Carbachol-Induced Potassium Release in Rat Parotid Acini: Comparison of the Roles of Cytosolic Ca$^{2+}$ and Protein Kinase C

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Received July 29, 1993 Accepted September 3, 1993

ABSTRACT—Carbachol (CCh) stimulated K$^+$ release from rat parotid acini. Treatment with the intracellular Ca$^{2+}$ antagonist 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) or the intracellular Ca$^{2+}$ chelator 1,2-bis(O-aminophenoxy)ethane-N,N',N''-tetraacetic acid (BAPTA) strongly suppressed the CCh-induced K$^+$ release. Combined addition of the Ca$^{2+}$ ionophore ionomycin and the microsomal Ca$^{2+}$-ATPase inhibitor thapsigargin caused a rapid increase in cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) and resulted in a marked release of K$^+$. In the absence of extracellular Ca$^{2+}$, CCh or a combination of ionomycin and thapsigargin caused a transient release of K$^+$ which correlated well with the transient change in [Ca$^{2+}$]$_i$. On the other hand, phorbol 12-myristate 13-acetate (PMA) did not potentiate the CCh-induced K$^+$ release, although the CCh-induced amylase release was significantly enhanced in the presence of PMA. Stauroporine, a protein kinase C-inhibitor, did not inhibit the CCh-induced K$^+$ release, which was in contrast with its inhibitory effect on amylase release. These results suggest that the K$^+$ release from rat parotid acini induced by CCh stimulation is mediated by a rapid increase in [Ca$^{2+}$]$_i$, but is not associated with activation of protein kinase C. This signal pathway is different from that for amylase release where activation of protein kinase C plays an important role.

Keywords: Parotid acini, Cytosolic Ca$^{2+}$ concentration, K$^+$ release, Protein kinase C, Muscarinic-cholinergic receptor

In rat parotid acinar cells, activation of muscarinic-cholinergic, $\alpha$-adrenergic, or substance P receptors stimulates phosphoinositide breakdown leading to production of inositol 1,4,5-trisphosphate and causes a rapid increase in cytosolic free calcium concentration ([Ca$^{2+}$]$_i$) (see Refs. 1–3 for reviews). Stimulation of these receptors results in fluid secretion and relatively limited amylase release. We recently reported the relationship between [Ca$^{2+}$]$_i$ and amylase release following muscarinic stimulation (4). Incubation of parotid acinar cells with the intracellular Ca$^{2+}$ antagonist 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) or the intracellular Ca$^{2+}$ chelator 1,2-bis(O-aminophenoxy)ethane-N,N',N''-tetraacetic acid (BAPTA) strongly attenuated the increase in [Ca$^{2+}$]$_i$ evoked by carbachol (CCh), a cholinergic agonist, but CCh-induced amylase release was not significantly suppressed by the incubation with TMB-8 and BAPTA. Furthermore, the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) stimulated amylase release without increasing [Ca$^{2+}$]$_i$ (4, 5), and the protein kinase C inhibitor staurosporine strongly inhibited the CCh- and PMA-induced amylase release (4). Thus we concluded that amylase release by muscarinic stimulation was mainly mediated by activation of protein kinase C but not by Ca$^{2+}$-mobilization.

Activation of muscarinic receptors is strongly associated with fluid secretion in addition to amylase release. Fluid secretion in parotid slices or acini has been studied in vitro by monitoring K$^+$ or $^{86}$Rb release, which is thought to reflect ionic transport (see Ref. 6 for a review). Patch-clamp studies have identified Ca$^{2+}$-sensitive K$^+$ channels in the basolateral membrane of salivary acinar cells (see Ref. 7 for a review), and the opening of the K$^+$ channels by an increase in [Ca$^{2+}$]$_i$ is suggested to result in a dramatic loss of cellular K$^+$. If Ca$^{2+}$ plays a central role in triggering K$^+$ release, the treatment of acini with TMB-8 and BAPTA would be expected to block the agonist-stimulated K$^+$ release, and the Ca$^{2+}$ ionophore ionomycin and the microsomal Ca$^{2+}$-ATPase inhibitor thapsigargin, which bypass the receptor-mediated elevation of [Ca$^{2+}$]$_i$, would mimic the effect of receptor stimulation on K$^+$ release. Here, using these agents, we examined the
relationship between \([\text{Ca}^{2+}]_{i}\) and \(K^+\) release in rat parotid acini following muscarinic stimulation. Furthermore, to assess whether activation of protein kinase C is involved in fluid secretion, the effects of PMA and staurosporine on \(K^+\) release were examined.

