Acetylcholine increases intracellular Ca\(^{2+}\) in the rat pituitary folliculostellate cells in primary culture

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Acetylcholine increases intracellular Ca\(^{2+}\) in the rat pituitary folliculostellate cells in primary culture. Am J Physiol Endocrinol Metab 280: E608–E615, 2001.—Pituitary folliculostellate cells (FSCs) are thought to partially inhibit pituitary hormone secretion through a paracrine mechanism. In this process, one of the important questions is what factors regulate the function of FSCs. Because ACh is synthesized in and possibly released from the corticotrophs and lactotrophs, we examined whether FSCs respond to ACh by the method of Ca\(^{2+}\) imaging in primary cultured FSCs from male Wistar rats. ACh (30 nM, 3 μM) increased intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) of FSCs in a concentration-dependent manner, with an initial rapid rise followed by a relatively sustained increase. The complete block of the response by atropine and pirenzepine suggests involvement of muscarinic receptors. Depletion of the stored Ca\(^{2+}\) by thapsigargin blocked the response completely. Blockers of phospholipase C, U-73122 and neomycin, suppressed significantly the rise in [Ca\(^{2+}\)]\(_i\). These results suggest that ACh increases [Ca\(^{2+}\)]\(_i\) in FSCs by activating phospholipase C, presumably through activation of M\(_i\) receptors. The rise in [Ca\(^{2+}\)]\(_i\) could trigger a variety of Ca\(^{2+}\)-dependent cellular processes, including the synthesis and release of bioactive substances, which in turn act on endocrine cells.

cholinergic modulation; paracrine mechanism; calcium imaging; M\(_i\) receptor

FOLLICULOSTELLATE CELLS (FSCs) in the anterior pituitary do not secrete any known traditional pituitary hormones by themselves but exert inhibitory effects on the secretion of growth hormone (GH), prolactin (PRL), luteinizing hormone, or adrenocorticotropic hormone (ACTH) (1, 18). FSCs share similar characteristics with glial cells and, to some extent, with immune cells. For example, the glial protein S-100 is a specific marker for FSCs in the anterior pituitary (15). FSCs produce basic fibroblast growth factor (6) and possess nitric oxide synthase (4) as well as interleukin-6 (19). Several recent reports suggest that FSCs modulate hormone secretion through a paracrine mechanism within the anterior pituitary (17). To date, it is poorly understood how the activity of FSCs is regulated. In the present study, we focused on the role played by ACh. ACh is synthesized by corticotrophs and by a subpopulation of lactotrophs in the anterior pituitary (3). Cholinergic modulation of GH and PRL release in the anterior pituitary has been demonstrated (2, 11, 21). Besides, muscarinic receptors have been found in rat anterior pituitary cells; however, their precise localization remains to be resolved (14, 16). From these lines of evidence, we hypothesized that ACh released from the endocrine pituitary cells could increase intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) through muscarinic receptors on the FSCs and cause a variety of Ca\(^{2+}\)-dependent cellular processes. A major question here is whether FSCs express functional cholinergic receptors and increase [Ca\(^{2+}\)]\(_i\) in response to ACh. To test this possibility, we examined the effect of ACh on primary-cultured FSCs by means of Ca\(^{2+}\) imaging.

METHODS

Primary culture of rat anterior pituitary cells. The anterior pituitary was excised from male Wistar rats (250–300 g body wt) after decapitation by a guillotine. The pituitary was minced and incubated in 10 ml of Dulbecco’s phosphate-buffered saline (–) (PBS, Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% trypsin (type III, Sigma, St. Louis, MO) and 0.25% collagenase (type I, Sigma) for 20 min at 37°C with a gentle stirring. After the incubation, pieces of the pituitary were transferred into 10 ml of PBS supplemented with 0.1 mg/ml trypsin inhibitor (type II, Sigma) and 4 U/ml deoxyribonuclease I (Sigma) and were dispersed by triturating with a 5-ml plastic pipette for 5 min. After washing with PBS, the cells were plated on poly-L-lysine-coated glass coverslips and incubated in MEM (Nissui Pharmaceutical) supplemented with 2 mM l-glutamine, 4% normal rat serum, and 0.2% BSA (fraction V, Sigma) for 3–5 days at 37°C in a humidified atmosphere of 5% CO\(_2\)-95% air. Identification of FSCs is described in RESULTS.

