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Halothane counteracts acetylcholine-induced increase in Ca^{2+} sensitivity of the contractile apparatus in airway smooth muscle

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Abstract

The direct relaxing effect of halothane on airway smooth muscle has been reported to involve the reduction of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and the $[Ca^{2+}]_i$-independent inhibitory mechanism. To clarify the extent of the contribution of these mechanisms, the effect of halothane on the $[Ca^{2+}]_i$-tension relationship in porcine tracheal smooth muscle strips was evaluated, using fura-2 fluorometry. The control $[Ca^{2+}]_i$-tension relationship was constructed from data of $[Ca^{2+}]_i$ and tension during the contractions induced by the stepwise increment of extracellular Ca^{2+} concentration under high K^+ depolarization. In the presence of acetylcholine (1 μM), the $[Ca^{2+}]_i$-tension relationship shifted upward, which indicated the acetylcholine-induced increase in the Ca^{2+} sensitivity of the contractile apparatus. Halothane (0.034 mM), in the absence of acetylcholine, did not alter the increases in either $[Ca^{2+}]_i$ or tension, hence no change in the $[Ca^{2+}]_i$-tension relationship. However, in the presence of acetylcholine, halothane did attenuate the acetylcholine-induced upward shift of the $[Ca^{2+}]_i$-tension relationship. Halothane proved to have a potent attenuating effect on the acetylcholine-induced increase in Ca^{2+} sensitivity of the contractile apparatus with little influence on $[Ca^{2+}]_i$. This desensitization of the contractile apparatus to $[Ca^{2+}]_i$ may play a major role in the direct airway relaxing effect of halothane.

Keywords: Halothane; Smooth muscle, airway; Ca^{2+}, intracellular; Ca^{2+} sensitivity

1. Introduction

In vivo (Hirshman et al., 1982; Warner et al., 1990) and in vitro (Korenaga et al., 1984; Brichant et al., 1991) studies reveal that halothane, a volatile inhalational anesthetic, produces a potent airway relaxing effect by inhibition of the neural pathway and by a direct effect on the airway smooth muscle. Recently it was reported that the direct effect involved both a reduction in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and a Ca^{2+}-independent inhibitory mechanism (Yamakage, 1992). It was proposed that the Ca^{2+}-independent relaxation might occur with a high concentration of halothane because the extent of reduction in tension was greater than the reduction of $[Ca^{2+}]_i$ seen with a high concentration of this anesthetic (Yamakage, 1992). However, the relative importance of these two Ca^{2+}-dependent or -independent inhibitory mechanisms have not been fully revealed.

In this study, we examined the effect of halothane on $[Ca^{2+}]_i$ and on Ca^{2+} sensitivity of the contractile apparatus in porcine tracheal smooth muscle using our original experimental model (Kai et al., 1993). In this model, smooth muscle is constantly depolarized by high K^+, so that the voltage-dependent Ca^{2+} channel is sufficiently opened. Under these conditions, $[Ca^{2+}]_i$ can be changed independently by a change of extracellular Ca^{2+} concentration. Therefore, we can obtain the $[Ca^{2+}]_i$-force relationship under constant stimulation except for Ca^{2+} concentration. These two Ca^{2+}-dependent and -independent mechanisms can then be analysed separately.

2. Materials and methods

2.1. Tissue preparation

Tracheas dissected from adult pigs at a local slaughterhouse, under a protocol approved by the animal research committee of our institute, were brought to our laboratory in preaerated ice-cold physiological saline solution (PSS).

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The lower end of the trachea, just above the first bronchus branching, 3 tracheal rings in length, was used for the experiments. The posterior portion of the trachea was excised longitudinally, and the cartilage was removed. The mucosa and adventitial tissue were carefully excised, under microscopic observation. The muscle sheets were transversely cut into rectangular strips of approximately 3 mm in length, 1 mm in width (Kai et al., 1993).

