethylation [3-deazaadenosine (3-DAA) plus homocysteine thiolactone (HCTL); 1, 9] on ACh- and high KCl-induced Ca²⁺ influx in isolated uterine
segments of 17 β-estradiol-3-benzoate (estradiol)-treated ovariectomized rats. These influxes are detectable as contractile responses of the muscle to ACh and high KCl in Ca-depleted Ringer's solution on addition of CaCl₂. We also showed that the [³H]-methyl group of L-[methyl-³H] methionine is incorporated into both proteins (carboxyl groups) and PE of the muscle in modified Locke-Ringer (Ringer) and Ca-depleted Ringer's solution, and we found that these methylations are blocked by 3-DAA plus HCTL. Preliminary reports have appeared in an abstract form (10) and a short communication (11).

Materials and Methods

Uterine segment of estradiol-treated ovariectomized rat was prepared as described previously (8, 12–14).

Measurement of contractile responses: The isotonic contractile responses of isolated rat uterine segment to ACh and high KCl in Ca-depleted Ringer's solution on addition of CaCl₂ were measured as described previously (8). However, several conditions were different from the previous work (8): (a) The uterine horns from estradiol-treated ovariectomized rats were divided longitudinally into several segments (about 1.5 × 12 mm; the uterine segments were not dissected away from the endometrium); (b) The uterine segments were each placed in a one-ml organ bath containing Ca-depleted Ringer's solution with the ionic composition described previously (8); (c) The isotonic muscle tension was adjusted to 100 mg. For measurement of the contractile response to ACh, ACh (3 × 10⁻⁶–3 × 10⁻⁴ M) was added to the muscle bath 2 min before addition of CaCl₂ (5 mM). While for measurement of the contractile response to high KCl, the uterine segment was immersed in Ca-depleted high KCl (25–80 mM) Ringer's solution with the ionic composition described previously (8) 10 min before addition of CaCl₂ (5 mM). The contractile responses to ACh and high KCl were expressed as percentages of the maximal response to 3 × 10⁻⁴ M ACh and 40 mM KCl, respectively, unless otherwise indicated. The effects of L-methionine and/or 3-DAA plus HCTL were examined by adding these compounds to the muscle bath 15 min and 10 min, respectively, before addition of CaCl₂ (5 mM), unless otherwise indicated. After the effects of L-Met and/or 3-DAA plus HCTL were examined once, we generally changed to new muscles and further investigated the effect. As a control, the same volume of Ca-depleted Ringer's solution was added instead of L-methionine and/or 3-DAA plus HCTL. The contractile responses of the control were measured three to five times until the standard error of means was less than two percent.

Atropine at 60 nM inhibited 94 and 97% the contractile responses of uterine segment to 3 × 10⁻⁶ and 3 × 10⁻⁴ M ACh in Ca-depleted Ringer's solution on addition of CaCl₂ (5 mM), respectively. In every experiment, we have usually examined whether the contractile response of uterine segment in Ca-depleted Ringer's solution was induced by adding CaCl₂ (5 mM) alone. The degree of the contractile response induced by adding CaCl₂ (5 mM) alone was 1.5 ± 0.4 (n = 123) % of the contractile response to 3 × 10⁻⁴ M ACh in Ca-depleted Ringer's solution on addition of CaCl₂ (5 mM). The contractile responses to ACh and high KCl were stable for 7 hr at least.

