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Activation of Inositol 1,4,5-Trisphosphate Receptor Is Essential for the Opening of Mouse TRP5 Channels

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ABSTRACT

We studied the opening mechanism of Ca^{2+}-permeable channels formed with mouse transient receptor potential type 5 (mTRP5) using *Xenopus* oocytes. After stimulation of coexpressed muscarinic M_1 receptors with acetylcholine (ACh) in a Ca^{2+}-free solution, switching to 2 mM Ca^{2+}-containing solution evoked a large Cl^- current, which reflects the opening of endogenous Ca^{2+}-dependent Cl^- channels following Ca^{2+} entry through the expressed channels. The ACh-evoked response was not affected by a depletion of Ca^{2+} store with thapsigargin but was inhibited by preinjection of antisense oligodeoxynucleotides (ODNs) to G_q, G_{11}, or both. The mTRP5 channel response was also induced by a direct activation of G proteins with injection of guanosine 5′-3-*O*-(thio)triphosphate (GTPγS). The ACh- and GTPγS-evoked responses were inhibited by either pretreatment with a phospholipase C inhibitor, U73122, or an inositol-1,4,5-trisphosphate (IP_3) receptor inhibitor, xestospongin C (XeC). An activation of IP_3 receptors with injection of adenophostin A (AdA) evoked the mTRP5 channel response in a dose-dependent manner. The AdA-evoked response was not suppressed by preinjection of antisense ODNs to $G_{q/11}$ or U73122 but was suppressed by either preinjection of XeC or a peptide mimicking the IP_3 binding domain of *Xenopus* IP_3 receptor. These findings suggest that the activation of IP_3 receptor is essential for the opening of mTRP5 channels, and that neither G proteins, phosphoinositide metabolism, nor depletion of the Ca^{2+} store directly modifies the IP_3 receptor-linked opening of mTRP5 channels.

In most mammalian cells, stimulation of G_q-couple or tyrosine kinase-type receptors with an agonist leads to the activation of phospholipase C (PLC) followed by a biphasic increase in the cytosolic Ca^{2+} concentration. The first transient phase is due to inositol 1,4,5-trisphosphate (IP_3) that causes the release of Ca^{2+} from intracellular stores, and the second late phase is caused by a sustained Ca^{2+} entry across the plasma membranes. Particularly in nonexcitable cells, the Ca^{2+} entry can be activated without receptor stimulation experimentally by depleting the intracellular Ca^{2+} stores under Ca^{2+}-free conditions or in the presence of inhibitors for endoplasmic reticulum Ca^{2+}-ATPase pump, such as thapsigargin. This is known as capacitative Ca^{2+} entry, which is mediated by store-operated nonselective cation channels or by Ca^{2+}-release-activated Ca^{2+} (CRAC) channels with a high selectivity of channel pores to Ca^{2+}. However, depending on cell types, Ca^{2+} entry is observed by activation of G_q-couple receptors even under Ca^{2+} store depletion or by diffusible second messengers, which may be generally referred to as receptor-activated Ca^{2+} channels (RACCs) (Fasolato et al., 1994).

Mammalian homologs of the *Drosophila* transient receptor potential (TRP) protein form Ca^{2+}-permeable cation channels putatively having six transmembrane segments. Since the original *Drosophila* TRP and TRP-like (TRPL) channels are involved in the light-induced signal transduction via G protein-mediated activation of PLC, the mammalian homologs have been considered as the candidates for Ca^{2+} channels activated downstream of phosphoinositide (PI) metabolism. Although previous studies have demonstrated that mammalian TRP channels are sensitive to store depletion in exogenously expressed cells, recent studies have provided growing evidence for the differences between recombinant TRP channels and native CRAC channels (Harteneck et al., 2000). Most significantly, none of the TRP channels has been shown to encode a channel with the precise ion-conduction properties or selectivity to Ca^{2+} expected of CRAC channels.

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ABBREVIATIONS: PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; ACh, acetylcholine; TRP, transient receptor potential; TRPL, TRP-like; mTRP5, mouse TRP type 5; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; ODN, oligodeoxynucleotide; XeC, xestospongin C; AdA, adenophostin A; PI, phosphoinositide; DAG, diacylglycerol; OAG, oleyl-2-acetyl-*sn*-glycerol; RACC, receptor-activated Ca^{2+} channel; FR, frog Ringer; CRAC channel, Ca^{2+}-release activated Ca^{2+} channel.

Recently, recombinant CaT1, a protein distantly related to the classical TRPs, has been shown to manifest the CRAC channel properties (Yue et al., 2001).