2.2. Fura-2 loading

Tracheal strips were loaded with the Ca^{2+} indicator dye, fura-2, in the form of acetoxymethyl ester (fura-2/AM), as previously described (Kai et al., 1993). The strips were incubated in aerated (95% O_2/5% CO_2) Dulbecco-modified Eagle's medium containing 50 μM fura-2/AM dissolved in dimethyl sulphoxide and 5% fetal bovine serum for 3 h at 37°C. After loading with fura-2, the strips were washed with normal PSS to remove dye in the extracellular space, and then were further incubated in normal PSS for at least 1 h to facilitate the de-esterification of intracellular fura-2/AM and to equilibrate the strips before starting the measurements.

2.3. Measurement of tension development

Each strip was mounted vertically in a 6-ml quartz organ bath, which was maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. The lower end of the strip was fixed by a small clip and the upper end of the strip was then attached by a small clip and thread to a force transducer (TB-612T, Nihon Koden, Japan) in order to record the isometric tension. During a 1-h fura-2 equilibration period, the strips were stimulated with 40 mM K^+ at 5–10-min intervals, and the muscle length was increased stepwise after each stimulation until the developed tension reached a maximum. When exposed to 40 mM K^+, strips showed a stable tension within 5 ~ 10 min. The responsiveness of each strip to 40 mM K^+ was recorded before starting the protocol. The developed tension was expressed as a percentage, assuming the values in the normal (5.9 mM K^+) PSS and steady state of 40 mM K^+ PSS to be 0% and 100%, respectively.

2.4. Measurement of fura-2 fluorescence

Changes in the fluorescence intensity of the fura-2-Ca^{2+} complex were monitored with a front-surface fura-2 fluorometer (model CAM-OF) specifically designed in collaboration with Japan Spectroscopic (Tokyo, Japan). The details of our front-surface fluorometry system have been described elsewhere (Hirano et al., 1990; Kai et al., 1993). In brief, two wavelengths of excitation light (340 and 380 nm) are obtained spectroscopically from a Xenon light source. The strips are illuminated by guiding the two alternating (400 Hz) wavelengths of excitation light through quartz optic fibers. The surface fluorescence of the strip is collected by glass optic fibers and introduced through a 500-nm band pass filter into a photomultiplier. We were thus able to measure the fura-2 fluorescence intensity of 500 nm emission light which was induced by alternating two wavelengths of excitation light (340 and 380 nm).

The ratio of the fluorescence intensities at 340-nm excitation to that at 380-nm excitation (fluorescence ratio) was monitored to estimate the changes in $[Ca^{2+}]_i$ and then was expressed as a percentage, taking the values in normal PSS (5.9 mM K^+) and steady state of 40 mM K^+ PSS observed before each measurement as 0% and 100%, respectively. When the absolute value of $[Ca^{2+}]_i$ was determined using the equation of Grynkiewicz et al. (1985) (in separate measurements), $[Ca^{2+}]_i$ in normal PSS (0%) and steady state of 40 mM K^+ PSS (100%) were 90 ± 14 nM and 499 ± 54 nM ($n = 8$), respectively (Kai et al., 1993). For reference purposes, these absolute $[Ca^{2+}]_i$ levels are shown together with the percentage fluorescence ratio in Figs. 1–3.

2.5. Solutions and drugs

Normal PSS was of the following composition (in mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25 and D-glucose 11.5. High K^+ PSS was identical to normal PSS, except for an equimolar substitution of KCl for NaCl. Ca^{2+}-free version of PSS was produced by exclusion of $CaCl_2$ from the composition of normal PSS. PSS was bubbled with 95% O_2/5% CO_2, with a resulting pH of 7.4 at 37°C. Fura-2/AM and EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid] were purchased from Dojindo (Kumamoto, Japan). Acetylcholine chloride was from Sigma (St. Louis, MO, USA). Halothane was from Hoechst Japan (Tokyo, Japan).

Halothane was applied by bubbling the bath solution with 3% halothane containing gas delivered by a calibrated vaporizer (Fluotec 3, Cyprane, UK). The halothane concentration in the bath solution was 0.034 ± 0.006 mM ($n = 6$), measured by gas chromatography.