**MATERIALS AND METHODS**

**Materials**

\(\text{CCh},\) hyaluronidase (type I-S), bovine serum albumin (BSA), TMB-8, ionomycin, thapsigargin and staurosporine were purchased from Sigma (St. Louis, MO, USA). Collagenase (CLS II) was from Cooper Biochemical (Malvern, PA, USA). Hanks' balanced salt solution was from Gibco (Chagrin Falls, OH, USA). Fura-2 acetoxymethyl ester (fura-2/AM), BAPTA acetoxymethyl ester (BAPTA/AM), EGTA and Hepes were from Dojin Laboratories (Kumamoto). All other reagents were from Wako Pure Chemicals (Osaka).

**Preparation of parotid acini**

Male Wistar-strain rats, weighing about 300 g, were anesthetized with diethyl ether and killed by cardiac puncture. Dispersed parotid acini were prepared by enzyme digestion with collagenase and hyaluronidase as described elsewhere (8). After digestion, the acini were washed twice and suspended in a Hanks' balanced salt solution of the following composition: 137 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl_2, 0.41 mM MgSO_4, 0.49 mM MgCl_2, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.5 mM glucose, 0.2% bovine serum albumin, and 20 mM Hepes. The medium used was adjusted with NaOH to pH 7.4.

**Assay of potassium release**

The acini prepared from two rats were resuspended in 20 ml of fresh Hank's balanced salt solution. Aliquots (0.5 ml) of the acini suspension were placed in glass tubes and stimulated with secretagogues for various intervals at 37°C. After incubation, the suspension was centrifuged at 3400 rpm for 3 sec. The supernatant was removed and subsequently used for determining \(K^+\) concentrations. To measure the total \(K^+\) content of the cell suspension, a portion of the suspension was homogenized with a Polytron homogenizer, and the supernatant was removed by centrifugation. The amount of \(K^+\) released was expressed as a percentage of the total cellular \(K^+\). Calculation of \(K^+\) release was performed with the following formula, as described by Quissell (9): \((K_i - K_o)/(K_i - K_o) \times 100\). \(K_i\) is the \(K^+\) concentration of the supernatant after stimulation; \(K_o\), the \(K^+\) concentration at time zero; \(K_s\), the total \(K^+\) concentration of the cell suspension. The \(K^+\) concentrations were analyzed with a flame photometer (Corning 480; Halstead, Essex, UK).

**Assay of amylase release**

The amylase release was assayed by a protocol similar to that for \(K^+\) release. The amylase activity was measured by the method of Bernfeld (10).

**Measurement of \([\text{Ca}^{2+}]_{i}\)**

Parotid acini were incubated with 2 \(\mu\)M fura-2/AM for 45 min at 37°C. The fura-2-loaded cells were washed twice, resuspended in a Hanks' balanced salt solution and then transferred into a quartz cuvette. The cuvette was thermostatically controlled at 37°C, and the cell suspension was continuously stirred. The fluorescence of the fura-2-loaded cells was measured with a Hitachi F-2000 spectrofluorimeter (Hitachi, Tokyo) with excitation at 340 and 380 nm and emission at 510 nm. The \([\text{Ca}^{2+}]_{i}\) was calculated from the ratio of fluorescence, as described by Grynkiewicz et al. (11). Maximum fluorescence was determined by lysing the cells with 0.1% Triton X-100, and minimum fluorescence was determined by the addition of 30 mM Tris base and 5 mM EGTA.