The activation mechanism of TRP channels remains controversial and appears not to be universal for all TRP channels. Based on phylogenetic sequence similarity, the TRP family can be classified into four subfamilies: TRP1, TRP2, TRP3/6/7, and TRP4/5. For TRP3/6/7, diacylglycerols (DAGs) have been identified as a common, membrane-anchored activator of the recombinant channels (Hofmann et al., 1999; Okada et al., 1999). In addition, the protein-protein interaction between IP_3 receptor and TRP3 (or TRP6) (Boulay et al., 1999) causes the opening of TRP3 channels by interrupting the inhibitory action of calmodulin bound to TRP3 channels (Ma et al., 2000; Zhang et al., 2001). TRP4/5 are abundantly expressed in neural tissues and colocalized in some neurons (Philipp et al., 1998). Although potentiation of store-operated Ca^{2+} entry has been observed in cells expressing TRP4 or TRP5 (Philipp et al., 1998; Kinoshita et al., 2000), recent studies have shown that TRP4/5 channels are activated after stimulation of $G_{q/11}$-couple receptors independently of Ca^{2+} store depletion (Okada et al., 1998; Schaefer et al., 2000). However, different from TRP3/6/7 channels, TRP4 and TRP5 channels are not activated by DAGs or DAG-lipase inhibitor (Hofmann et al., 1999; Schaefer et al., 2000). Thus, it remains unclear what is needed for the opening of TRP4/5 channels.

In the present study, we coexpressed mouse TRP5 (mTRP5) channels with muscarinic M_1 receptors in *Xenopus* oocytes and recorded ACh-evoked RACC responses that were clearly distinguishable from the native CRAC channel response of oocytes. We have investigated the opening and modulatory mechanism of mTRP5 channels using oocyte model in combination with antisense oligodeoxynucleotides (ODNs) to *Xenopus* $G_{q/11}$, a potent IP_3 receptor agonist adenophostin A (AdA), and several enzyme inhibitors. The present findings suggest that mTRP5 channels are opened by activation of IP_3 receptors.

Experimental Procedures

Materials. The mTRP5 cDNA fragment isolated by digesting pCI-neo-mTRP5 (Okada et al., 1998) with *Not*I and *Sal*I was blunted and inserted into the *Sma*I site of pGEMHE (Liman et al., 1992), which contains the 5′- and 3′-untranslated regions of the *Xenopus laevis* β-globin gene for yielding a high expression in *X. laevis* oocytes. The plasmid mTRP5/pGEMHE was linearized by *Sph*I, blunted, and used as the template for in vitro RNA transcription using T7 RNA polymerase. Plasmid pSPM10 carrying porcine muscarinic M_1 receptor (Fukuda et al., 1987) was linearized by *Xba*I and used as the template for in vitro RNA transcription using SP6 polymerase. Antisense ODNs to *X. laevis* G_{i1} protein (XAG_{i1}, 5′-CCCATGGCGACGGTTCTCCG-3′), G_q protein (XAG_q, 5′-GTCATGCCTCCTTGACTAGT-3′), and G_{11} protein (XAG_{11}, 5′-GTCATCCCTTCCCCCCGGCA-3′) were designed to span the start codons of the published cDNA sequences for *X. laevis* G proteins (Olate et al., 1989; Shapira et al., 1994). Peptides mimicking the IP_3 binding site (488–498, NRERQKLMREQ) of *X. laevis* IP_3 receptor (Kume et al., 1993) and the N-terminal region (2–17, GCTLSAGERAALERSK) of *X. laevis* $G\alpha_o$ protein (Olate et al., 1989) were synthesized and designated as IP_3RF and PGON1, respectively. IP_3RF and PGON1 were dissolved at 135 and 100 μM, respectively, in water and stored at −80°C. Xestospongin C (XeC; Calbiochem-Novabiochem, San Diego, CA), thapsigargin (Wako Pure Chemicals, Osaka, Japan), and 1-oleyl-2-acetyl-*sn*-glycerol (OAG; Calbiochem-Novabiochem) were dissolved in dimethylsulfoxide as stock solutions at 1, 2, and 10 mM, respectively, and stored at −20°C. U73122 and U73343 (BIOMOL Research Laboratories, Plymouth Meeting, PA) were dissolved at 10 mM in chloroform and stored at −20°C. D-*myo*-IP_3 K^+ salt (Sigma, St. Louis, MO), AdA phosphate (a gift from Dr. M. Takahashi, Sankyo Co., Tokyo, Japan), and guanosine 5′-3-*O*-(thio)triphosphate (GTPγS) Li^+ salt (Roche, Tokyo, Japan) were dissolved in water at 1, 10, and 50 mM, respectively, and stored at −80°C.

