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Functional expression of Ca^{2+}-mobilizing α-thrombin receptors in mRNA-injected *Xenopus* oocytes

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α-Thrombin (TH) initiates a program of intracellular events that lead to DNA replication in quiescent CCL39 Chinese hamster lung fibroblasts via membrane receptors that have yet to be characterized at a molecular level. Functional TH receptors were expressed in *Xenopus laevis* oocytes following injection of poly(A)$^+$ RNA from TH-responsive CCL39 cells; their presence was demonstrated by TH-stimulated $^{45}Ca^{2+}$ efflux or Ca^{2+}-dependent Cl^- channel activation. In voltage clamp experiments on microinjected oocytes a Ca^{2+}-activated Cl^- current was detected in response to TH (0.2–10 U/ml). The TH response was blocked by a specific TH inhibitor, and potentiated by addition of FGF or intracellular injection of GTP-γ-S.

α-Thrombin receptor; Ca^{2+} mobilization; Ca^{2+}-activated Cl^- channel; G-protein coupled receptor; (*Xenopus* oocyte)

1. INTRODUCTION

Over the past several years studies from our laboratory have focused on analyzing the signal-transducing and effector mechanisms involved in growth factor activation of cell division using a line of Chinese hamster lung fibroblasts, CCL39, as a model system. The serine protease TH is a potent mitogen for CCL39 cells and several post-receptor events stimulated by TH have been identified and characterized in detail, including Na^+/H^+ exchange and intracellular pH changes [1,2], phosphoinositide hydrolysis [3], protein phosphorylation [4,5], gene activation [6] and adenylate cyclase inhibition [7]. To date however, little is known about the putative TH receptor(s) involved in mediating these events as well as those triggering platelet aggregation. We previously identified TH binding sites on the surface of CCL39 cells; however, these sites were found not to be responsible for transmitting TH's mitogenic effect [8].

To study functional TH receptors, and as a first step towards their isolation, we have attempted to express CCL39 TH receptors in a heterologous system, the *Xenopus* oocyte. This system has proven useful in the characterization of various neuropeptide and hormone receptors (for reviews see [9–11] and their molecular cloning, as in the case of 5HT type 1C [12,13] and substance K [14] receptors. Here we demonstrate for the first time that microinjection of CCL39 cell poly(A)$^+$ RNA leads to expression of TH receptors that are coupled, as expected for a bona fide TH receptor, via a G-protein-mediated mechanisms, to intracellular Ca^{2+}-mobilization and Ca^{2+}-dependent Cl^- channel activation.

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Abbreviations: CCH, carbachol; EGTA, (ethylenebis(oxyethylenenitrilo)) tetraacetic acid; FGF, fibroblast growth factor; G-protein, GTP-binding regulatory protein; GTP-γ-S, guanosine 5′-O-(3-thiotriphosphate); Hepes, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid; 5HT, 5-hydroxytryptamine; IP_3, D-*myo*-inositol 1,4,5-triphosphate; MBS, modified Barth's solution; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; TH, α-thrombin

2. MATERIALS AND METHODS

2.1. *Materials*

Purified TH (3209 NIH units/mg) was generously provided by Dr. J.W. Fenton II (New York State Department of Health, Albany, NY). Recombinant basic FGF was a gift from Dr. D. Gospodarowicz (University of California, Medical Center, San Francisco, CA). 5HT and CCH were purchased from Sigma, PPACK from Calbiochem, and GTP-γ-S (tetralithium salt) from Boehringer-Mannheim.

2.2. *RNA preparation*

Total RNA was extracted from CCL39 Chinese hamster lung fibroblasts (ATCC) by the guanidium thiocyanate/cesium chloride gradient method of Chirgwin et al. [15] and poly (A)$^+$-selected by oligo d(T)-cellulose affinity chromatography. Fractionation of poly(A)$^+$ RNA was achieved by sucrose density gradient centrifugation (10–30%) for 16 h at 4°C. RNA for microninjection was resuspended in filtered diethylpyrocarbonate-treated H_2O at a concentration of 2mg/ml for total poly (A)$^+$ and 1 mg/ml for fractionated RNA.