2.6. Experimental protocols

To examine the effect of halothane on Ca^{2+} sensitivity of the contractile apparatus, we determined the $[Ca^{2+}]_i$-tension relationship of contractions induced by the cumulative application of extracellular Ca^{2+} during 40 mM K^+ depolarization, either with or without halothane treatment, as follows: after a 10-min incubation in Ca^{2+}-free PSS containing 2 mM EGTA and then 5-min incubation in Ca^{2+}-free PSS without EGTA, the tracheal strips were immersed in Ca^{2+}-free 40 mM K^+ solution, then, the extracellular Ca^{2+} concentration was increased stepwise by the cumulative addition of $CaCl_2$. In the case of halothane treatment, 3% halothane gas was applied from

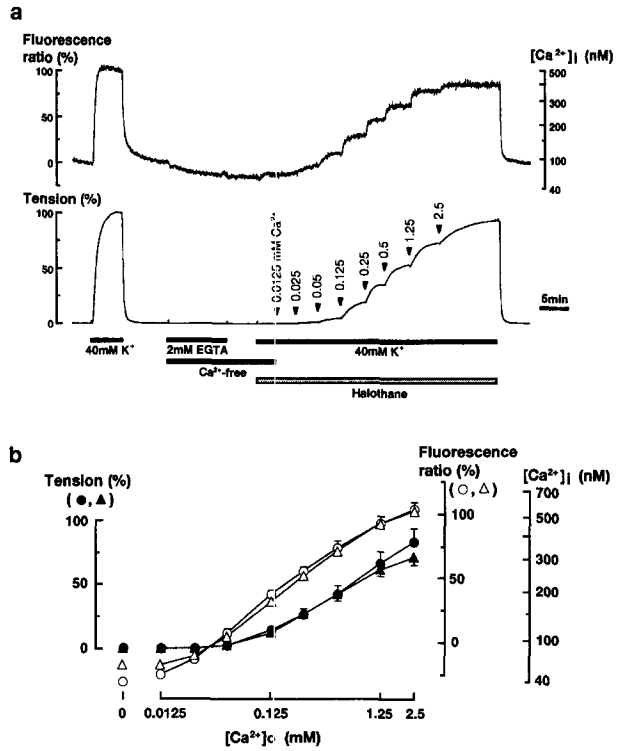

Fig. 1. The effect of halothane on $[Ca^{2+}]_i$ and tension of the contraction induced by the cumulative application of extracellular Ca^{2+} during 40 mM K^+ depolarization. (a) A representative recording of the changes in $[Ca^{2+}]_i$ and tension induced by the cumulative application of $CaCl_2$ in Ca^{2+}-free 40 mM K^+ solution with halothane treatment. (b) The effect of halothane on the increase in $[Ca^{2+}]_i$ (△) and tension (▲) induced by the increase of extracellular Ca^{2+} during 40 mM K^+ depolarization. Controls: $[Ca^{2+}]_i$ (○) and tension (●) without halothane. The plots represent the means of 10 (control) or 5 (with halothane) preparations, with the S.E.M. shown by vertical bars.

the time of replacement with Ca^{2+}-free PSS without EGTA. To examine the effect of halothane on the enhancement of the contractions induced by 1 μM acetylcholine, a similar protocol was used, except that acetylcholine was applied after a 5-min incubation in Ca^{2+}-free PSS containing 2 mM EGTA. Acetylcholine at the concentration of 1 μM produced maximum and reproducible responses in $[Ca^{2+}]_i$ and tension in porcine tracheal smooth muscle.

2.7. Data analysis

The values measured were expressed as means ± S.E.M. (n = number of observations). A strip from a different animal was used for each observation. A one-way analysis of variance for repeated measurements was used to determine the concentration dependency. A two-way analysis of variance for repeated measurements was used to determine the statistical significance of the drug effects. In addition, an analysis of covariance was used to determine the statistical significance of the difference in the $[Ca^{2+}]_i$-tension relationship. P values less than 0.05 were considered to be significant.