Preparation of RNA-Injected Oocytes. Small pieces of ovarian lobes were dissected out from cold-anesthetized *X. laevis* and shaken gently at 22°C for 90 min in a solution (88 mM NaCl, 1 mM KCl, 1 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 7.5 mM Tris-HCl, pH 7.6) containing 1.1 mg/ml collagenase (Wako Pure Chemicals). Defolliculated oocytes were selected and incubated overnight at 20°C in modified Barth's saline (MBS; 88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 7.5 mM Tris-HCl, pH 7.6, supplemented with 10 U/ml penicillin and 10 μg/ml streptomycin). Healthy-looking oocytes were injected in Ca^{2+}-free MBS with 50 nl of sterile solution containing mRNA for mTRP5 together with M_1 receptor mRNA (each 13.3 ng/oocyte) and incubated further for 1 to 3 days. When needed, oocytes were injected either with one of the antisense ODNs to *X. laevis* G proteins (50 ng/oocyte), synthetic peptides IP_3RF (10 ng/oocyte), or PGON1 (8 ng/oocyte) on the next day of mRNA injection.

Electrophysiological Recordings. Oocytes were voltage-clamped at a holding potential of −80 mV in Ca^{2+}-free frog Ringer (FR) solution (115 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, and 1 mM EGTA, pH 7.4, with NaOH) with two intracellular glass electrodes (1–2 MΩ with 3 M KCl) connected to an OC-725C amplifier (Warner Instrument, Hamden, CT). The current and voltage outputs were monitored using a flat pen recorder and a MacLab A/D converter with Scope software (ADInstruments Pty Ltd., Castle Hill, Australia). The I-V relationship was evaluated by applying a voltage ramping command ranging between −120 mV and + 60 mV for a duration of 1 s using the D/A output from MacLab. For the detection of Ca^{2+} influx, the perfusion line was switched for 30 s to Ca^{2+}-containing FR solution (1 mM EGTA was substituted to 2 mM $CaCl_2$). ACh was dissolved in Ca^{2+}-free FR to be 100 μM and applied similarly by perfusion. GTPγS (250 pmol) and AdA (0.5 fmol–50 pmol) were injected into oocytes during recording with another glass micropipette in 1% oocyte volume. Depletion of the intracellular Ca^{2+} store was done by pretreatment of the oocytes at room temperature with 2 μM thapsigargin in a Ca^{2+}-free FR solution for 2 h before recording. Treatments of oocytes with XeC, U73122, and U73343 were done in a Ca^{2+}-containing FR for 30 min before current recording. The amplitude of current trace was measured as the maximal deflection from the baseline to the peak of response. Values are shown as means ± S.E.M. where *n* is the number of experiments. Statistical significance was evaluated by one-way analysis of variance with post hoc tests using Prism 3.04 software (GraphPad Software, San Diego, CA).

Results

Mouse TRP5 Channels Were Opened after Stimulation of Muscarinic M_1 Receptors. In the present study, mTRP5 was coexpressed with muscarinic M_1 receptors stimulation of which with ACh evokes a G protein-mediated PI turnover in *X. laevis* oocytes (Fukuda et al., 1987). To evaluate the ACh-induced RACC-related response, we recorded the whole-cell current at a holding potential of −80 mV from the oocytes perfused with Ca^{2+}-free FR and applied 2 mM Ca^{2+}-containing FR for 30 s before and after a stimulation of coexpressed M_1 receptors with ACh (100 μM).

In the control, noninjected oocytes (Fig. 1A), no current response was evoked when recording solution was switched from Ca^{2+}-free FR to 2 mM Ca^{2+}-containing FR, or to Ca^{2+}-

free FR containing 100 μM ACh. In oocytes injected with mRNA for M_1 receptor only (Fig. 1B), application of ACh in Ca^{2+}-free solution evoked a typical transient inward current, which is known to be caused by an activation of the PLC pathway (Nomura et al., 1987). Although switching of the recording solution to Ca^{2+}-containing FR did not evoke a current response before ACh, a small Ca^{2+}-induced current was observed after ACh (0.018 $\pm$ 0.009 μA, $n = 6$), which reflects an endogenous RACC-related response. In oocytes coinjected with mRNAs for mTRP5 and M_1 receptors (Fig. 1C), the second application of Ca^{2+} after ACh evoked a large (4.81 $\pm$ 1.45 μA, $n = 6$; Fig. 1D) inward current response. The I-V relationships of the oocytes coexpressing mTRP5 and M_1 receptors was evaluated by ramping the holding potential in the presence of external Ca^{2+} before and after ACh (Fig. 1E). The reversal potential of the response (−25 mV) indicated that the Ca^{2+}-evoked response after ACh was mediated by the increase in Ca^{2+}-activated Cl^- channel current, $I_{Cl(Ca)}$, which is a characteristic of Ca^{2+} influx into *X. laevis* oocytes (Barish, 1983). Therefore, the $I_{Cl(Ca)}$ evoked by external Ca^{2+}-perfusion after stimulation of M_1 receptors with ACh was used as a reporter of Ca^{2+} influx in the following experiments.