Measurement of protein carboxylmethylation: Protein carboxylmethyltransferase activity was determined by analysis of trichloroacetic acid-precipitated, baselabile, volatile radioactivity as described by others (15–17). Uterine segment from estradiol-treated ovariectomized rat was prepared as described above. The muscle was blotted with filter paper, weighed and equilibrated for a minimum of 60 min in Ringer with the ionic composition described previously (8) or Ca-depleted Ringer's solution. The solution was then added to a muscle, weighed and equilibrated for a minimum of 60 min in Ringer with the ionic composition described previously (8) or Ca-depleted Ringer's solution. The solution was changed every 5 min and bubbled with 5% CO₂ in O₂ at 30°C. The muscle was then placed in 2 ml of Ringer or Ca-depleted Ringer's solution bubbled with 5% CO₂ in O₂ at 30°C. Then, L-[methyl-³H] methionine (5 μCi/tube, 15 Ci/mmol) was added, and the mixture was incubated for 60 min at 30°C. For experiments on the inhibitory effect of 3-DAA plus HCTL, 3-DAA plus HCTL (300 μM, each) in a small volume of less than 1% of the reaction system was added 20 min
after addition of L-[methyl-3H] methionine. As a control, the same volume of Ringer or Ca-depleted Ringer's solution was added to the reaction tube instead of 3-DAA plus HCTL. Incubations were stopped by separating the muscles from the reaction medium. The muscles were then rinsed three times with 3 ml of ice-cold 10% trichloroacetic acid as rapidly as possible and placed in liquid nitrogen. The frozen muscle was homogenized in one ml of ice-cold 10% trichloroacetic acid in a glass homogenizer, and the homogenizer was washed twice with one ml of ice-cold 10% trichloroacetic acid. The homogenate and washing fluid were combined and centrifuged at 1,600×g for 10 min. The supernatant was carefully aspirated, and the pellet was hydrolyzed by incubation for 20 min in 0.5 ml of 1 M Na borate buffer (pH 11) at 37°C. The radioactivity was then extracted into 3 ml of toluene/isoamyl alcohol/methanol (3:2:0.15, v/v) by vigorous vortexing for 30 sec. The organic phase was separated by centrifugation, and two 1.0 ml aliquots were transferred to scintillation vials. One aliquot was counted directly in 10 ml of ACS-liquid scintillation counting solution (Amersham). The other aliquot was evaporated by heating in an oven at 95°C for 1.5 hr and then mixed with 10 ml of fluor and counted. Protein carboxymethyl transferase activity was determined from the difference between the total and nonvolatile radioactivities.

Measurement of phospholipid methylation: Preparation of uterine segment and phospholipid methylation were carried out as for measurement of protein carboxymethylation, except that 10 μCi L-[methyl-3H]methionine (15 Ci/mmole) was added to the reaction tube. Methylated phospholipids in the muscle were extracted by the method of Hirata et al. (18) and separated by the method of Prasad and Edwards (19). The muscle was frozen in liquid nitrogen and homogenized in one ml of ice-cold chloroform/methanol/hydrochloric acid (2:1:0.02, v/v) in a glass homogenizer, and then the homogenizer was washed twice with one ml volumes of the same solution. The combined mixture was incubated for 5 min at 30°C. Then 2 ml of 0.1 M KCl in 50% (v/v) methanol was added, and the tube was vigorously shaken in a shaker for 15 min and centrifuged at 1,600×g for 10 min. The aqueous phase was aspirated, and the chloroform/methanol phase was washed with 2 ml of 0.1 M KCl in 50% methanol. Then the aqueous phase was removed, and 0.1 ml of the chloroform/methanol phase was transferred to a scintillation vial for measurement of methylation. The remainder of the phospholipid-containing phase (1.6 ml) was evaporated under a stream of N2 gas at 30°C. The residue was dissolved in a small volume of chloroform/methanol (2:1, v/v), and an aliquot was applied to a silica gel G plate. The chromatogram was developed with chloroform/propionic acid/n-propyl alcohol/water (3:2:6:1, v/v), and the spots of phospholipids separated were located by exposing the plate to iodine vapor. Then the spots were scraped off the plate and transferred to counting vials, and 6 ml of ACS-II liquid scintillation counting solution (Amersham) was added. The radioactivity was counted in a liquid scintillation counter (Packard). The spots of phospholipids were identified by comparison of their Rf values with those of authentic PE, L-phosphatidyl-N-monomethyl-ethanolamine (PME), L-phosphatidyl-N,N-dimethyl-ethanolamine (PM2E) and phosphatidylcholine (PC). The Rf values of PE, PME, PM2E and PC were 0.42±0.01 (n=22), 0.23±0.01 (n=22), 0.11±0.01 (n=22) and 0.05±0.01 (n=22), respectively.

