brief communication

Activation of two different receptors mobilizes calcium from distinct stores in *Xenopus* oocytes

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ABSTRACT Acetylcholine (ACh) and thyrotropin-releasing hormone (TRH) utilize inositol 1,4,5-trisphosphate (IP_3) as a second messenger and evoke independent depolarizing membrane electrical responses accompanied by characteristic ^{45}Ca efflux profiles in *Xenopus* laevis oocytes injected with GH_3 pituitary cell mRNA. To determine whether this could be accounted for by mobilization of calcium from functionally separate stores, we measured simultaneously ^{45}Ca efflux and membrane electrical responses to ACh and TRH in single oocytes. We found that depletion of ACh-sensitive calcium store did not affect the membrane electrical response to TRH and the TRH-evoked ^{45}Ca efflux. Our data suggest that ACh and TRH mobilize calcium from distinct cellular stores in the oocyte. This is the first demonstration in a single cell of strict subcellular compartmentalization of calcium stores coupled to two different populations of cell membrane receptors that utilize the same second messenger.

INTRODUCTION

In native *Xenopus* oocytes, acetylcholine (ACh) evokes a characteristic membrane electrical response (1, 2). This intrinsic response to ACh is mediated by an increase in inositol 1,4,5-trisphosphate (IP_3) (3) that releases Ca^{2+} from a cellular store(s) to elevate cytoplasmic free Ca^{2+} (4–7). Oocytes injected with mRNA isolated from GH_3 pituitary cells acquire responsiveness to thyrotropin-releasing hormone (TRH) (8–10). The acquired response to TRH is similar to the intrinsic response to ACh and is mediated by breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate IP_3 and release cell calcium (10). Despite these similarities, acquired responses to TRH are usually much larger than intrinsic responses to ACh and display a distinct pattern with a much longer latency period (11). The present report demonstrates that these responses are generated independently in *Xenopus* oocytes, although they proceed by apparently identical signal transduction pathway. We provide evidence that the activation of ACh and TRH receptors mobilizes calcium from distinct cellular stores in the oocyte.

MATERIALS AND METHODS

Xenopus frogs were obtained from South African Snake Farm (Fish Hoek, Republic of South Africa). The frogs were maintained at 18–20° in a 12 h light/12 h dark cycle and fed diced beef liver twice weekly. Oocytes were excised from ovary fragments as previously described (3, 4).

Electrophysiologic measurements were performed on two-electrode voltage-clamped oocytes in a 0.3-ml perfusion chamber, essentially as described (3, 4).

For calcium efflux studies, oocytes were incubated with 0.2 mCi/ml $^{45}CaCl_2$ (specific radioactivity 0.1 mCi/μmol) 18 h before the experiment. The perfusion bath was connected to a fraction collector and the medium changed every min. This allowed to record simultaneously membrane electrical responses and ^{45}Ca efflux from a single cell. Oocytes were homogenized at the end of each experiment to determine residual label. Results were calculated either as percent of total label released or as fractional efflux rates due to application of agonists. These methods are described in detail elsewhere (5, 10).

TRH receptors were expressed after injection of total cytosolic GH_3 pituitary tumor cell RNA (150–200 ng/oocyte) 48 h before experiment (8, 10).

All experiments were performed in ND96 medium and oocytes were maintained in NDE96 medium (4). ACh and TRH were from Sigma Chemical Co. (Saint Louis, MO) and $^{45}CaCl_2$ from Amersham (Aylesbury, UK). All other chemicals were of analytical grade. All experiments were performed several times on oocytes from different donors.

RESULTS

Oocytes that displayed characteristic membrane electrical responses to ACh were injected with GH_3 cell RNA. After 48 h, the oocyte preserved its intrinsic response to ACh (Fig. 1 *A*) and acquired a response to TRH (Fig. 1 *B*). Simultaneous exposure to both agonists resulted in a superimposition of the two responses (Fig. 1 *C*), which preserved their characteristic latencies (1.1 ± 0.5 s for ACh and 14.0 ± 1.2 s for TRH, $n = 7$) (11), amplitudes, and patterns. These results imply that responses to ACh and TRH are evoked independently in a single oocyte.