Mouse TRP5 Channels Were Not Opened by Depletion of Ca^{2+} Stores. In several studies (Philipp et al., 1998; Kinoshita et al., 2000), TRP proteins have been shown to form Ca^{2+}-permeable channels linked to a depletion of intracellular Ca^{2+} stores with endoplasmic reticulum Ca^{2+}-ATPase inhibitors. To test whether the opening of mTRP5 channels is triggered or modified by the depletion of Ca^{2+} stores, we pretreated oocytes for 2 h in Ca^{2+}-free FR with 2 μM thapsigargin, and tested the effects on ACh-Ca^{2+}-induced $I_{Cl(Ca)}$ response.

In noninjected oocytes pretreated with thapsigargin (Fig. 2A), the first perfusion with 2 mM Ca^{2+}-containing FR evoked a small $I_{Cl(Ca)}$ response (0.062 $\pm$ 0.027 μA, $n = 6$). The intrinsic CRAC channel response was not potentiated after stimulation with ACh (0.079 $\pm$ 0.013 μA). Complete depletion of intracellular Ca^{2+} stores was confirmed by the finding that ACh evoked no response alone in oocytes expressing M_1 receptors. In oocytes coexpressing mTRP5 with M_1 receptors and treated with thapsigargin (Fig. 2B), the amplitude of the CRAC-related response (0.097 $\pm$ 0.026 μA, $n = 6$) was not significantly different from that of noninjected oocytes. However, the second exposure to 2 mM Ca^{2+} after ACh evoked a large $I_{Cl(Ca)}$, and the amplitude (4.75 $\pm$ 1.03 μA, Fig. 2C) was not different from that of thapsigargin-untreated, mTRP5-expressing cells (see Fig. 1). These findings demonstrate that activation of Ca^{2+}-permeable channels formed by mTRP5 is triggered by PLC-coupled receptor stimulation, which is not triggered or modified by depletion of intracellular Ca^{2+} stores.

Mouse TRP5 Channels Were Opened by Direct Activation of G Proteins with GTPγS. Heterotrimeric G pro-