3. Results

3.1. Effect of halothane on the extracellular Ca^{2+}-induced increases in $[Ca^{2+}]_i$ and tension during high K^+ depolarization

Fig. 1a shows a representative recording of the changes in $[Ca^{2+}]_i$ and tension induced by the cumulative application of $CaCl_2$ in Ca^{2+}-free 40 mM K^+ solution during halothane application. In response to the stepwise incre-

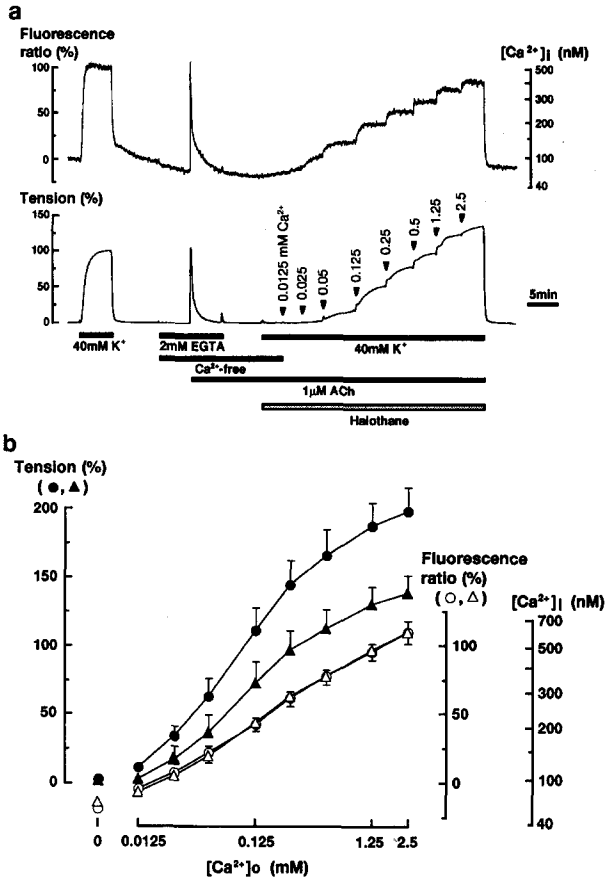

Fig. 2. The effect of halothane on $[Ca^{2+}]_i$ and tension of the contraction induced by the cumulative application of extracellular Ca^{2+} during 40 mM K^+ depolarization in the presence of 1 μM acetylcholine. (a) A representative recording of the changes in $[Ca^{2+}]_i$ and tension induced by the cumulative application of $CaCl_2$ in Ca^{2+}-free 40 mM K^+ solution in the presence of 1 μM acetylcholine with halothane treatment. (b) The effect of halothane on the increase in $[Ca^{2+}]_i$ (△) and tension (▲) induced by the increase of extracellular Ca^{2+} during 40 mM K^+ depolarization in the presence of 1 μM acetylcholine. Controls: $[Ca^{2+}]_i$ (○) and tension (●) without halothane. The plots represent the means of 6 (control) or 5 (with halothane) preparations, with the S.E.M. shown by vertical bars.

ment of extracellular Ca^{2+} concentration (0–2.5 mM), the $[Ca^{2+}]_i$ and tension increased concentration dependently (Fig. 1b). Under control conditions (without halothane), $[Ca^{2+}]_i$ increased from $-26.1 \pm 2.0\%$ to $108.9 \pm 6.1\%$, and tension increased from $0 \pm 0\%$ to $83.1 \pm 11.2\%$ ($n = 10$). In the halothane-treated strips, $[Ca^{2+}]_i$ increased from $-13.8 \pm 1.1\%$ to $107.0 \pm 2.3\%$, and tension increased from $0 \pm 0\%$ to $71.2 \pm 6.2\%$ ($n = 5$). There was no significant difference between the $[Ca^{2+}]_i$ or tension under either set of conditions. Therefore, halothane did not alter the increases in the $[Ca^{2+}]_i$ and tension induced by the cumulative application of extracellular Ca^{2+} during K^+ depolarization.