**RESULTS**

**Effect of TMB-8 and BAPTA on \(K^+\) release stimulated by \(\text{CCh}\)**

As shown in Fig. 1, stimulation of parotid acini with 10 \(\mu\)M \(\text{CCh}\) caused a release of about 40% of the total intracellular \(K^+\) within 30 sec. Subsequently, the released \(K^+\) was taken up by the cells and decreased to a sustained level of about 14% by 3 min. When 30 \(\mu\)M TMB-8, which is thought to inhibit \(\text{Ca}^2+\)-mobilization from intracellular stores (12), was added 5 min before stimulation with \(\text{CCh}\), the release of \(K^+\) induced by \(\text{CCh}\) was markedly suppressed (Fig. 1). The amount of \(K^+\) released within 30 sec of stimulation in the presence of TMB-8 was only 6% of the total intracellular \(K^+\), and this level was maintained up to 5 min after the stimulation.

In addition, we examined whether the \(\text{CCh}\)-induced \(K^+\) release is blocked by previously loading parotid acini with BAPTA, a \(\text{Ca}^2+\) chelator. For loading with BAPTA, parotid acini were pre-incubated for 30 min with 10 \(\mu\)M BAPTA/AM in dimethylsulphoxide (DMSO), washed by centrifugation, and then re-suspended to fresh Hanks' balanced salt solution without BAPTA/AM. The control acini were pre-incubated for 30 min in the presence of 0.2% DMSO alone. In the control acini, stimulation with 10 \(\mu\)M \(\text{CCh}\) induced a rapid release of \(K^+\) within 30 sec followed by a subsequent uptake of \(K^+\) into the cells (Fig. 2). In contrast, in acini loaded with BAPTA, the release of \(K^+\) in response to \(\text{CCh}\) was almost completely blocked (Fig. 2).
Effects of ionomycin and thapsigargin on secretory responses and $[Ca^{2+}]_i$

The $Ca^{2+}$ ionophore ionomycin and the microsomal $Ca^{2+}$-ATPase inhibitor thapsigargin cause an elevation of $[Ca^{2+}]_i$ without formation of inositol phosphates (4, 13, 14). We examined the ability of ionomycin and thapsigargin to evoke the $K^+$ release from parotid acini. Addition of 0.5 $\mu$M ionomycin or 0.5 $\mu$M thapsigargin alone to the fura-2-loaded acini slowly increased $[Ca^{2+}]_i$ from a resting level of $92.3 \pm 6.4$ nM (means $\pm$ S.E., n = 9)
to a maximum value of 235.2 ± 25.8 nM (n=4) or 254.0 ± 12.3 nM (n=5), respectively (Fig. 3, A and B), but the effect of these agents on K⁺ release was very modest (Fig. 4). Even if the concentration of ionomycin or thapsigargin was increased to 1 μM, it did not mimic the CCh-induced K⁺ release (data not shown). However, when a combination of 0.5 μM ionomycin and 0.5 μM thapsigargin was added to the acini suspension, there was a marked release of K⁺ (Fig. 4). The amount of K⁺ released 1 min after stimulation was about 36% of the total intracellular K⁺, which was similar to the CCh-induced K⁺ release. As shown in Fig. 3C, the combined addition of ionomycin and thapsigargin elicited a biphasic increase in [Ca²⁺], comparable with that by CCh stimulation (Fig. 3D), and the response was more rapid than that induced by ionomycin or thapsigargin alone. The peak value (457.4 ± 28.9 nM, n=4) of [Ca²⁺] induced by a combination of ionomycin and thapsigargin was almost similar to that (392.1 ± 34.6 nM, n=5) observed by CCh stimulation.

Furthermore, the amounts of amylase released during incubation for 5 min were measured using the same samples as employed for K⁺ release. As shown in Table 1, the addition of 0.5 μM ionomycin or 0.5 μM thapsigargin alone had no effect on amylase release. Although a combined addition of these agents caused a slight release of amylase, the release was not statistically significant when compared with the basal release.

Table 1. The effects of ionomycin and thapsigargin on amylase release

| Treatments                  | Amylase release (% of total) |
|-----------------------------|------------------------------|
| None                        | 5.42±1.11                    |
| Ionomycin (0.5 μM)          | 5.09±0.60                    |
| Thapsigargin (0.5 μM)       | 5.03±0.77                    |
| Ionomycin (0.5 μM) + Thapsigargin (0.5 μM) | 6.82±1.58          |

Parotid acini were incubated for 5 min with 0.5 μM ionomycin, 0.5 μM thapsigargin or a combination of these agents. The released amylase was expressed as a percent of the total amylase in the acini. Values are means ± S.E. of 4 experiments.