Statistical analysis: Statistical analyses were carried out by Student's t-test, and differences giving P<0.05 were considered as significant.

Materials: Estradiol was purchased from Teikokuzoki Hormone Mfg. Co. (Osaka, Japan). HCTL, L-methionine, PC, PE, PM2E were from Sigma Chemical Co. (St. Louis, MO). 3-DAA was from Southern Research Institute (Birmingham, AL; Dr. J.A. Montgomery). All drugs were prepared in Ringer, Ca-depleted Ringer's solution or chloroform/methanol (2:1, v/v) on the day of use and were neutralized when necessary.

Results

Effects of L-methionine and/or 3-DAA plus HCTL on the contractile response to ACh in Ca-depleted Ringer's solution on addition of CaCl2: The contractile response of
rat uterine segment to $3 \times 10^{-6} - 3 \times 10^{-4}$ M ACh in Ca-depleted Ringer's solution on addition of CaCl$_2$, which was used as an indicator of Ca$^{2+}$ influx through ACh-operated Ca$^{2+}$ channels, was significantly increased by adding 3 mM L-methionine to the muscle bath 15 min before addition of CaCl$_2$ (5 mM) (Fig. 1A). The contractile responses to $3 \times 10^{-6}$ and $3 \times 10^{-4}$ M ACh were increased 21.4 (n=8) and 5.4 (n=8) %, respectively, by 3 mM L-methionine.

The effect of 3 mM L-methionine depended upon the time of treatment (Fig. 1B). The effect of 3 mM L-methionine on the contractile response to $10^{-5}$ M ACh was maximal 15 min after addition of L-methionine, and the time for half-maximal saturation was about 5.7 min.

The effect of the L-methionine concentration on the contractile response to $10^{-5}$ M ACh on addition of CaCl$_2$ (5 mM) is shown in Fig. 1C. The effect of L-methionine was dose-dependent, reaching a plateau at a concentration of 3 mM, and the concentration

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**Fig. 1.** Enhancing effect of L-methionine on the contractile response to ACh. Isolated uterine segments were equilibrated in Ca-depleted Ringer's solution for a minimum of 60 min. A) Effect of L-methionine on the contractile responses to various concentrations of ACh. The contractile responses to various concentrations of ACh in the absence and presence of 3 mM L-methionine on addition of CaCl$_2$ (5 mM) were measured as described under "Materials and Methods". The ordinate indicates the percentage of the maximal response (to $3 \times 10^{-4}$ M ACh) of the control. Points and bars are means and S.E.s for preparations from 5 to 9 animals. *P<0.02, **P<0.01: significance of difference from the control. B) Contractile response to ACh as a function of time of treatment with L-methionine. The effect of the time of treatment with 3 mM L-methionine on the contractile response to $10^{-6}$ M ACh on addition of CaCl$_2$ (5 mM) was examined as described under "Materials and Methods". Points and bars are means ±S.E. of values for 10 to 13 animals. C) Effect of the concentration of L-methionine on the contractile response to ACh. The contractile response to $10^{-6}$ M ACh in the presence of the indicated concentrations of L-methionine on addition of CaCl$_2$ (5 mM) were measured as described under "Materials and Methods". Points and bars are means ±S.E. of values for 5 to 6 animals.
Fig. 2. Effects of L-methionine and/or 3-DAA plus HCTL on the contractile responses to various concentrations of ACh. Isolated uterine segments were equilibrated in Ca-depleted Ringer's solution for a minimum of 60 min, and the contractile responses to the indicated concentrations of ACh in the absence and presence of 3 mM L-methionine and/or 3-DAA (100, 300 or 500 nM) plus HCTL (100, 300 or 1000 nM) on addition of CaCl\(_2\) (5 mM) were measured as described under "Materials and Methods". Ordinates indicate percentages of the maximal response (to 3x10^{-4} M ACh) in controls. Points and bars are means±S.E.s. of the values in 6 to 16 animals. *P<0.05, **P<0.005: significance of difference from the value with 3-DAA plus HCTL.