3.2. Effect of halothane on the extracellular Ca^{2+}-induced increases in $[Ca^{2+}]_i$ and tension during high K^+ depolarization in the presence of acetylcholine

Fig. 2a shows a representative recording of the changes in $[Ca^{2+}]_i$ and tension induced by the cumulative application of $CaCl_2$ during depolarization with 40 mM K^+ in the presence of 1 μM acetylcholine and with the application of halothane. In response to the stepwise increase of extracellular Ca^{2+} concentration (0–2.5 mM), $[Ca^{2+}]_i$ and tension increased concentration dependently (Fig. 2b). In the presence of acetylcholine, the extent of the increase in $[Ca^{2+}]_i$ was similar to that in the absence of acetylcholine, however, the extent of the increase in tension was more than twice as great as that in the absence of acetylcholine (Fig. 1b and Fig. 2b). Without halothane application, the $[Ca^{2+}]_i$ increased from $-19.3 \pm 3.2\%$ to $109.7 \pm 8.0\%$, while the tension increased from $2.0 \pm 0.9\%$ to $199.2 \pm 17.9\%$ ($n = 6$). In the halothane-treated strips, the $[Ca^{2+}]_i$ increased from $-14.2 \pm 1.2\%$ to $109.4 \pm 8.3\%$, while the tension increased from $1.0 \pm 0.6\%$ to $138.8 \pm 12.5\%$ ($n = 5$). The application of halothane somewhat inhibited the increase in tension, but did not affect the increase in $[Ca^{2+}]_i$.

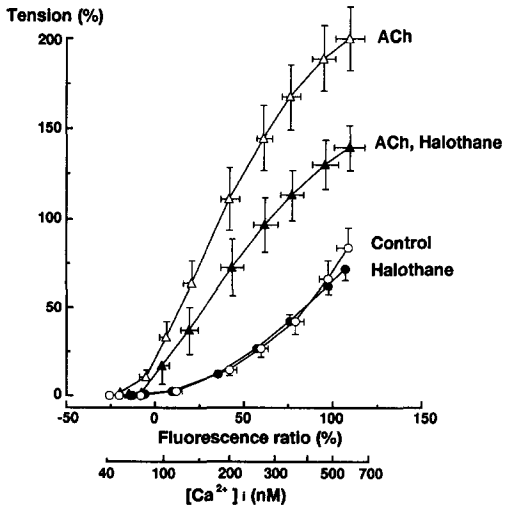

Fig. 3. The effect of halothane on $[Ca^{2+}]_i$-tension relationship. The $[Ca^{2+}]_i$-tension relationship was obtained based on the data in Figs. 1b and 2b. (○,●) indicate the values obtained from contractions induced by the increase of extracellular Ca^{2+} during 40-mM K^+ depolarization, with (●) or without (○) treatment with halothane. (△,▲) indicate the values obtained from contractions induced by the increment of extracellular Ca^{2+} during 40-mM K^+ depolarization in the presence of 1 μM acetylcholine, either with (▲) or without (△) halothane treatment.

3.3. Effect of halothane on the $[Ca^{2+}]_i$-tension relationship

The $[Ca^{2+}]_i$-tension relationships in the case of the extracellularly applied Ca^{2+}-induced contraction during high K^+ depolarization, in the presence or absence of acetylcholine, either with or without halothane treatment, were obtained from the data in Fig. 1b and Fig. 2b, and are shown in Fig. 3. The $[Ca^{2+}]_i$ (abscissa)-tension (ordinate) relationship in the presence of acetylcholine significantly shifted upward compared to that in the absence of acetylcholine, and the higher the $[Ca^{2+}]_i$, the greater the increase in tension. Treatment with halothane significantly attenuated this upward shift in the $[Ca^{2+}]_i$-tension relationship induced by acetylcholine. However, the $[Ca^{2+}]_i$-tension relationship in the absence of acetylcholine was not affected by halothane.

4. Discussion

In the present study, halothane was observed to attenuate the acetylcholine-induced upward shift of the $[Ca^{2+}]_i$-tension relationship without affecting the 'native' $[Ca^{2+}]_i$-tension relationship during the depolarization- and Ca^{2+}-induced contraction. This means that halothane attenuated the acetylcholine-induced enhancement of the increase in tension. In other words, acetylcholine increases the Ca^{2+} sensitivity of the contractile apparatus, while halothane attenuates it. The precise mechanisms controlling the change in Ca^{2+} sensitivity of the contractile apparatus by acetylcholine are unknown. Several undetermined biological steps may be involved between the receptor stimulation and the increase in Ca^{2+} sensitivity. In any event, it is plausible that halothane attenuates the acetylcholine-induced increase in Ca^{2+} sensitivity by acting on certain intracellular signal transduction systems, without affecting the $[Ca^{2+}]_i$ levels. Consistent with our observation, Jones et al. (1993, 1994) showed that halothane attenuates the canine tracheal smooth muscle contraction stimulated by acetylcholine without any detectable changes in $[Ca^{2+}]_i$.