K⁺ release in the absence of extracellular Ca²⁺

In Ca²⁺-free medium containing 1 mM EGTA, the K⁺ release induced with 10 μM CCh reached a maximum 30 sec after stimulation and then fell to the control level within 2 min (Fig. 5A). A transient release of K⁺ was also observed by a simultaneous addition of 0.5 μM

Fig. 4. K⁺ release induced by ionomycin and thapsigargin. Ionomycin, thapsigargin or a combination of them was added to parotid acini (at time zero). (△—△), 0.5 μM ionomycin; (●—●), 0.5 μM thapsigargin; (●—○), 0.5 μM ionomycin plus 0.5 μM thapsigargin; (●), none. Values are means ± S.E. of 4 experiments.

Fig. 5. K⁺ release in the absence of extracellular Ca²⁺. Parotid acini were suspended in a Ca²⁺-free medium containing 1 mM EGTA and then stimulated (at time zero). (A) CCh-induced K⁺ release. (●—●), 10 μM CCh; (○—○), basal release. (B) K⁺ release induced by a combination of ionomycin and thapsigargin. (●—○), 0.5 μM ionomycin plus 0.5 μM thapsigargin; (○—○), basal release. The results in (A) and (B) are means ± S.E. of 4 experiments.
ionomycin and 0.5 μM thapsigargin (Fig. 5B), although the release took 3 min to return to the control level. The addition of CCh or a combination of ionomycin and thapsigargin to fura-2-loaded acini caused a transient increase in $[\text{Ca}^{2+}]_i$ which declined to a resting level (Fig. 6), indicating that the stimulation mobilized $\text{Ca}^{2+}$ from intracellular $\text{Ca}^{2+}$ stores. The changes in $[\text{Ca}^{2+}]_i$ corresponded well to the patterns of $K^+$ release in the absence of extracellular $\text{Ca}^{2+}$.

Fig. 6. Effects of CCh and a combination of ionomycin and thapsigargin on $[\text{Ca}^{2+}]_i$ in the absence of extracellular $\text{Ca}^{2+}$. Fura-2-loaded acini were suspended in a $\text{Ca}^{2+}$-free medium containing 1 mM EGTA and then stimulated by 10 μM CCh or a combination of 0.5 μM ionomycin (Iono) and 0.5 μM thapsigargin (ThG). These results are representative of 3 experiments.

ionomycin and 0.5 μM thapsigargin (Fig. 5B), although the release took 3 min to return to the control level. The addition of CCh or a combination of ionomycin and thapsigargin to fura-2-loaded acini caused a transient increase in $[\text{Ca}^{2+}]_i$, which declined to a resting level (Fig. 6), indicating that the stimulation mobilized $\text{Ca}^{2+}$ from intracellular $\text{Ca}^{2+}$ stores. The changes in $[\text{Ca}^{2+}]_i$, corresponded well to the patterns of $K^+$ release in the absence of extracellular $\text{Ca}^{2+}$.

**Effect of PMA and staurosporine on $K^+$ and amylase release**

It has been reported that the protein kinase C activator PMA induces amylase release in rat parotid acini (4, 5), and we examined the ability of PMA to stimulate the $K^+$ release. PMA itself had no effect on the $K^+$ release. Figure 7 shows the $K^+$ release evoked by CCh in the presence and absence of 100 nM PMA. The $K^+$ release induced by 10 μM CCh was not significantly affected by pre-incubation with PMA. However, when the concentration of CCh was decreased to 1 μM, the response of $K^+$ release was partially but significantly suppressed by PMA. We measured the amounts of amylase released during this incubation. Unlike $K^+$ release, amylase release in response to CCh was significantly enhanced by PMA (Table 2A).

We also examined the effect of the protein kinase C inhibitor staurosporine (100 nM) on the CCh-induced $K^+$ release. This inhibitor itself had no effect on basal $K^+$ release. When acini were stimulated with 10 μM CCh, the early response of $K^+$ release seen within 1 min was unaffected by pre-incubation with staurosporine (Fig. 8).