Measurement of [Ca\(^{2+}\)]\(_i\). Details of the imaging technique and superfusion system have been described previously (9). In brief, cultured cells were loaded by incubation with 1 μM Fura PE-3 AM (TefLabs, Austin, TX) for 60 min at 37°C. The coverslip was placed in a small superfusion chamber on the stage of a Nikon Diaphot microscope. [Ca\(^{2+}\)]\(_i\) was recorded by using the QuantiiCell 700 system (Applied Imaging, Sunderland, UK). The cells were illuminated alternately at 340-nm
and 380-nm excitation wavelengths, and then 510-nm emission light images were captured by an image-intensifying charge-coupled device camera (Photonics Science, Turnbridge Wells, UK). The time interval of each 340- to 380-nm ratio frame was 6 s. Ratios were converted to Ca\(^{2+}\) concentrations by the following equation (7): \[ [\text{Ca}^{2+}]_i = k_d \beta (R - R_{\text{min}})/(R_{\text{max}} - R) \], where \( k_d \) is the dissociation constant for Fura PE-3 Ca\(^{2+}\), \( R \) is the ratio, \( R_{\text{min}} \) and \( R_{\text{max}} \) are the ratio values of Fura PE-3 at zero and saturating \([\text{Ca}^{2+}]_i\), respectively, and \( \beta \) is the ratio of fluorescence at 380 nm for Fura PE-3 in saturating and zero \([\text{Ca}^{2+}]_i\).

Superfusion was performed with a control solution containing (in mM): 137.5 NaCl, 5 KCl, 2.5 CaCl\(_2\), 0.8 MgCl\(_2\), 0.6 NaHCO\(_3\), 10 glucose, 20 HEPES, and 0.1% BSA, and the pH was adjusted to 7.4 with NaOH. Nominal Ca\(^{2+}\)-free solution was prepared by replacing Ca\(^{2+}\) with Mg\(^{2+}\) in the control solution. This solution contained 10–20 \(\mu\)M Ca\(^{2+}\) (10). Excess K\(^+\) solution was prepared by replacing Na\(^+\) with K\(^+\) in the control solution. The cells were continuously superfused at 37°C throughout the experiment, and the flow rate was ~1 ml/min. All drugs were applied through superfusion.

**Drugs.** The following drugs were purchased from Wako Chemicals (Osaka, Japan): ACh, thapsigargin, U-73343, U-73122, and pirenzepine. Nifedipine, neomycin, and ryanodine were purchased from Sigma. SKF-96365 was from Biomol Research Laboratories (Plymouth Meeting, PA).

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**Fig. 1.** Ca\(^{2+}\) imaging of folliculostellate cells (FSCs). Arrows indicate FSCs separated from endocrine cells in A–E. A: phase contrast microscopy of FSCs and other pituitary cells. B: same group of cells shown in immunofluorescent microscopy with Cy3-labeled anti-S-100 and FITC-labeled anti-growth hormone antibodies. C: Ca\(^{2+}\) images of the same cells under resting condition. D: Ca\(^{2+}\) images of the same cells observed with 300 nM ACh. FSCs increased \([\text{Ca}^{2+}]_i\). E: Ca\(^{2+}\) images of the cells observed with 50 mM K\(^+\). FSCs hardly responded in contrast to other pituitary cells. It should be noted that the FSCs located at upper right aggregated with endocrine cells. This disturbed the Ca\(^{2+}\) signal of FSCs in response to ACh and to excess K\(^+\). In this case, we excluded the response of FSCs from the data. Scale bars in A–E, 20 \(\mu\)m. Color bar at bottom indicates the intracellular calcium concentration (\([\text{Ca}^{2+}]_i\)), 0–1,000 nM.
RESULTS