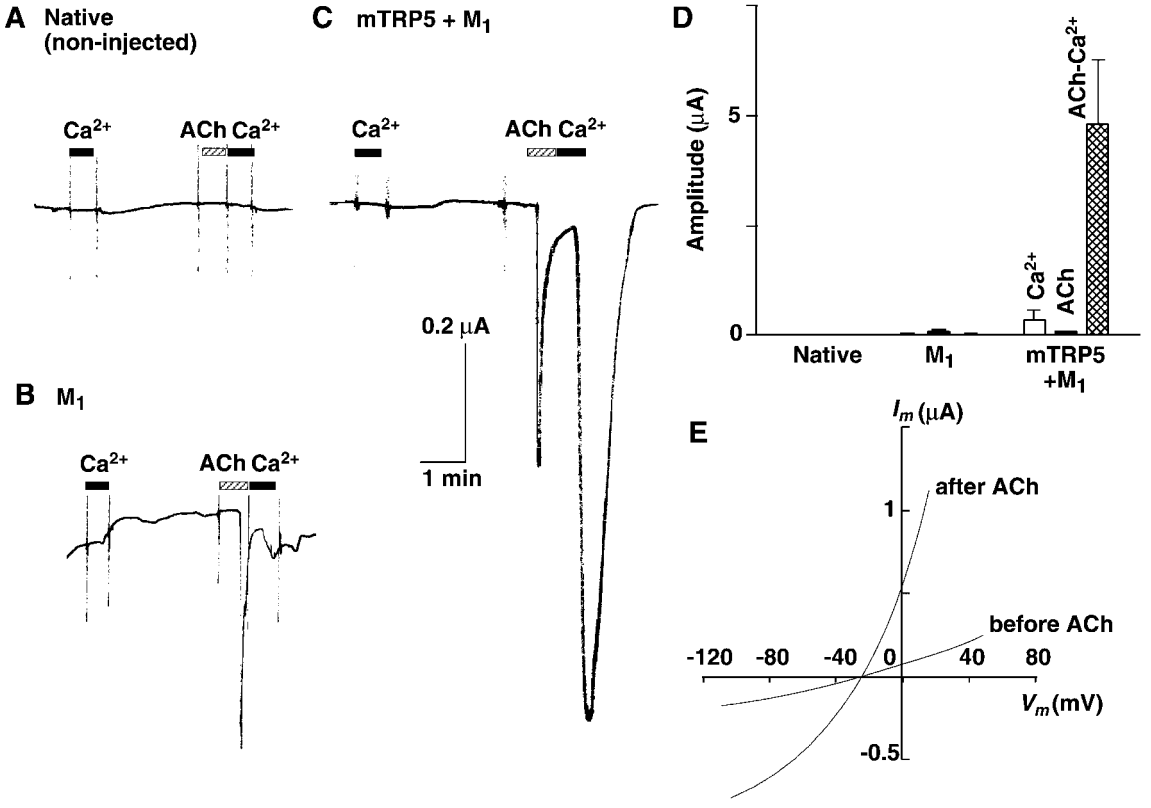

Fig. 1. Activation of mTRP5 channels after stimulation of coexpressed muscarinic M_1 receptors with ACh. Whole-cell current traces were recorded at a holding potential of −80 mV in Ca^{2+}-free FR from noninjected (A), M_1-mRNA-injected (B), and mTRP5-plus-M_1-mRNA-injected (C) oocytes. Two millimolar Ca^{2+}-containing FR (closed bar) or Ca^{2+}-free ACh solution (100 μM, hatched bar) was applied for 30 s as indicated by bars. Vertical spikes on the traces, of which outward (upward) deflections were truncated, indicate the times at which the I-V relation was examined by applying a 1-s ramping voltage command. D, average ($\pm$S.E.M., $n = 6$) amplitudes of the current responses of oocytes to the first application of Ca^{2+}-containing FR (open column), the application of 100 μM ACh in Ca^{2+}-free FR (closed column), and the subsequent application of Ca^{2+}-containing FR (cross-hatched column). E, current-voltage relationship of the responses to Ca^{2+}-containing FR before and after ACh stimulation indicating the mediation by $I_{Cl(Ca)}$. Leak-subtracted I-V traces are shown.

teins may have participated in the TRP5 channel response not only as transducers of the activation signal but also as regulators of the channel activity, because *Drosophila* TRPL channels have been shown to be activated by constitutively active $G\alpha_{11}$ protein (Obukhov et al., 1996). To evaluate the effects of direct G protein activation, we injected GTPγS, which causes irreversible activation of G proteins, and recorded the GTPγS-induced change in the $I_{Cl(Ca)}$ response to Ca^{2+} perfusion (Fig. 3).

In control oocytes (Fig. 3A), injection of GTPγS (250 pmol) did not evoke $I_{Cl(Ca)}$ responses by itself or to Ca^{2+} perfusion. In oocytes coexpressing mTRP5 and M_1 receptors (Fig. 3B), injection of the same dose of GTPγS induced the $I_{Cl(Ca)}$ response to the second Ca^{2+} perfusion (2.10 ± 0.58 μA, $n = 6$). The preloading with GTPγS did not eliminate the subsequent ACh-induced $I_{Cl(Ca)}$ to the third Ca^{2+} perfusion (4.47 ± 0.99 μA, $n = 6$; Fig. 3C). These findings suggest that mTRP5 channels are opened by direct activation of G proteins. The limited activation of mTRP5 by GTPγS may be explained by an incomplete diffusion of GTPγS within 2 min after 1% oocyte volume injection.

Antisense ODNs to *X. laevis* G_q and G_{11} Inhibited the Opening of mTRP5 Channels by GTPγS and ACh. From *X. laevis* oocytes, cDNAs for G protein α-subunits G_q, G_{11} (Shapira et al., 1994), G_{14} (Shapira et al., 1998), G_s, G_{i1}, G_{i3} (Olate et al., 1990), and G_o (Olate et al., 1989) have been cloned. In the oocytes, however, activation of PLC-β and subsequent PI turnover are known to be caused mainly by Gβγ-subunits (Stehno-Bittel et al., 1995), whereas $G\alpha_{q/11}$ family proteins determine the specificity of coupling between muscarinic receptors and the PI signaling (Berstein et al., 1992). To distinguish the roles of Gα subtypes in the transduction of the GTPγS- and ACh-evoked opening of mTRP5 channels, we synthesized 20-mer antisense ODNs to *X. laevis* G_{i1} (XAG_{i1}), G_q (XAG_q), and G_{11} (XAG_{11}), and 50 ng/oocyte antisense ODN was injected on the following day of mRNA injection to reduce the amount of a specific subtype of endogenous Gα (Kaneko et al., 1992; Shapira et al., 1998).