2.3. *Microinjection*

Ovarian fragments were surgically removed from adult female *Xenopus leavis* (C.R.B.M., Montpellier, France) and follicular cells

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surrounding the oocytes were removed either by manual dissection (to retain the endogenous muscarinic response) or by treatment (2 h) at room temperature with 2 mg/ml collagenase (Type IA) and 0.4 mg/ml soybean trypsin inhibitor (both from Sigma) in MBS (in mM: 88.0 NaCl, 1.0 KCl, 2.4 $NaHCO_3$, 15.0 Hepes pH 7.6, 0.3 $Ca(NO3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$) containing penicillin (50 units/ml) and streptomycin (50 μg/ml). Dumont stage V and VI oocytes were microinjected with approximately 50 nl RNA solution.

2.4. *$^{45}Ca^{2+}$-efflux assay*

To measure stimulation of $^{45}Ca^{2+}$ efflux by Ca^{2+}-mobilizing agents, oocytes were incubated for 2 to 6 h at 18°C in MBS containing 50 μCi/ml $^{45}Ca^{2+}$ (Amersham France). Following $^{45}Ca^{2+}$-loading oocytes were rinsed thouroughly and placed in individual perfusion chambers. Eluate was collected for determination of radioactivity at the indicated times.

2.5. *Electrophysiology*

Voltage clamp experiments were performed with a standard 2-electrode voltage clamp amplifier in the virtual ground mode. Whole cell currents were recorded on single oocytes held in a 1-ml perfusion chamber at room temperature in ND96 solution (in mM: 96.0 NaCl, 2.0 KCl, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 5.0 Hepes pH 7.4). Microelectrodes (Jencons Scientific) were filled with 3 M KCl and had resistances between 0.2 and 2 MΩ. Holding potential for the responses to agonists was -80 mV; the reversal potential was determined by the ramp method. Computerized stimulation of the preparation, data acquisition and analyses were performed using the pCLAMP software (AXON Instruments, CA). Bath application of agonists were performed (20 μl of a 50× stock solution) while the perfusion was stopped for the indicated time. For intracellular injections of GTP-γ-S, a third micropipet was inserted in the oocyte.

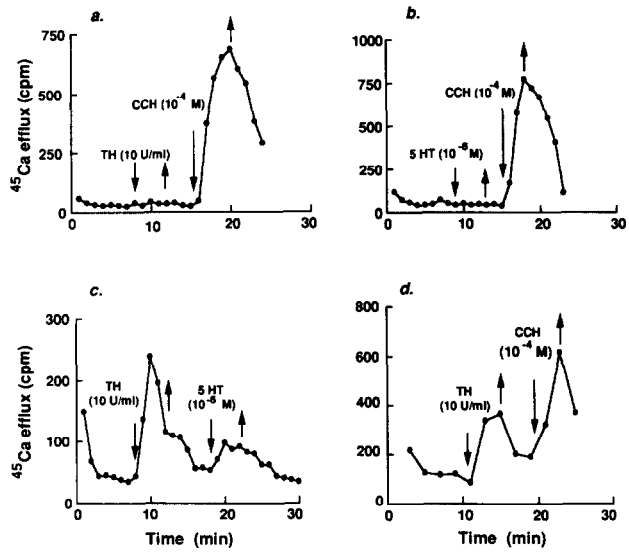

Fig.1. $^{45}Ca^{2+}$ efflux stimulated by Ca^{2+}-mobilizing agonists. $^{45}Ca^{2+}$ efflux was determined on single, manually defolliculated oocytes either non-injected (a and b) or microinjected with CCL39 cell poly $(A)^+$ RNA 3 or 4 days prior to assay (c and d, respectively). TH (10 U/ml), CCH (10^{-4} M), or 5HT (10^{-6} M) were added and removed where indicated by arrows. Oocytes shown in experiments a–c were continuously perfused with MBS and eluate was collected at 30 s intervals whereas in d, incubation medium (100 μl MBS plus 200 μl wash) was collected every 2 min.