Identification of FSCs in the cultured cells. It has been demonstrated that FSCs have distinctive morphological characteristics in the pituitary (15). In primary culture, FSCs extended thin cytoplasm (Fig. 1A, arrows). The thin cytoplasmic extension often formed processes in FSCs in culture. FSCs were confirmed by immunocytochemical staining of S-100 protein, a specific marker of FSCs in the anterior pituitary (Fig. 1B, arrows). Ca^{2+} images are shown in pseudo-color (Fig. 1, C-E). FSCs responded to 300 nM ACh (Fig. 1D) but not to 50 mM K^+ (Fig. 1E). On the other hand, non-FSCs responded to 50 mM K^+ but not to 300 nM ACh. The time course of the responses is shown in Fig. 2, A and B. FSCs responded weakly to 50 mM K^+ compared with non-FSCs. The average of peak Δ[Ca^{2+}]_i induced by 50 mM K^+ was 42 ± 28 nM (n = 59 in 3 independent experiments) in FSCs and 646 ± 234 nM (n = 85 in 3 independent experiments) in non-FSCs (Fig. 2C). Additionally, non-FSCs did not respond to 300 nM ACh in these 85 cells. In the present experiment, therefore, cells were designated as FSCs if they showed distinctive morphology and relative unresponsiveness to excess K^+ (peak Δ[Ca^{2+}]_i < 100 nM). We tested the validity of these criteria in 85 cells and found that 84 of them were stained by antisera to S-100 protein.

ACh-induced rise in [Ca^{2+}]_i. Representative responses to various concentrations of ACh are shown (Fig. 3A). FSCs responded to ACh in a concentration-dependent manner at concentrations between 30 and 3,000 nM (Fig. 3B). No cells responded to 10 nM ACh. However, 30 nM and 100 nM ACh elicited responses in a substantial portion of the cells examined. Among 45 cells examined, 28 cells responded to 30 nM ACh with peak Δ[Ca^{2+}]_i of 53 ± 75 nM; 37 cells among 42 responded to 100 nM ACh with peak Δ[Ca^{2+}]_i of 120 ± 111 nM. All cells examined responded to ACh at a concentration of ≥300 nM. The averages of peak Δ[Ca^{2+}]_i induced by 300, 1,000, and 3,000 nM ACh were 229 ± 177, 301 ± 200, and 384 ± 235 nM, respectively.

ACh elicited a rapid initial increase of [Ca^{2+}]_i followed by a sustained phase (Fig. 4A). Similar responses were replicated by two sequential applications of ACh at an interval of 30 min. The peak Δ[Ca^{2+}]_i of the first application (211 ± 115 nM) was similar to that in the second application (194 ± 115 nM). The histogram for the frequency distribution of Δ[S2/S1] was ~1.0, with an average of 0.941 ± 0.327 (n = 90 in 4 independent experiments) as shown in Fig. 4B. This result was used as a control for the following experiments.

Muscarinic receptor antagonists. Two muscarinic antagonists, atropine (10 nM) and pirenzepine (10 nM), were applied 3 min before a second application of ACh. Typical examples are shown in Fig. 5. Both atropine (n = 54) and pirenzepine (n = 49) abolished the ACh-induced response in all cells examined. The responses recovered after a 30-min washout.

Ca^{2+}-free medium and Ca^{2+} channel blockers. To determine whether an influx of extracellular Ca^{2+} or mobilization of intracellular Ca^{2+} contributed to
changes in $[Ca^{2+}]_i$, $Ca^{2+}$-free solution replaced the control solution 3 min before the second application of ACh. The $Ca^{2+}$-free solution did not affect the initial rise of $[Ca^{2+}]_i$ but reversibly suppressed the late sustained increase (Fig. 6A). The peak $\Delta[Ca^{2+}]_i$ was $152 \pm 86$ nM in the control and $134 \pm 55$ nM in the $Ca^{2+}$-free solution. The frequency distribution of $S_2/S_1$ remained similar to that of control, and the average was $1.002 \pm 0.436$ ($n = 53$ in 5 independent experiments), as shown in Fig. 6B.

The effects of $Ca^{2+}$ channel blockers are shown in Fig. 7. All blockers were applied to the bath 3 min before the second application of ACh. Gadolinium (100 μM), a blocker of store-operated $Ca^{2+}$ channels, did not affect the initial rise of $[Ca^{2+}]_i$ but reversibly suppressed the following sustained increase (Fig. 7A). The values of peak $\Delta[Ca^{2+}]_i$ were $220 \pm 105$ nM in the control and $239 \pm 143$ nM with gadolinium. $S_2/S_1$ distributed similarly to the control, with an average of $1.068 \pm 0.508$ ($n = 54$ in 4 independent experiments) as shown in Fig. 7C. SKF-96365 (30 μM), a blocker of store-operated $Ca^{2+}$ channels, also reversibly suppressed the sustained increase that followed (Fig. 7B). It was noted that the initial rise was partially inhibited ($P < 0.01$). The peak $\Delta[Ca^{2+}]_i$ was $244 \pm 100$ nM in the control and $181 \pm 98$ nM after SKF-96365. The distribution of $S_2/S_1$ shifted to the left, with an average of $0.813 \pm 0.399$ ($n = 59$ in 4 independent experiments) as shown in Fig. 7D. An L-type $Ca^{2+}$ channel blocker, nifedipine (10 μM), had no effect. The peak $\Delta[Ca^{2+}]_i$ was $288 \pm 206$ nM in the control and $318 \pm 214$ nM with nifedipine. No difference was detected in the distribution of $S_2/S_1$ ($n = 47$ in 3 independent experiments).