As shown in Fig. 4, XAG_{i1} showed no inhibitory effect on the responses to GTPγS and ACh or on the mTRP5-mediated $I_{Cl(Ca)}$ response evoked after GTPγS and ACh. However, in oocytes preloaded with XAG_q, XAG_{11}, or both at the same total amount, GTPγS or ACh evoked no current response by itself. Moreover, there was a significant reduction in the amplitudes of mTRP5-mediated $I_{Cl(Ca)}$ responses evoked after GTPγS and ACh. These findings suggest the primary role of $G_{q/11}$ in the transduction of the opening signal to mTRP5. The finding that both XAG_q and XAG_{11} were equipotent in inhibiting the mTRP5 response indicated that an insufficient signal was transduced for mTRP5 channels when the majority of either subtype of $G_{q/11}$ family proteins was knocked down.

Mouse TRP5 Channels Were Opened by Stimulation of IP_3 Receptors with Adenophostin A. AdA, a compound isolated from the culture broth of *Penicillium brevicompactum*, is the most potent known agonist for the IP_3 receptor (Takahashi et al., 1994), which causes Ca^{2+} mobilization in *X. laevis* oocytes when injected (Hartzell et al., 1997). To evaluate the contribution of IP_3 receptors in the opening of mTRP5 channels, we injected various amounts of AdA into oocytes and recorded $I_{Cl(Ca)}$ responses to the subsequent external Ca^{2+} perfusion (Fig. 5).

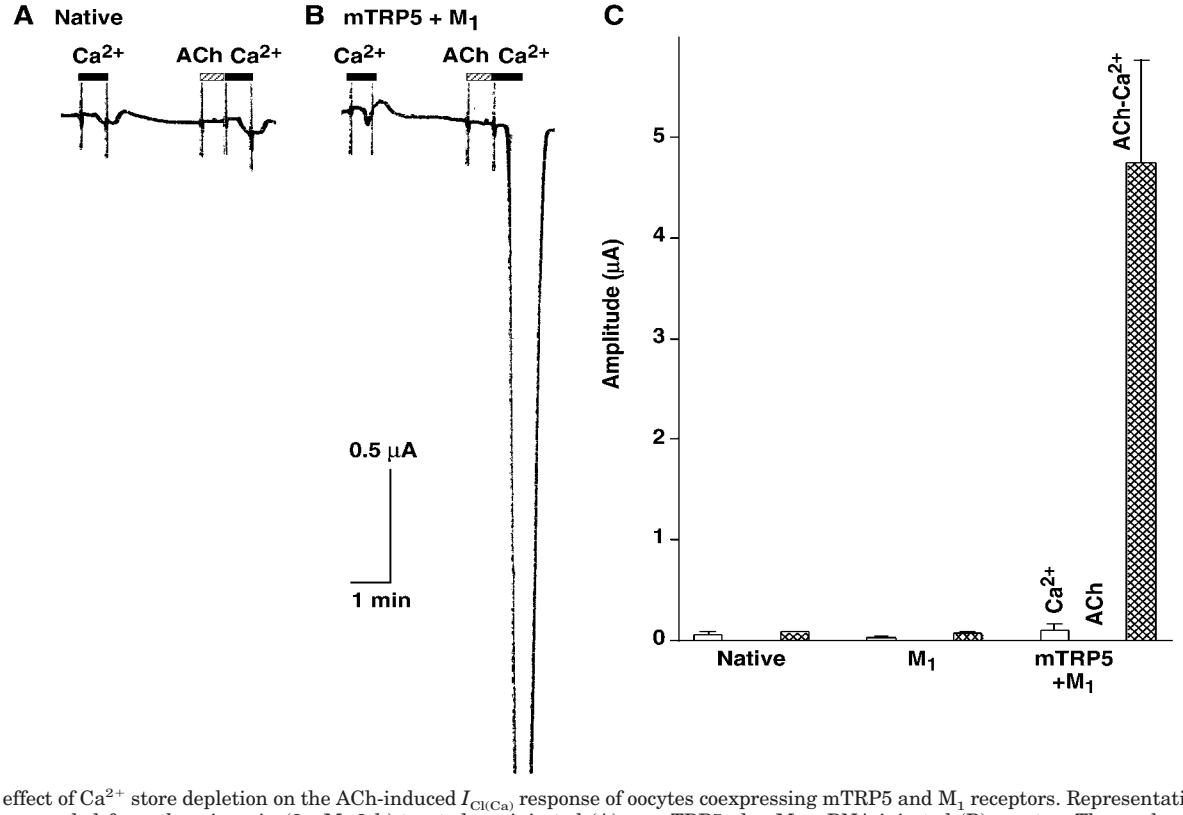

Fig. 2. No effect of Ca^{2+} store depletion on the ACh-induced $I_{Cl(Ca)}$ response of oocytes coexpressing mTRP5 and M_1 receptors. Representative current traces were recorded from thapsigargin (2 μM, 2 h)-treated noninjected (A) or mTRP5-plus-M_1-mRNA-injected (B) oocytes. The peak of the large current response of the mTRP5-expressing oocyte was truncated. C, average ($n = 6$) amplitudes of $I_{Cl(Ca)}$ evoked by exposure to external 2 mM Ca^{2+} before (open column) and after (cross-hatched column) stimulation of M_1 receptors with ACh (100 μM), which in itself evoked no $I_{Cl(Ca)}$ response.