3. RESULTS

3.1. *TH-stimulated Ca^{2+} efflux*

In order to study expression of exogeneous receptors induced by mRNA injection, outer follicular layers must be removed from oocytes either by manual dissection or by treatment with collagenase. In our initial $^{45}Ca^{2+}$-efflux experiments, collagenase treatment of oocytes was avoided in order to conserve the endogenous muscarinic response. However, under these conditions we occasionally encountered donors whose oocytes had detectable responses to elevated TH concentrations; subsequently, we found that ≥15 min of collagenase treatment abolished endogenous TH responsiveness. In each oocyte preparation the absence of endogenous TH receptors was verified prior to RNA microinjection. An experiment using manually defolliculated oocytes is shown in fig.1a,b, in which addition of TH, or 5HT, to $^{45}Ca^{2+}$-loaded oocytes had no effect on $^{45}Ca^{2+}$ release, whereas the muscarinic agonist CCH elicited a rapid and transient efflux of $^{45}Ca^{2+}$. The effect of CCH on release of $^{45}Ca^{2+}$ from pre-loaded oocytes was similar to that previously described [16–18] and is mediated by an endogenous muscarinic receptor coupled via a GTP-binding protein to inositol triphosphate production which in turn induces Ca^{2+} mobilization from intracellular stores. In oocytes injected with poly$(A)^+$ RNA isolated from CCL39 cells, TH induced an increase in $^{45}Ca^{2+}$ efflux (fig.1c,d). Co-expression of the CCl39 5HT2 receptor could also be detected (fig.1c), in agreement with the presence of 5HT type 2 receptors coupled to phospholipase C activation in CCL39 cells [19]. Following TH stimulation, oocytes remained responsive to a second agonist, such as CCH or 5HT (fig.1c,d); however, further stimulation of $^{45}Ca^{2+}$ efflux was not possible due to limited amounts of intracellular $^{45}Ca^{2+}$ in the exchangeable pool. Increase in TH-induced $^{45}Ca^{2+}$ efflux in microinjected oocytes (ratio of cpm in the first TH-stimulated fraction to cpm in the preceding fraction) varied among oocytes and from donor to donor, but routinely ranged from 1.2 to 12.5 fold (mean = 2.73 ± 0.36 (SE); n = 56). Expression could be detected by 24 h but was maximal after 2 to 3 days. Hirudin, a specific TH inhibitor, completely blocked the effect of TH (n = 4; not shown).

To enrich injected RNA in TH receptor message, and estimate the size of the latter, total CCL39 cell poly$(A)^+$ RNA was fractionated by sucrose gradient centrifugation and individual fractions were tested for TH receptor activity in the $^{45}Ca^{2+}$ efflux assay following 2–3 days of expression. TH receptor mRNA was present in sucrose gradient fractions >3 kilobases (not shown).

3.2. *Effect of TH on Cl^- channel activity in microinjected oocytes*

Since stimulation of a number of Ca^{2+}-mobilizing neurotransmitter and hormone receptors expressed in oocytes have been shown to evoke oscillatory Cl^- cur-

rents via Ca^{2+}-activated Cl^- channels, we examined the effect of TH on Cl^- channel activity in oocytes microinjected with CCL39 RNA. Detection of receptor expression by this method has several advantages over the $^{45}Ca^{2+}$-efflux assay including increased sensitivity, immediate visualization of the results and furthermore, the same oocyte can be used repeatedly which allows for more detailed characterization of receptor responses (e.g. desensitization, inhibition, synergy) without exhausting exchangeable stores of intracellular $^{45}Ca^{2+}$.

Indeed, TH stimulated an inward current in voltage-clamped oocytes, as indicated by the downward deflection of the trace (fig.2). Oocytes used for voltage clamp experiments in the present study were defolliculated by collagenase treatment, therefore, endogeneous muscarinic and, more importantly, TH receptors could not be detected in non-injected oocytes (n = 12) from each donor (n = 3; not shown). In oocytes voltage clamped at −80 mV, the reversal potential for the TH response was found to be −25 mV which corresponds to the equilibrium potential for Cl^- ions in native oocytes [10]. In oocytes microinjected with total CCL39 cell poly(A)$^+$ RNA (fig.2a,b) the threshold for the TH response was observed at TH concentrations of 5–10 U/ml; this response was specific for TH since addition of the TH inhibitor, PPACK, to the bath reversed the response (fig.2b). Injection of sucrose gradient-fractionated RNA (>3 kb) increased the sensitivity and the magnitude of the TH response; 10-fold lower TH concentrations were required to evoke Cl^- currents and the amplitude of the peak deflection height was generally greater (fig.2c,d). We found that oocytes were refractory to repeated stimulation by TH yet they remained responsive to 5HT following TH treatment. Such homologous desensitization has been observed for TH activation of phospholipase C in intact CCL39 fibroblasts [20].