**Table 2. The effects of PMA and staurosporine on carbachol (CCh)-induced amylase release**

|               | Amylase release (% of total) |       |
|---------------|------------------------------|-------|
|               | control                      | CCh (1 μM) | CCh (10 μM) |
| None          | 7.93±1.29                   | 10.99±1.78 | 12.81±0.99 |
| PMA (100 nM)  | 10.08±1.20                  | 18.36±1.38* | 21.31±2.35* |
| Staurosporine (100 nM) | 6.63±0.75 | 12.92±1.05 |

(A) Parotid acini were pre-incubated for 5 min in the presence or absence of 100 nM PMA and then stimulated for 5 min with 1 or 10 μM CCh. The released amylase was expressed as a percent of the total amylase in the acini. Values are means±S.E. of 3 experiments. (B) Parotid acini were pre-incubated for 5 min in the presence or absence of 100 nM staurosporine and then stimulated for a further 10 min with 10 μM CCh. Values are means±S.E. of 5–7 experiments. *P<0.05, as compared with the value obtained in the absence of PMA or staurosporine.
However, the increased K\(^+\) release was maintained throughout the stimulation and did not decrease to the sustained level. On the other hand, the amounts of amylase released during this incubation were significantly inhibited by staurosporine (Table 2B).

**Fig. 8.** Effect of staurosporine on CCh-induced K\(^+\) release. Parotid acini were incubated for 5 min with or without 100 nM staurosporine and then stimulated by adding 10 nM CCh (at time zero). (○—○), CCh alone; (●—●), staurosporine plus CCh; (▲—▲), none; (●—▲), staurosporine alone. Values for the CCh-induced K\(^+\) release are means±S.E. of 6 experiments, and for the basal release, they are means±S.E. of 3 experiments.

**DISCUSSION**

In the present study, treatment of rat parotid acini with the intracellular Ca\(^{2+}\) antagonist TMB-8 and the intracellular Ca\(^{2+}\) chelator BAPTA almost completely suppressed the K\(^+\) release induced by CCh. Since the same treatment strongly attenuates [Ca\(^{2+}\)]\(\text{i}\), elevation following CCh stimulation (4), it is reasonable to conclude that the inhibition of K\(^+\) release is due to a blocking of Ca\(^{2+}\)-mobilization or buffering of [Ca\(^{2+}\)]\(\text{i}\). Furthermore, the combined addition of ionomycin and thapsigargin caused both a noticeable increase in [Ca\(^{2+}\)], and a marked release of K\(^+\), which were comparable to the responses induced by CCh. These results strongly support the view that an increase in [Ca\(^{2+}\)], is an essential and sufficient signal for K\(^+\) release from rat parotid acini.

Stimulation of muscarinic receptors causes amylase release in addition to K\(^+\) release. The amylase release induced by CCh was not significantly inhibited by treatment with TMB-8 and BAPTA (4), and a combination of ionomycin and thapsigargin had little or no effect on amylase release, as shown here. These results suggest that an increase in [Ca\(^{2+}\)], is not a crucial signal for stimulating amylase release. Thus it seems that there are considerable differences in the Ca\(^{2+}\)-dependence of K\(^+\) and amylase release. It has been shown that removal of Ca\(^{2+}\) from extracellular medium reduces amylase release induced by muscarinic agonists (15, 16). However, the removal of Ca\(^{2+}\) also inhibits amylase release induced by PMA (4) that does not stimulate Ca\(^{2+}\)-mobilization (4). Accordingly, the decrease in secretory response in a Ca\(^{2+}\)-free medium may be due to mechanisms independent of the attenuation of the [Ca\(^{2+}\)]\(\text{i}\) response. Lengthy incubation of cells in a Ca\(^{2+}\)-free medium results in a decrease in resting [Ca\(^{2+}\)], and the unphysiologically decreased [Ca\(^{2+}\)], may affect cellular components required for maintaining amylase release.