Although previous studies have shown that a transient $I_{Cl(Ca)}$ response can be observed immediately after the injection of AdA into fresh oocytes (DeLisle et al., 1997; Hartzell et al., 1997), in the present oocytes cultured for several days with or without injection of mRNA, an intracellular application of AdA in a range from 0.5 fmol to 50 pmol per oocyte

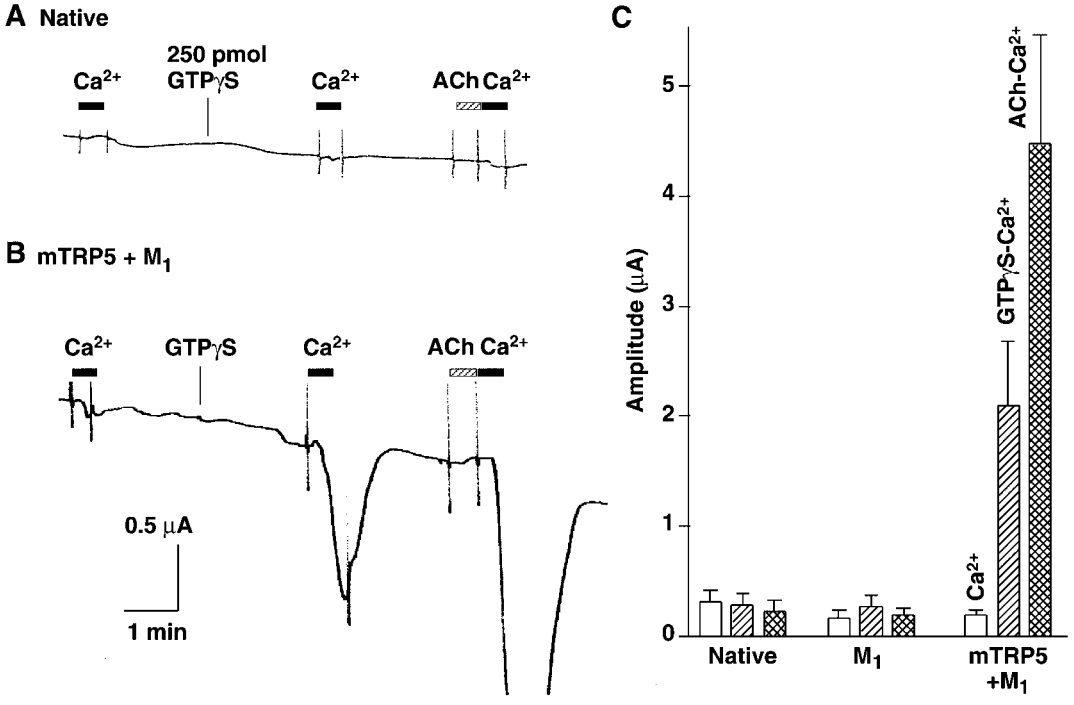

Fig. 3. Activation of mTRP5 channels after injection of GTPγS. A, native responses of a noninjected oocyte to an injection of GTPγS (250 pmol) and the following Ca^{2+} perfusion 2 min after the injection. B, representative current responses to the GTPγS injection recorded from an oocyte coexpressing mTRP5 and M_1 receptors. Functional expression of mTRP5 was confirmed by application of ACh (100 μM) and the subsequent perfusion with Ca^{2+}. C, average (n = 6) amplitudes of $I_{Cl(Ca)}$ in response to the first application of Ca^{2+} (open column), the second application of Ca^{2+} 2 min after injection of GTPγS (hatched column), and the third application of Ca^{2+} after ACh stimulation (cross-hatched column).