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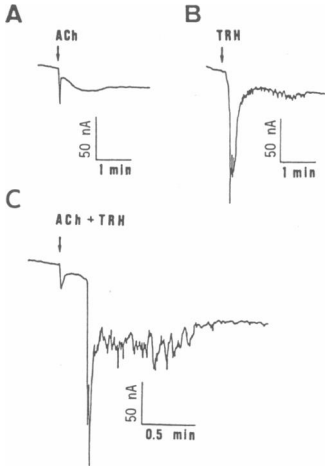

FIGURE 1 Independent responses to ACh and TRH in oocytes. (*A*) Intrinsic response to 0.1 mM ACh; (*B*) acquired response to 0.1 μM TRH; (*C*) response to simultaneous application of 0.1 mM ACh and 0.1 μM TRH. Arrows denote the beginning of exposure to agonists. Similar results were obtained with 1 μM TRH (see also Fig. 2). The concentration of TRH was reduced to allow recording of both responses with the same sensitivity and the chart speed increased to more clearly separate the early rapid response to ACh. Agonists were applied for 1 min. This experiment was repeated more than 15 times in oocytes of 10 donors.

Oocytes were injected with 150 ng total GH_3 cell RNA, maintained in NDE96, and responses were recorded 48 h later. Oocytes were clamped at −100 mV. Downward deflection of the tracing denotes a depolarizing current.

Characteristic ^{45}Ca efflux profiles (Fig. 2, *A* and *B*, *upper panel*) were associated with membrane electrical responses to ACh or TRH (Fig. 2, *A* and *B*, *lower panel*). In oocytes injected with GH_3 cell mRNA, ACh caused a moderate increase in ^{45}Ca efflux (2.0 ± 0.5% of total label, $n = 5$), which was similar to the ACh-induced efflux in uninjected controls in this series of experiments (2.6 ± 0.5% of total label, $n = 5$). These values were similar to the values reported by us previously for uninjected oocytes in response to ACh (5). As previously reported (10), the markedly greater membrane electrical response to TRH was associated with a large, prolonged increase in ^{45}Ca efflux. In this series of experiments, the TRH-stimulated increase efflux was 34.4 ± 2.8% of total label ($n = 13$). Stimulus-evoked membrane electrical responses and ^{45}Ca efflux were measured simultaneously in a single oocyte exposed first to TRH and then to ACh. ACh caused a full membrane electrical response (Fig. 2 *C*, *lower panel*) within the period of enhanced ^{45}Ca efflux evoked by TRH (Fig. 2 *C*, *upper panel*). Hence, the continuous mobilization of cellular Ca^{2+} caused by TRH did not interfere with the response to ACh.

These data were consistent with the idea that ACh and TRH mobilize Ca^{2+} from separate stores in the oocyte. To more directly demonstrate the existence of separate stores, ^{45}Ca-prelabeled oocytes were incubated in medium containing nonradioactive $CaCl_2$ (2 mM) and were repeatedly challenged with ACh at 30 min intervals. The second and third membrane electrical responses to ACh were similar in magnitude to the first response (Fig. 3, *A–C*, *lower panel*) whereas the efflux of ^{45}Ca associated with each consecutive response was progressively smaller (Fig. 3, *A–C*, *upper panel*). The preservation of an undiminished membrane electrical response to repeated challenges with ACh is consistent with refilling of the ACh-releasable store with calcium from the medium. This accounts as well for the decrease in ^{45}Ca efflux due to dilution of specific ^{45}Ca radioactivity with nonradioactive calcium. Because the third response to ACh was associated with ^{45}Ca efflux that was only 21 ± 3% ($n = 11$, $N = 4$) of that evoked during the first response, the specific radioactivity of the ACh-sensitive store was apparently diluted five-fold. Subsequent challenge with TRH caused a membrane electrical response that was similar to control responses obtained in oocytes not previously exposed to ACh (Fig. 3 *D*, *lower panel*). This response to TRH was associated with efflux of ^{45}Ca (32.5 ± 3.3% of total label) that was apparently unchanged when compared with matched controls not exposed to ACh (28.8 ± 2.7% of total label, $n = 7$, $N = 2$). This suggests that the specific radioactivity of calcium in the TRH-responsive store was not diluted at all by previous exposures to ACh. These data are consistent with ACh and TRH utilizing separate stores of cellular calcium.

To directly demonstrate that the ACh- and TRH-responsive calcium stores were distinct, the store responsive to ACh was specifically depleted by repeated stimulation with ACh in Ca^{2+}-free medium. The response to ACh virtually disappeared after the third challenge with the agonist (Fig. 4, *A–C*). Nevertheless, subsequent addition of TRH evoked a full membrane electrical response (Fig. 4 *D*). The amplitude of this response (2,177 ± 490 nA, $n = 11$, $N = 4$) was similar to the amplitude of responses to TRH obtained in oocytes of the same batches not exposed previously to ACh (1519 ± 166 nA, $n = 29$, $N = 4$). Thereafter, readdition of $CaCl_2$ to the medium was sufficient to completely restore the response to ACh (Fig. 4 *E*), thereby showing that the abolition of the ACh response was due to calcium depletion. Hence, depletion of the ACh-responsive store to a level that abolished the membrane electrical response to ACh had little effect on the response to TRH.

