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

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Received 10 July 2000; accepted in final form 15 December 2000

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 \(\mu\)M) 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 of [Ca\(^{2+}\)]\(_i\). These results suggest that ACh increases [Ca\(^{2+}\)]\(_i\) in FSCs by activating phospholipase C, presumably through activation of M\(_1\) 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.

METHODOLOGY

**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 \(\mu\)M 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 QuantiCell 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): 
\[
[Ca^{2+}]_i = k_d \beta (R - R_{\text{min}})/(R_{\text{max}} - R),
\]
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 \([Ca^{2+}]_i\), respectively, and \(\beta\) is the ratio of fluorescence at 380 nm for Fura PE-3 in saturating and zero \([Ca^{2+}]_i\).

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 [Ca\(^{2+}\)]. 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 ([Ca\(^{2+}\)], 0–1,000 nM.
Gadolinium chloride was from Nacalai Tesque (Kyoto, Japan).

Statistical analysis. ACh-induced maximum changes in $[\text{Ca}^{2+}]_i$, $\Delta[\text{Ca}^{2+}]_i$, were considered significant when they exceeded 10 nM from basal $[\text{Ca}^{2+}]_i$. At least three independent experiments were made to draw conclusions. In each experiment, ACh was applied twice to the same cells with a 30-min wash period. The peak values of $\Delta[\text{Ca}^{2+}]_i$, in response to the first application and to the second challenge were designated as $S_1$ and $S_2$, respectively, and the $S_2$-to-$S_1$ ratio ($S_2/S_1$) was determined. In some experiments, the effects of drugs introduced between the initial and second applications of ACh were evaluated by frequency histograms constructed for $S_2/S_1$. The data were expressed as means ± SD. A paired $t$-test was used for statistical analysis. The significance level was set at $P < 0.05$.

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). 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 $\Delta[\text{Ca}^{2+}]_i$, 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 $\Delta[\text{Ca}^{2+}]_i$, <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 $[\text{Ca}^{2+}]_i$. 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 $\Delta[\text{Ca}^{2+}]_i$, of 53 ± 75 nM; 37 cells among 42 responded to 100 nM ACh with peak $\Delta[\text{Ca}^{2+}]_i$, of 120 ± 111 nM. All cells examined responded to ACh at a concentration of ≥300 nM. The averages of peak $\Delta[\text{Ca}^{2+}]_i$, 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 $[\text{Ca}^{2+}]_i$, 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 $\Delta[\text{Ca}^{2+}]_i$, 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 $S_2/S_1$ 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.

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

The effects of \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) channels, did not affect the initial rise of \([\text{Ca}^{2+}]_i\) but reversibly suppressed the following sustained increase (Fig. 7A). The values of peak \(\Delta[\text{Ca}^{2+}]_i\) were 220 ± 105 nM in the control and 239 ± 143 nM with gadolinium. \(S_2/S_1\) distributed similarly to the control, with an average of 1.068 ± 0.508 (\(n = 54\) in 4 independent experiments) as shown in Fig. 7B.

It was noted that the initial rise was partially inhibited \((P < 0.01)\). The peak \(\Delta[\text{Ca}^{2+}]_i\) was 244 ± 105 nM in the control and 181 ± 98 nM after SKF-96365. The distribution of \(S_2/S_1\) shifted to the left, with an average of 0.813 ± 0.399 (\(n = 59\) in 4 independent experiments) as shown in Fig. 7D. An L-type \(\text{Ca}^{2+}\) channel blocker, nifedipine (10 μM), had no effect. The peak \(\Delta[\text{Ca}^{2+}]_i\) was 288 ± 206 nM in the control and 318 ± 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 \(\text{Ca}^{2+}\) stores was examined by experiments with thapsigargin, an inhibitor of endoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase (8). Thapsigargin (1 μM) was applied 22 min before and throughout the second application of ACh. Thapsigargin...
gin alone caused a small and transient increase in 
$[Ca^{2+}]_i$ (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 $[Ca^{2+}]_i$ to 149 ± 115 nM from the control of 246 ± 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 ± 0.419 ($n = 61$ in 4 independent experiments) as shown in Fig. 9D. U-73343 (10 μM, 50-min prior superfusion), an inactive analog of U-73122, did not affect the response (Fig. 9B). The peak $Δ[Ca^{2+}]_i$ was 179 ± 117 nM in the control and 180 ± 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 μM, 50-min prior superfusion), an inhibitor of phos-

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**Fig. 5.** Inhibition by muscarinic antagonists. A: atropine (10 nM) completely blocked the response ($n = 54$ in 4 independent experiments). B: pirenzepine (10 nM) also completely blocked the response ($n = 49$ in 4 independent experiments). These responses were reversed after a 30-min wash.

**Fig. 6.** Effect of Ca$^{2+}$-free medium. A: removal of extracellular Ca$^{2+}$ did not affect the initial rise of $[Ca^{2+}]_i$ but reversibly suppressed the following sustained increase. B: distribution of $S_2/S_1$ of peak $Δ[Ca^{2+}]_i$ was similar to control, and the average was 1.002 ± 0.436 ($n = 53$ in 5 independent experiments).
Phospholipase C, suppressed the response to 96 ± 27 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 [Ca2+]i of FSCs and did not affect the peak Δ[Ca2+]i induced by ACh. The peak Δ[Ca2+]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 [Ca2+]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.
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 \([\text{Ca}^{2+}]_i\) of FSCs and did not affect the peak \(\Delta[\text{Ca}^{2+}]_i\) 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 M2 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 \([\text{Ca}^{2+}]_i\) in FSCs may stimulate a variety of \(\text{Ca}^{2+}\)-dependent cellular processes, including synthesis and release of bioactive substances, which in turn act on the endocrine cells.

In conclusion, ACh increased \([\text{Ca}^{2+}]_i\) of FSCs by activating phospholipase C through M1 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.

We are grateful to Professor V. N. Luine for critical reading of the manuscript.

This work was supported in part by Grants-in-Aid 08680872, 10670071, and 10480227 from the Ministry of Education, Science, Sports and Culture of Japan.

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