The contribution of intracellular $Ca^{2+}$ stores was examined by experiments with thapsigargin, an inhibitor of endoplasmic reticulum $Ca^{2+}$-ATPase (8). Thapsigargin (1 μM) was applied 22 min before and throughout the second application of ACh. Thapsigargin decreased $[Ca^{2+}]_i$ by 10.2 ± 0.3 μM.

Fig. 3. ACh-induced rise in $[Ca^{2+}]_i$. A: representative responses to different concentrations of ACh. ACh was applied for 3 min as indicated by horizontal bar. B: values are means ± SD of peak $\Delta[Ca^{2+}]_i$ in no. of cells indicated beside each symbol from 3–7 independent experiments.

Fig. 4. Responses to two sequential applications of ACh. A: cells were stimulated twice with 300 nM ACh. A second stimulation, performed after a 30-min wash period, produced a similar response. ACh increased $[Ca^{2+}]_i$ with an initial rapid rise followed by a relatively sustained increase. B: the population of the ratio of $S_2$ to $S_1$ ($S_2/S_1$) distributed ~1.0, and the average was $0.941 \pm 0.277$ ($n = 90$ in 4 independent experiments).
gin alone caused a small and transient increase in $[\text{Ca}^{2+}]_i$ (Fig. 8). Application of ACh, repeated after a 30-min interval, failed to induce any response in the presence of thapsigargin ($n = 60$ in 3 independent experiments).

Phospholipase C inhibitors and ryanodine. Inhibitors of phospholipase C, neomycin and U-73122, were used to examine whether ACh activates phospholipase C in the FSCs. Prior superfusion with neomycin (3 mM) for 1 h inhibited the initial rise in $[\text{Ca}^{2+}]_i$ to $149 \pm 115$ nM from the control of $246 \pm 135$ nM ($P < 0.01$), which was partially reversed as shown in Fig. 9A. The distribution of $S_2/S_1$ was shifted to the left with an average of $0.617 \pm 0.419$ ($n = 61$ in 4 independent experiments) as shown in Fig. 9D. U-73343 (10 $\mu$M, 50-min prior superfusion), an inactive analog of U-73122, did not affect the response (Fig. 9B). The peak $\Delta[\text{Ca}^{2+}]_i$ was $179 \pm 117$ nM in the control and $180 \pm 120$ nM with U-73343. No difference was detected in the distribution of $S_2/S_1$ as shown in Fig. 9E ($n = 63$ in 4 independent experiments). U-73122 (10 $\mu$M, 50-min prior superfusion), an inhibitor of phos-
pholipase C, suppressed the response to 96 ± 47 nM from a control of 177 ± 66 nM (P < 0.01), which was reversed as shown in Fig. 9C. The distribution of S2/S1 was shifted to the left, and the average was 0.570 ± 0.230 (n = 48 in 3 independent experiments) as shown in Fig. 9E.

Ryanodine (20 μM) did not increase [Ca^{2+}]_i of FSCs and did not affect the peak Δ[Ca^{2+}]_i induced by ACh. The peak Δ[Ca^{2+}]_i was 233 ± 130 nM in the control and 227 ± 127 nM with ryanodine. No difference was detected in the distribution of S2/S1 (n = 47 in 4 independent experiments).

**DISCUSSION**

The present experiments demonstrated that ACh increased [Ca^{2+}]_i of FSCs in a concentration-dependent manner. Because this response was blocked completely by atropine and pirenzepine, the response is likely to be mediated by a muscarinic receptor. Piren-
zepine is relatively specific to the M₁ receptor subtype but also blocks M₄ receptors with a similar potency (5).