In order to demonstrate the effect of L-methionine more clearly, we investigated the effects of 3-DAA plus HCTL on the contractile responses to various concentrations of ACh on addition of CaCl\(_2\) (5 mM) in the absence and presence of 3 mM L-methionine. As shown in Fig. 2, the contractile responses to various concentrations of ACh on addition of CaCl\(_2\) (5 mM) were inhibited dose-dependently by 3-DAA plus HCTL: 100 μM 3-DAA plus 100 μM HCTL, 300 μM 3-DAA plus 300 μM HCTL, and 500 μM 3-DAA plus 1 mM HCTL decreased the contractile response to 3x10^{-4} M ACh by 25, 53 and 89%, respectively. The inhibition by 3-DAA plus HCTL was non-competitive and was significantly attenuated in the presence of 3 mM L-methionine.

Effects of L-methionine and/or 3-DAA plus HCTL on the contractile response to high KCl in Ca-depleted Ringer's solution on addition of CaCl\(_2\): The contractile response of rat uterine segment to 30–60 mM KCl in Ca-
depleted Ringer's solution on addition of CaCl₂ (5 mM) was used as an indicator of Ca²⁺ influx through voltage-operated Ca²⁺ channels. As shown in Fig. 3, addition of 3 mM L-methionine to the muscle bath 15 min before CaCl₂ (5 mM) significantly increased this response. The contractile responses to 30, 35 and 40 mM KCl were increased 90.6 (n=12), 8.2 (n=10) and 3.1 (n=13) %, respectively, by 3 mM L-methionine.

As reported previously (8), we have observed that the contractile response to high KCl was often biphasic, especially at low KCl concentrations; at final concentrations of less than 35 mM KCl, it was consistently biphasic. In this work we measured only the contraction height of the second phase, because this second phase was observed over a wide concentration range of KCl (25–80 mM), and the contractile response was of more constant height than the first phase of contraction.

Figure 4 shows the effect of 3-DAA plus HCTL on the contractile responses to various concentrations of KCl on addition of CaCl₂ (5 mM) in the absence and presence of 3 mM L-methionine. 3-DAA plus HCTL inhibited these responses dose dependently. For example, 30 µM 3-DAA plus 30 µM HCTL and 100 µM 3-DAA plus 100 µM HCTL caused 28 and 75% inhibition, respectively, of the contractile response to 60 mM KCl. The inhibition by 3-DAA plus HCTL was non-competitive and was significantly attenuated

Fig. 4. Effect of L-methionine and/or 3-DAA plus HCTL on the contractile responses to various concentrations of KCl. Isolated uterine segments were equilibrated in Ca-depleted Ringer's solution for a minimum of 60 min, and then the contractile responses to various concentrations of KCl in the absence and presence of 3 mM L-methionine and/or 3-DAA (30 or 100 µM) plus HCTL (30 or 100 µM) on addition of CaCl₂ (5 mM) were measured as described under "Materials and Methods". The ordinates indicate the percentages of the maximal response (40 mM KCl) in controls. Points and bars are means±S.E.s of values for 5 to 10 animals. *P<0.05, **P<0.001: significance of difference from the value with 3-DAA plus HCTL.

Table 1. Effect of 3-DAA plus HCTL on protein carboxyvmethyltransferase activity of uterine segments

| Conditions | Ringer | Ca-depleted Ringer |
|------------|--------|--------------------|
| Control    | 1.99±0.18 | 1.22±0.16          |
| 300 µM 3-DAA + 300 µM HCTL | 0.80±0.07* | 0.42±0.05*         |