Halothane did not affect the increases in the $[Ca^{2+}]_i$ or tension induced by the cumulative application of extracellular Ca^{2+} during K^+ depolarization. Since these increases in $[Ca^{2+}]_i$ depend exclusively on extracellular Ca^{2+}, and hence, Ca^{2+} influx through the voltage-dependent Ca^{2+} channel, halothane, at the concentration used in the present

study, probably has little effect on the voltage-dependent Ca^{2+} influx. Consistent with our observations, Tagliente et al. (1992) reported that halothane did not affect the KCl-induced contraction of the isolated guinea pig trachea. Yamamoto et al. (1993) also found that halothane had no significant effect on the KCl-induced contractions of canine tracheal smooth muscle strips, at a higher tension.

The aqueous concentration of halothane obtained by bubbling a bath solution using 3% halothane gas was 0.034 mM in this study, and this was thought to be an extremely low concentration. This low concentration resulted from our use of an organ bath open to the atmosphere. Even at this low concentration, halothane attenuated the acetylcholine-induced increase in Ca^{2+} sensitivity of the contractile apparatus. It has been reported that halothane, at concentrations over 0.33 mM, reduced both the tension and $[Ca^{2+}]_i$ in the contraction induced by carbachol (Yamakage, 1992). In a preliminary study, we also observed that a high concentration of halothane (0.309 ± 0.019 mM), which could be obtained in a solution first bubbled in a closed container and then applied in the organ bath, reduced both the $[Ca^{2+}]_i$ (from $45.5 \pm 10.4\%$ to $22.8 \pm 6.6\%$) and tension (from $184.5 \pm 20.2\%$ to $53.0 \pm 23.0\%$) of 1 μM carbachol-induced contractions ($n = 4$). It has been shown that halothane inhibits voltage-dependent Ca^{2+} channels in porcine tracheal smooth muscle cells at the concentration of 1.45 mM (Yamakage et al., 1995). Thus, it is obvious that high concentrations of halothane reduce $[Ca^{2+}]_i$, and this may contribute to the direct relaxation of airway smooth muscle. However, at a low concentration, as in the present study, halothane by itself did not affect $[Ca^{2+}]_i$, but instead attenuated the acetylcholine-induced increase in Ca^{2+} sensitivity of the contractile apparatus. The latter mechanism may be more important than the direct reduction of $[Ca^{2+}]_i$ with regard to mechanisms of the airway relaxing effect observed clinically.

Several in vivo studies have shown that the major action of halothane in airway dilation was due to the block of vagal activity, since this effect was mimicked by atropine (Shah and Hirshman, 1986; Brown et al., 1993). The block of vagal activity can include the block of vagal nerve transmission and the block of intracellular signal transductions from membrane muscarinic receptors to effectors in the airway smooth muscle. An in vivo study which was designed to estimate separately the effects of halothane on neural transmission (evoked by electrical vagal nerve stimulation) and on smooth muscle (stimulated by acetylcholine) revealed that both mechanisms contributed approximately equally to halothane-induced airway dilation (Warner et al., 1990). The latter, so called direct, effect on smooth muscle might be rather a specific inhibition of the receptor-mediated intracellular signal transduction system than a non-specific mechanism, because in our present study halothane only attenuated acetylcholine-induced enhancement of tension without affecting the contraction induced by depolarization and Ca^{2+} alone. Therefore we consider that halothane acts on the muscarinic receptor and/or intracellular signal transduction system originating from the receptor, and this action probably also contributes to the block of the vagal activity.

In conclusion, halothane apparently attenuates the acetylcholine-induced increase in Ca^{2+} sensitivity of the contractile apparatus without affecting the $[Ca^{2+}]_i$ levels. This desensitization of the contractile apparatus to $[Ca^{2+}]_i$ may play a major role in the direct airway-relaxing effect of halothane.

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