As we will discuss, the response of FSCs to ACh is likely to involve an activation of phospholipase C via Gq/11. Because M₁ but not M₄ receptors are coupled to Gq/11 (20), the present action of ACh is most likely mediated by the M₁ receptor.

The initial rise in [Ca²⁺]ᵢ was not affected by the removal of extracellular Ca²⁺ but was completely blocked by an inhibitor of the endoplasmic reticulum Ca²⁺-ATPase, thapsigargin (8). These results indicate that the initial rise in [Ca²⁺]ᵢ was caused by mobilization of Ca²⁺ from internal stores. However, the late sustained increase in [Ca²⁺]ᵢ depended completely on the presence of extracellular Ca²⁺. This phase was almost completely blocked by gadolinium, which is known to block store-operated Ca²⁺ influx (12). Another inhibitor of store-operated Ca²⁺ entry, SKF-96365 (13), also caused a partial suppression. Thus the late sustained phase is probably due to an influx of extracellular Ca²⁺. Involvement of voltage-gated Ca²⁺ channels, however, is unlikely, because FSCs are not excitable cells and express few of these channels. Indeed, FSCs weakly increased the [Ca²⁺]ᵢ in response to 50 mM K⁺. In the present study, nifedipine, a blocker of the L-type Ca²⁺ channel, did not affect the response at all. Based on the observation that two different inhibitors of phospholipase C, neomycin and U-73122, both suppressed the ACh-induced response, we propose that ACh activates phospholipase C and promotes production of D-myo-inositol 1,4,5-trisphosphate in the FSCs.

Fig. 9. Effects of phospholipase C inhibitors. A: neomycin (3 mM), a blocker of phospholipase C, was introduced 1 h before ACh application and continuously superfused as indicated by horizontal solid bar. The exact time of application is indicated in parenthesis. Neomycin inhibited the rise of [Ca²⁺]ᵢ. This inhibition was partially reversed. B: U-73343 (10 μM), structural analog of U-73122 without inhibitory action, was applied for 50 min, as indicated in parenthesis, and washed with a control solution for 10 min before second ACh application. U-73343 did not affect the response. C: U-73122 (10 μM), an inhibitor of phospholipase C, was applied in the same time course as U-73343, and it reversibly suppressed the rise in [Ca²⁺]ᵢ. D: distribution of S₂/S₁ of the peak Δ[Ca²⁺] was shifted to the left by neomycin, and the average was 0.617 ± 0.419 (n = 61 in 4 independent experiments). E: distribution of S₂/S₁ of the peak Δ[Ca²⁺] was shifted to the left by U-73122 with an average of 0.570 ± 0.230 (n = 48 in 3 independent experiments). The distribution was not affected by U-73343, and the average was 1.066 ± 0.403 (n = 63 in 4 independent experiments).
It should be noted, however, that we found that U-73343, an inactive analog of U-73122, inhibited the response to ACh when U-73343 was present simultaneously with ACh (data not shown). This nonspecific action of U-73343 may be due to competitive inhibition on ACh action, because the inhibition was reversed by increasing the concentration of ACh (our preliminary results). We did not further investigate this point but simply washed out U-73343 before ACh application and found that the 10-min wash completely removed the nonspecific action of U-73343. Therefore, we applied U-73122 in the same time course as U-73343, which must reveal a specific action of U-73122 to inhibit phospholipase C.

An involvement of ryanodine receptors is unlikely, because ryanodine did not increase [Ca\(^{2+}\)]\(_i\) of FSCs and did not affect the peak \(\Delta[Ca^{2+}]\)\(_i\) induced by ACh.

In the anterior pituitary, ACh is synthesized by corticotrophs and by a small proportion of lactotrophs (3). ACh may be secreted alone or with ACTH and PRL. To date, ACh has been considered to act on somatotrophs and lactotrophs through their \(M_1\) receptors to decrease the secretion of GH and PRL (2). The present findings indicate that FSCs are another target of ACh action in the anterior pituitary. The rise in [Ca\(^{2+}\)]\(_i\) in FSCs may stimulate a variety of Ca\(^{2+}\)-dependent cellular processes, including synthesis and release of bioactive substances, which in turn act on the endocrine cells.

In conclusion, ACh increased [Ca\(^{2+}\)]\(_i\) of FSCs by activating phospholipase C through \(M_1\) receptor activation. The present results, taken together with previous findings, suggest that ACh could function as a paracrine factor acting on FSCs in the anterior pituitary.

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