L-[methyl-³H]Methionine (5 µCi/tube, 15 Ci/mmol) was added to the reaction tube and after incubation for 20 min at 30°C, the blockers were added. After further incubation for 40 min, the muscle was removed and frozen in liquid nitrogen, and the activity of the enzyme in the muscle was determined as described under "Materials and Methods". Values are means±S.E.s for preparations from 5 to 7 animals. *P<0.002, significantly different from the control. **[³H]Methyl incorporation, fmol/mg wet weight.
Table 2. Effect of 3-DAA plus HCTL on phospholipid methyltransferase activity of uterine segments

| Conditions      | Phospholipid methyltransferase activity** |                      | Ca-depleted Ringer |                      |
|-----------------|------------------------------------------|----------------------|--------------------|----------------------|
|                 | Phospholipid                        | PME | PM2E | PC | Phospholipid | PME | PM2E | PC |
| Control         | 85.9±9.8 | 8.11±0.61 | 16.07±2.31 | 1.42±0.22 | 54.7±5.0 | 5.49±0.39 | 12.14±0.77 | 1.57±0.35 |
| 300 μM 3-DAA    | 31.6±3.0* | 1.76±0.27* | 2.72±0.53* | 0.53±0.09* | 22.4±1.8* | 1.45±0.11* | 2.64±0.28* | 1.09±0.29* |
| + 300 μM HCTL   |                      |      |     |     |                      |      |      |     |

L-[methyl-³H]Methionine (10 μCi/tube, 15 Ci/mmol) was added to the reaction tube, and after incubation for 20 min at 30°C, the blockers were added. After further incubation for 40 min, the muscle was removed and frozen in liquid nitrogen, and the activity of the enzyme in the muscle was determined as described under "Materials and Methods". Values are means±S.E.s. for preparations from 6 to 9 animals. *P<0.001, significantly different from the control. **[³H]Methyl incorporation, fmol/mg wet weight.
by 3 mM L-methionine.

Presence of protein carboxylmethyltransferase and phospholipid methyltransferase in rat uterine smooth muscle and effect of 3-DAA plus HCTL on these activities: If the effect of L-methionine on the contractile responses to ACh and high KCl in Ca-depleted Ringer's solution on addition of CaCl₂ can be explained by increased carboxymethylation and/or phospholipid methylation in rat uterine segment, SAM-dependent carboxymethylation and/or phospholipid methylation should occur in the muscle. Therefore, we tested for protein carboxylmethyltransferase and/or phospholipid methyltransferase in the muscle under conditions similar to those for the contractile responses to ACh and high KCl in Ca-depleted Ringer's solution (Tables 1 and 2). The muscle was incubated for 60 min at 30°C after addition of L-[³H]-methionine. Then the muscle was removed from the reaction medium, and its protein carboxylmethyltransferase activity was measured. The specific activities of the enzyme in the muscle in Ringer and in Ca-depleted Ringer's solution were found to be 1.99±0.18 (n=5) and 1.22±0.16 (n=7) fmol [³H]-methyl incorporation/mg wet weight, respectively (Table 1). The enzyme activities of the muscle in Ringer and Ca-depleted Ringer's solution were significantly inhibited (about 60 and 66%, respectively) by 300 µM 3-DAA plus 300 µM HCTL. As shown in Table 1, the protein carboxylmethyltransferase activity of the muscle in Ca-depleted Ringer's solution was 60-70% of that in Ringer's solution.

The amount of [³H]-methyl incorporation into total phospholipids (chloroform/methanol phase), PME, PM₂E and PC by phospholipid methyltransferase of the muscle in Ringer and in Ca-depleted Ringer's solution are shown in Table 2. The [³H]-methyl incorporation in these two solutions were inhibited 63-83 and 31-78%, respectively, by 300 µM 3-DAA plus 300 µM HCTL. The inhibition of [³H]-methyl incorporation into PM₂E being the greatest. As shown in Table 2, the [³H]-methyl incorporations into total phospholipids, PME and PM₂E by phospholipid methyltransferase of the muscle in Ca-depleted Ringer's solution were 64-76% of those in Ringer, but [³H]-methyl incorporation into PC was not less in Ca-depleted Ringer's solution.

These results in Tables 1 and 2 indicate the following: 1) Protein carboxylmethyltransferase and phospholipid methyltransferase are present in rat uterine segment. 2) These enzymes are inhibited by blockers (3-DAA plus HCTL) of SAM-mediated transmethylation. 3) These enzyme activities are also present in the muscle treated with Ca-depleted Ringer's solution, but in this muscle, their specific activities are lower than those in muscle in Ringer's solution.