DISCUSSION

In mRNA-injected *Xenopus* oocytes, two responses that result from activation of distinct cell membrane receptors

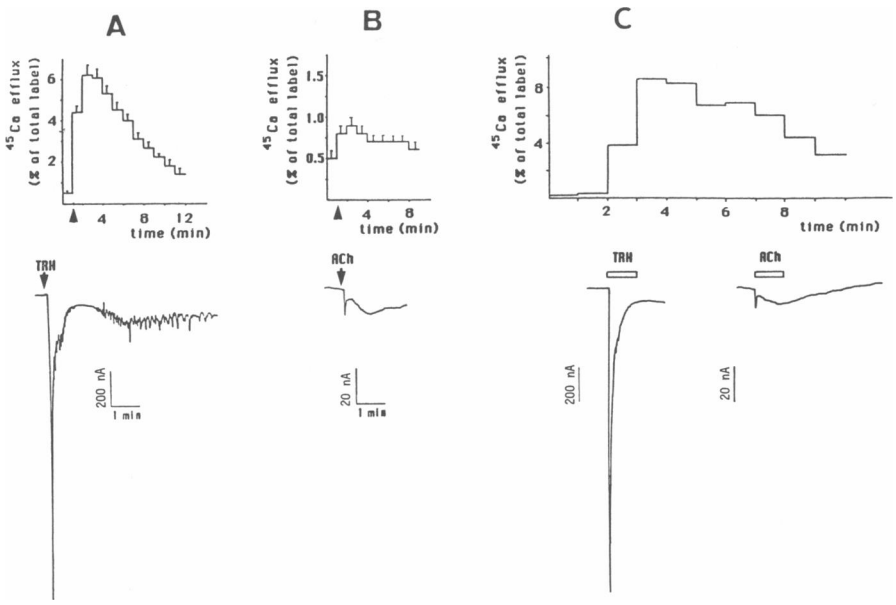

FIGURE 2 Characteristic profiles of ^{45}Ca efflux associated with membrane electrical responses to 1 μM TRH or 0.1 mM ACh. (*A*) Mean ± SEM ^{45}Ca efflux of 13 oocytes challenged with TRH for 1 min (*upper panel*). Representative response to TRH (*lower panel*); (*B*) mean ± SEM ^{45}Ca efflux of 5 oocytes challenged with ACh for 1 min (*upper panel*). Representative response to ACh (*lower panel*). Arrows mark the beginning of the exposure to agonists. (*C*) Representative simultaneous recording of membrane electrical responses and ^{45}Ca efflux caused by TRH and ACh in the same cell. (*Upper panel*) ^{45}Ca efflux. (*Lower panel*) Response to 1 μM TRH followed 5 min later by a response to 0.1 mM ACh in an oocyte clamped at −100 mV. Bars denote the duration of the exposure to agonists. This experiment was repeated in eight oocytes of four different donors with identical results.

Oocytes were injected with 150 ng RNA and assayed 48 h after injection of RNA and 18 h after addition to the medium of $^{45}CaCl_2$ (0.2 mCi/ml). Each oocyte was impaled with two conventional electrodes for voltage clamp measurements and intermittently perifused with 0.5 ml ND medium containing 2 mM $CaCl_2$ at 1 min intervals. The cell was homogenized at the end of the experiment to determine total ^{45}Ca (4,170 cpm).

are shown to be independent even though they are mediated by the same signal transduction pathway. Specifically, we have shown that independent responses to ACh and TRH are caused by mobilization of Ca^{2+} from discrete cellular stores. Although compartmentalization of cellular calcium stores observed in mRNA-injected *Xenopus* oocytes could have been peculiar to giant cells, studies in several mammalian cell populations have shown that responses to two different agonists that act via the PIP_2—IP_3—Ca^{2+} cascade appear to be independent also. For example, ACh and TRH act independently and additively to stimulate PIP_2 hydrolysis, IP_3 formation, and thyroid-stimulating hormone secretion in mouse pituitary TtT tumor cells (12). Similarly, Hughes et al. (13) described sequential, apparently independent mobilization of Ca^{2+} by methacholine and substance P, two signals that mobilize Ca^{2+} via the IP_3 pathway, in rat parotid acinar cells. Although these observations are consistent with compartmentalization of elements of signal transduction pathways in mammalian cells and localized elevation of calcium within the cytoplasm (14), they are not conclusive because individual cells within an apparently homogeneous cell population may exhibit different responses to external stimuli. By contrast, interpretation of our findings in single *Xenopus* oocytes is not complicated by potential variability of responses in individual cells in a population. Hence, this is the first demonstration of independent responses mediated by the PIP_2—IP_3—Ca^{2+} pathway in a single cell.