3.3. *Effect of FGF and GTP-γS on TH-stimulated Cl^- channel activity*

FGF and other tyrosine kinase-activating growth factors have been shown to potentiate the TH-induced breakdown of phosphoinositides in intact CCL39 cells [21]. When added to oocytes previously microinjected with CCL39 mRNA FGF had no effect on its own (fig.3, upper trace), but it markedly potentiated Cl^- channel activity stimulated by TH. As can be seen in fig.3, FGF appeared to prolong the oscillatory component of the TH response, when added either before or after TH. The response to 5HT in CCL39 RNA-injected oocytes was similarly amplified by FGF (not shown).

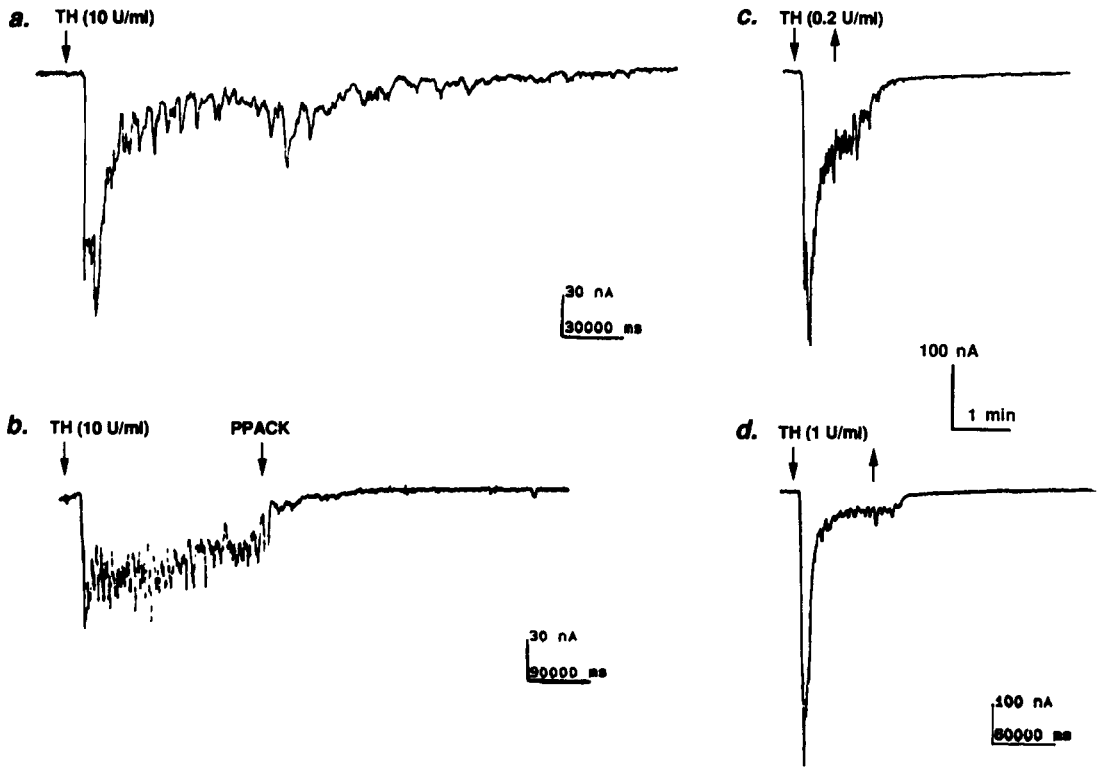

Fig.2. TH stimulation of Ca^{2+}-dependent Cl^- channel activity. Experiments were performed on oocytes from the same donor voltage clamped at −80 mV, 2 days after microinjection of total poly (A)$^+$ RNA (a and b) or sucrose gradient-fractionated poly (A)+ RNA (c and d) from CCL39 cells. Bath applications of TH and PPACK (50-fold molar excess) and their removal (start of perfusion) are indicated by arrows. Final TH concentrations in the 1 ml bath were 10 U/ml (a and b), 0.2 U/ml (c) and 1 U/ml (d)).