Discussion

The main findings in the present study were as follows: 1) L-Methionine significantly enhanced the contractile responses of isolated uterine segment from estradiol-treated ovariectomized rat to 3x10⁻⁶-3x10⁻⁴ M ACh in Ca-depleted Ringer's solution on addition of CaCl₂ (used as an indicator of Ca²⁺ influx through ACh-operated Ca²⁺ channels). 2) The effect of L-methionine was dependent upon both the duration time of L-methionine treatment and the concentration of L-methionine. 3) 3-DAA plus HCTL, which are inhibitors of SAM-dependent methylation, caused dose-dependent inhibition of the contractile responses to 3x10⁻⁶-3x10⁻⁴ M ACh in Ca-depleted Ringer's solution on addition of CaCl₂. The inhibitory effect of 3-DAA plus HCTL was significantly attenuated in the presence of L-methionine. 4) L-Methionine also enhanced the contractile responses of isolated uterine segment to 30-60 mM KCl in Ca-depleted Ringer's solution on addition of CaCl₂ (used as an indicator of Ca²⁺ influx through voltage-operated Ca²⁺ channels). The contractile responses to high KCl was also inhibited by 3-DAA plus HCTL, and the inhibitory effect of 3-DAA plus HCTL was significantly attenuated in the presence of L-methionine. 5) The activities of both protein carboxylmethyltransferase and phospholipid methyltransferase were observed in isolated uterine segment from estradiol-treated ovariectomized rat under conditions similar to those in which the contractile
responses of the muscle to ACh in Ringer or Ca-depleted Ringer's solution were observed. 3-DAA plus HCTL inhibited these enzyme activities.

The enhancing effect of L-methionine on the contractile responses of the uterine segment to ACh and high KCl in Ca-depleted Ringer's solution on addition of CaCl₂ can be explained by supposing that it causes some of the following changes in steps stimulating-contraction coupling: 1) Increase in release from the muscle of a substance(s) (that facilitates the contractile responses to ACh and high KCl). 2) Increase in the accessibility and/or the number of ACh receptors in the muscle. 3) Unmasking of ACh receptor in the muscle. 4) Change of a step(s) (Ca²⁺ influx, Ca²⁺ efflux, etc.) that increases the concentration of free Ca²⁺ in the muscle cytosol. 5) Improvement of the contractile system in the muscle.

Recently, we found that the enhancing effect of L-methionine on the contractile response to high KCl in Ca-depleted Ringer's solution on addition of CaCl₂ was also observed in the presence of 30 nM atropine (which inhibits the contractile response to 3×10⁻⁴ M ACh) or 30 μM eserine (which shifts the dose-response curves for ACh to the left). It was found that in Ca-depleted Ringer's solution from which uterine segment was preincubated with L-methionine for 10 min in the presence of 3-DAA plus HCTL, the enhancing effect of L-methionine on the contractile response of another uterine muscle to ACh was not observed (S. Ichida et al., unpublished data). These findings suggested that a substance(s) which facilitates the contractile responses to ACh and high KCl was not released from uterine segment during preincubation with L-methionine.

It seems unlikely that L-methionine increases the accessibility and/or number of ACh receptors or unmasks ACh receptors, because methionine also enhanced the contractile response to high KCl in Ca-depleted Ringer's solution on addition of CaCl₂ (irrespective of whether 3-DAA plus HCTL was present in the muscle bath, Figs. 3 and 4).

Recently, we also found that ACh- and high KCl-induced ⁴⁵Ca influx on rat uterine segment was inhibited by 3-DAA plus HCTL, and it was enhanced by L-methionine in the presence of 3-DAA plus HCTL (S. Ichida et al., unpublished data). Therefore, it seems likely that the enhancing effect of L-methionine on the contractile responses to ACh and high KCl is mainly due to increased Ca²⁺ influx of the muscle.