The morphological features of the cell that may endow it with such functional compartmentalization are unclear. We have previously reported that much greater responses to TRH had been obtained at the animal hemisphere of the oocyte, in contrast to opposite and less pronounced asymmetry for the native responses to ACh (11). Similarly, we have shown that IP_3-evoked responses appear to be five times greater at the animal hemisphere (15). A simple model would assume that the cell is grossly compartmentalized into animal and vegetable hemispheric compartments. However, experiments conducted according to the protocol described in Fig. 4, but including exposure of only the animal hemisphere to the hormones, yielded identical results (Shapira and Oron, unpublished results). These findings imply that the compartments associated with different receptors coexist in the same hemisphere and probably span dimensions smaller than

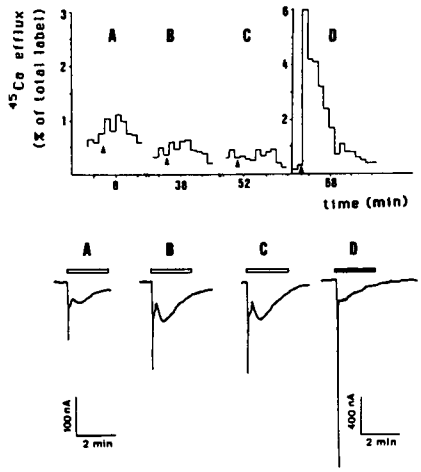

FIGURE 3 Simultaneous recording of ^{45}Ca efflux and membrane electrical responses in an oocyte repeatedly challenged with 0.1 mM ACh and then with 1 μM TRH. The oocyte was incubated in ND medium containing nonradioactive Ca^{2+} (2 mM) and exposed three consecutive times to 0.1 mM ACh at 30 min intervals (*A–C*) and then to 1 μM TRH (*D*). ^{45}Ca efflux (*upper panel*, arrows indicate the addition of agonists for the duration of 2 min) and membrane electrical responses (*lower panel*, bars indicate the duration of exposure to agonists) were determined in an oocyte clamped at −80 mV as described in the legend of Fig. 2. Due to the much longer duration of ^{45}Ca efflux, different time scales were used for upper and lower panels. Also, because of the disparity in the magnitudes of the responses, different calibration was used for ACh and TRH responses. This experiment was repeated 11 times in oocytes of four donors, yielding essentially identical results.

Oocyte injected with 150 ng RNA was incubated for 48 h in NDE96 medium. During the last 18 h before the experiment the oocyte was incubated with $^{45}CaCl_2$. Total oocyte ^{45}Ca was 14,500 cpm.

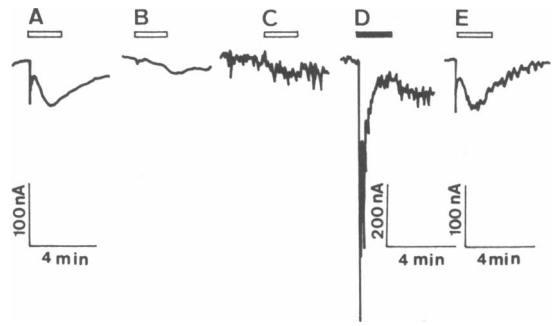

FIGURE 4 Depletion of ACh-responsive Ca^{2+} store in a single RNA-injected (150 ng) oocyte. The cell was clamped at −80 mV and incubated in Ca^{2+}-free ND (+ 0.1 mM EGTA) and challenged three consecutive times with 0.1 mM ACh at 30 min intervals (*A–C, open bars*) and then with 1 μM TRH (*D, solid bar*). It was reassayed for a response to ACh 8 min after the addition of $CaCl_2$ (2 mM) to the medium (*E, open bar*). Current fluctuations seen in panel *C* often develop after prolonged Ca^{2+} depletion. Note the different scales for ACh and TRH responses. This experiment was repeated 11 times in oocytes of four different donors.

the cell itself. We are currently looking for additional characteristics of these compartments.

Compartmentalization of elements of the signal transduction pathway may represent an important mode of regulation of physiologic responses to different stimuli that use the same second messengers.

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