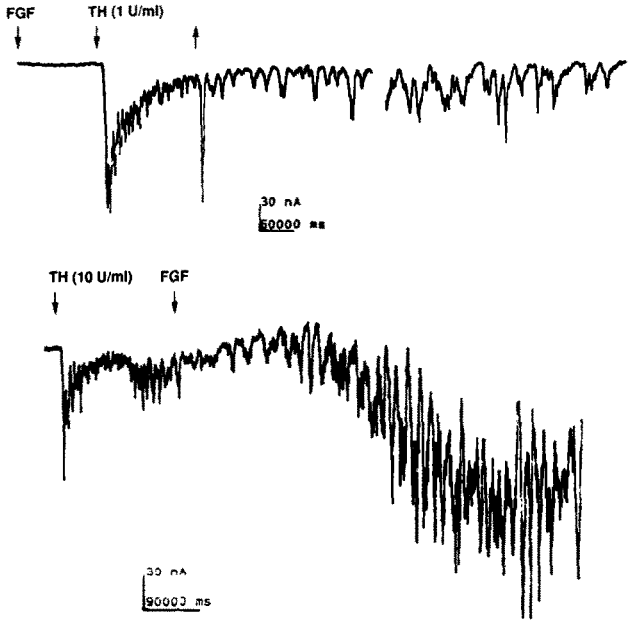

Fig.3. FGF effect on TH-stimulated Cl^- channel activity. Oocytes from different donors were microinjected with sucrose gradient fraction B RNA (upper trace), or total poly $(A)^+$ RNA from CCL39 cells (lower trace). Bath applications of FGF and TH (final concentrations of 125 ng/ml and 1 U/ml, respectively) and their removal (start of perfusion) are indicated by arrows.

In an attempt to demonstrate the involvement of a GTP-binding protein in coupling of the expressed TH receptor to subsequent post-receptor steps leading to Cl^- channel activation we injected the non-hydrolyzable GTP analog, GTP-γ-S, into oocytes prior to TH

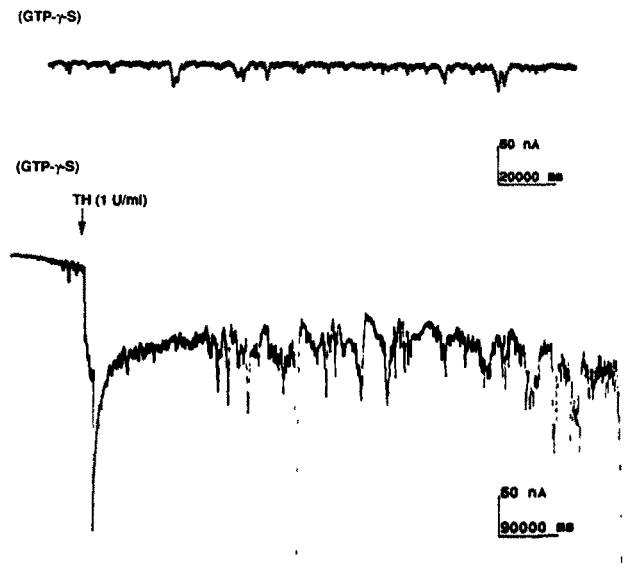

Fig.4. Effect of GTP-γ-S on TH-stimulated Cl^- currents. Oocytes from the same donor were microinjected with sucrose gradient fraction B RNA from CCL39 cells. Upper trace: Current fluctutations immediately following intracellular injection of GTP-γ-S (50 nl; 1 mM solution). Bottom trace: TH response with no prior nucleotide injection. Final bath concentration of TH was 1 U/ml.

addition. Injection of 50 nl of 1 mM GTP-γ-S (resulting in a final intracellular concentration of 50 μM assuming homogeneous distribution in a cytoplasmic volume of 1 μl) induced persistent inward current fluctuations as pictured in fig.4 (upper, trace). Addition of TH to GTP-γ-S-injected oocytes expressing TH receptor evoked an inward current with a relatively unchanged rapid transient component but with a potentiated delayed fluctuating component (fig.4, lower trace). The effect of GTP-γ-S injection on the 5HT response was once again similar to that oberserved on the TH response indicating a similar mechanism of action for the two agonist receptors (not shown).