The effects of L-methionine and 3-DAA plus HCTL on the contractile responses to ACh and high KCl in Ca-depleted Ringer's solution on addition of CaCl₂ can be explained by supposing that L-methionine and 3-DAA plus HCTL affect the contractile system(s) directly as described above. However, this possibility seems unlikely so far because 3 mM L-methionine and 500 μM 3-DAA plus 1 mM HCTL did not affect calcium-dependent superprecipitation of crude uterine actomyosin (S. Ichida et al., submitted for publication), although the possibility that L-methionine and 3-DAA plus HCTL have different effects on actomyosin and intact uterine muscle cannot be excluded.

We found that the inhibitory effect of 100 μM 3-DAA plus 100 μM HCTL on the contractile response to high KCl in Ca-depleted Ringer's solution on addition of CaCl₂ was much greater than that on the response to ACh (Figs. 2 and 4). This difference in the sensitivities to 3-DAA plus HCTL of the responses to ACh and high KCl may reflect differences between the contractile responses of the muscle produced by Ca²⁺ influxes through voltage (high KCl)- and receptor (ACh)-operated Ca²⁺ channels.

SAM-dependent methylations have been shown to be involved in posttranslational modification of proteins, in the biosynthesis of phospholipids, and in posttranscriptional modification of nucleic acids (1). Therefore, the effect of L-methionine on the responses to ACh and high KCl may be partly due to effects of SAM-dependent methyltransferases on protein(s) and/or phospholipid(s) in several steps of stimulation-contraction coupling. Indeed, as shown in Tables 1 and 2, we demonstrated SAM-dependent protein carboxymethylation and phospholipid methylation in the muscle. This finding at least indicates that protein carboxymethyltransferase and phospholipid methyltransferase
are present in the isolated uterine segment from ovariectomized rats. However, we cannot exclude the possibility that these methyltransferase activities were due to the methyltransferases of endometrium in the uterine segment because the smooth muscles were not dissected away from the endometrium as shown in the Materials and Methods section.

3-DAA has been shown to be both an efficient substrate and a potent inhibitor of S-adenosyl homocysteine (SAH) hydrolase (20–22). Therefore, treatment of cells with 3-DAA results in both accumulation of S-3-deazaadenosylhomocysteine and increase in intracellular SAH (20, 22, 23). This intracellular accumulation of SAH and/or S-3-deazaadenosylhomocysteine results, in turn, in inhibition of various cellular methylation reactions, because SAH and its close structural analog inhibit various SAH-utilized methyltransferases (1, 9). As shown in Figs. 2 and 4, 3-DAA plus HCTL caused dose-dependent inhibition of the contractile responses to ACh and high KCI in Ca-depleted Ringer's solution on addition of CaCl₂. This inhibitory effect of 3-DAA plus HCTL was significantly attenuated by the presence of L-methionine, again suggesting that the effect of L-methionine is partly due to SAM-dependent methylation catalyzed by protein carboxylmethyltransferase and/or phospholipid methyltransferase in the muscle.

Sastry et al. (24) have reported that L-methionine increased the contraction height of rat hemidiaphragm upon electrical stimulation of the nerve or the muscle and that phospholipid methylation played a significant role in the functional activity of the rat diaphragm. Recently, Landon et al. (25) also reported that the contractile response of rat aorta to high KCl was enhanced by L-methionine. In rat vas deferens, we also observed that L-methionine enhanced the contractile response to norepinephrine in the presence of methylation blockers (S. Ichida et al., submitted for publication). From their findings and ours, methylation of protein and/or phospholipid seems to be important in stimulation-contraction coupling in both skeletal and smooth muscle.

In this work, we found that L-methionine enhanced the contractile responses of rat uterine segment to ACh and high KCl in Ca-depleted Ringer's solution on addition of CaCl₂ with and without 3-DAA plus HCTL, and that both carboxylmethyltransferase and phospholipid methyltransferase are present in the muscle. However, further experiments are required to determine the exact step(s) in stimulation-contraction coupling in rat uterine segment at which L-methionine is effective. The correlation between the effect of L-methionine and carboxylmethyltransferase and/or phospholipid methyltransferase activity also needs investigation.

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