Addition of ionomycin or thapsigargin alone did not induce K\(^+\) release so effectively, which is different from the results that the Ca\(^{2+}\) ionophores, A23187 and ionomycin, readily evoke K\(^+\) or 86Rb release from salivary gland slices (17–20). In the previous studies, however, the slices were pre-incubated with the ionophores in the absence of extracellular Ca\(^{2+}\) and then stimulated by Ca\(^{2+}\) addition. If the ionophores were added to a medium already containing Ca\(^{2+}\), the K\(^+\) or 86Rb release is smaller than that obtained by receptor stimulation (17, 18, 21). This low ability of these agents to stimulate K\(^+\) release is probably related to the fact that the [Ca\(^{2+}\)]\(\text{i}\) response is slow compared with the receptor-mediated response, because the rapid increase in [Ca\(^{2+}\)], produced by a combined addition of ionomycin and thapsigargin resulted in a marked release of K\(^+\). It is likely that the initial rate of [Ca\(^{2+}\)], elevation in addition to the level of [Ca\(^{2+}\)], is important in stimulating K\(^+\) release.

CCh and a combination of ionomycin and thapsigargin caused a rapid and transient release of K\(^+\) even in Ca\(^{2+}\)-free medium. The patterns of K\(^+\) release correlated well with those of the transient response of [Ca\(^{2+}\)], suggesting that the K\(^+\) release is primarily mediated by the Ca\(^{2+}\) release from intracellular stores and does not require Ca\(^{2+}\) entry from the extracellular medium. Ionomycin causes a release of Ca\(^{2+}\) from the intracellular stores by acting as an ionophore at the intracellular membrane (22–24), while thapsigargin is believed to release Ca\(^{2+}\) by inhibition of the endoplasmic reticulum Ca\(^{2+}\) pump (13, 25). Therefore, it is possible that a combination of these two agents additively acts on intracellular stores and causes a rapid release of Ca\(^{2+}\) similar to the receptor-mediated [Ca\(^{2+}\)], response.

It has been suggested that activation of protein kinase C is important in accelerating amylase release from rat parotid acini (4, 5). However, it is not clear whether the
kinase plays a role in the fluid secretion of parotid glands. In this study, the protein kinase C activator PMA did not affect the basal release of K⁺ or potentiate the K⁺ release induced by CCh, which was in marked contrast to the effect of PMA on amylase release (4, 5). Putney et al. (26) reported that another phorbol ester, phorbol-dibutyrate, did not affect the ⁸⁶Rb efflux in rat parotid slices. Furthermore, the protein kinase C inhibitor staurosporine had no inhibitory effect on the CCh-induced K⁺ release, although the amylase release was suppressed by the inhibitor. At least, these results suggest that the activation of protein kinase C is unlikely to play a major role in inducing K⁺ release. Muscarinic stimulation causes a rapid decrease in cytosolic pH ([pH]) in rat parotid cells (27, 28), which is thought to be associated with ion transport in the fluid secretory process. We recently showed that PMA did not change basal [pH], or affect the CCh-induced decrease in [pH], in rat parotid cells (29). This finding supports the view that activation of protein kinase C does not stimulate fluid secretion.

However, we do not rule out the possibility that protein kinase C is involved in a feedback control mechanism for K⁺ release from rat parotid acini, because the CCh-induced K⁺ release was partially suppressed by PMA. It has been shown that PMA inhibits the agonist-induced elevation of [Ca²⁺], and the formation of inositol phosphates in a variety of cell types (30–33), suggesting that there is negative feedback of phosphoinositide metabolism through protein kinase C. Thus it is possible that pretreatment with PMA suppresses the CCh-induced K⁺ release by affecting phosphoinositide hydrolysis. In addition, the CCh-induced K⁺ release was maintained at high values in the presence of staurosporine. If this effect of staurosporine was caused through its action on protein kinase C, the result may be interpreted as further evidence that protein kinase C plays a role in down-modulation of K⁺ release. However, since the specificity of staurosporine is not great (34), further studies are necessary to determine whether the maintained release of K⁺ seen in the presence of staurosporine is due to inhibition of protein kinase C

In conclusion, the present study has demonstrated that the K⁺ release from rat parotid acini induced by muscarinic stimulation is mediated by a rapid increase in [Ca²⁺], but is not associated with activation of protein kinase C. This signal pathway is different from that in amylase release where activation of protein kinase C plays an important role.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (No. 03670869) from the Ministry of Education, Science and Culture of Japan.

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