4. DISCUSSION

TH, as a growth factor, is unique in that its proteolytic activity is required for mitogenic stimulation of cells. Although TH binds to platelets and cells with high affinity [8,22,23] the concept of a classical receptor, for which ligand occupancy triggers a biological response, is questionable since active site-blocked TH analogs antagonize binding but they do not antagonize the mitogenic action of TH [8]. Therefore, we have set out to identify a functional TH receptor and not TH binding sites on CCL39 fibroblasts. To our knowledge, the present study represents the first demonstration that TH responsiveness resides in a protein, whose messenger RNA(s) can be expressed in a heterologous cell system. This finding provides an approach for functional cDNA cloning of the receptor. The function that we have chosen to assay is Ca^{2+} mobilization which activates Ca^{2+}-dependent Cl^- channels; events leading to Ca^{2+} mobilization in the *Xenopus* oocyte have been clearly established for other agonists and include IP_3 formation following receptor activation of phospholipase C via a pertussis toxin-sensitive G-protein [16,17,24–26]. In light of the recent molecular evidence for the existence of a number of G-protein-coupled receptor families (e.g. α- and β-adrenergic, 5HT, muscarinic) [27,28] it has to be considered that more than one TH receptor may be involved in eliciting the complex biological actions of TH. Here we demonstrated expression of the TH receptor coupled to phospholipase C activation, which may not be the receptor involved in adenylate cyclase inhibition. Further studies will be necessary to resolve this issue.

The TH receptor we describe here was determined to be exogenous since its expression was dependent upon the injection of RNA. Control non-injected, or water-injected oocytes were nonresponsive to TH, immediately following collagenase treatment, or at the time of TH receptor assays. In our hands collagenase treatment also abolished the endogenous response to CCH.

The persistent current fluctuations induced by FGF in TH- or 5HT-treated microinjected oocytes resembled the large inward current with irregular delay and

amplitude (F-component) of the oocyte muscarinic response, previously described in detail [10,29]. Although the molecular mechanism underlying the F-component is not known, it has been shown to be carried by Cl^- ions and Ca^{2+}-dependent (inhibited by intracellular EGTA injection) [16].

The effect of FGF on TH-induced Cl^- channel activity in oocytes is similar to the potentiating effect of FGF and other growth factors with tyrosine kinase receptors on inositol phosphate formation in CCL39 cells [21]. The FGF receptor has recently been shown to possess tyrosine kinase activity [30] and, for fibroblasts it was proposed that the activated receptor tyrosine kinases exert their effect by inducing phosphorylation of some component of the phosphoinositide pathway, either directly or indirectly. The origin of the expressed FGF receptor in microinjected oocytes is unknown, either the hamster TH and FGF receptor mRNA's were co-injected (i.e. colocalized in mRNA preparations) or oocytes express endogenous FGF receptors resistant to collagenase treatment. The latter possibility is likely since *Xenopus* oocytes contain high levels of mRNA encoding a protein highly homologous to basic FGF [31].

Our results concerning the enhancing effect of injected effect of injected GTP-γ-S on TH stimulation of Cl^- channel activity confirm the involvement of a G-protein in mediation of the TH-induced response in oocytes in agreement with results obtained in mammalian cells [32,33]. Injection of GTP-γ-S into *Xenopus* oocytes has previously been shown to evoke a long-lasting inward current response [25,34] that has been shown by Kaneko et al. to involve Cl^- channels identical to the IP_3-stimulated or receptor-mediated transient current responses.

In conclusion, the results described here indicate that the *Xenopus* oocyte is a promising system for further characterization of functional TH receptors from different species and anatomical sources as well as for molecular cloning. We are currently pursuing these direction of investigation.

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