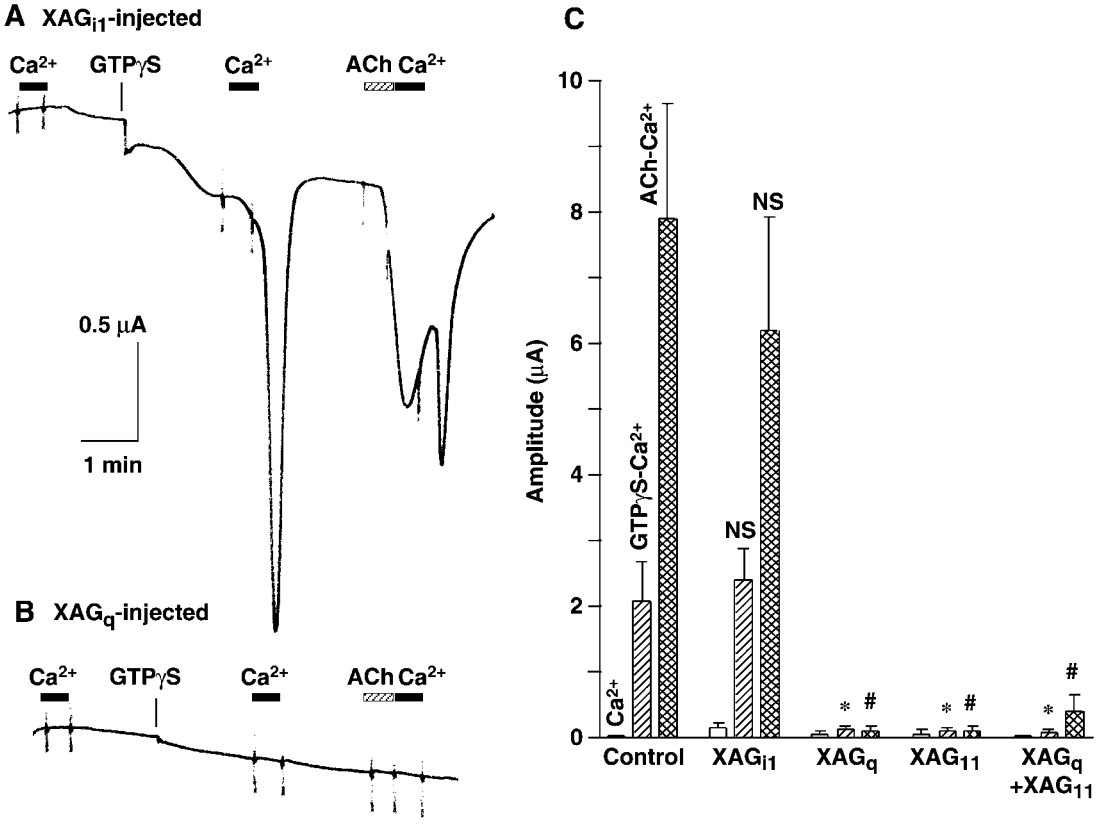

Fig. 4. Effects of preinjection with antisense ODNs to *X. laevis* G proteins on GTPγS-induced activation of mTRP5 channels. Representative current traces were recorded from oocytes coexpressing mTRP5 and M_1 receptors and preinjected with 50 ng of anti-G_{i1} antisense ODN, XAG_{i1} (A) or 50 ng of anti-G_q antisense ODN, XAG_q (B). GTPγS (250 pmol) was injected, and Ca^{2+}-containing FR was perfused 2 min after the injection. C, average (n = 6) amplitudes of $I_{Cl(Ca)}$ in response to the first application of Ca^{2+} (open column), the second application of Ca^{2+} 2 min after injection of GTPγS (hatched column), and the third application of Ca^{2+} after ACh stimulation (cross-hatched column). *, $P < 0.05$ versus control GTPγS-Ca^{2+}. #, $P < 0.05$ versus control ACh-Ca^{2+}. NS, not significant.

evoked a small (<0.1 μA) or virtually no transient current response by itself (Fig. 5, A and B). Instead, AdA at high concentrations up to 50 pmol elicited a gradual, irreversible increase in the Cl^- conductance in a Ca^{2+}-free condition, which is different from the slow increase in the Cl^- conductance through capacitative Ca^{2+} entry channels in the presence of external Ca^{2+} (DeLisle et al., 1997; Hartzell et al., 1997). Therefore, the slow increase in the Cl^- conductance is considered to reflect the Ca^{2+} release from stores by AdA.

In the control, no-mRNA-injected oocytes (Fig. 5A), only a small response to the second Ca^{2+} perfusion was observed 10 min after an injection of 50 pmol of AdA (0.053 ± 0.022 μA, $n = 6$). In oocytes coexpressing mTRP5 with M_1 receptors (Fig. 5B), 50 pmol of AdA evoked a large $I_{Cl(Ca)}$ in response to the second Ca^{2+} perfusion (1.14 ± 0.28 μA, $n = 6$). The preloading with AdA did not affect the subsequent ACh-induced $I_{Cl(Ca)}$ to the third Ca^{2+} perfusion (4.95 ± 0.64 μA, $n = 6$). The activating effect of AdA on the mTRP5 response was observed dose dependently from 0.5 fmol/oocyte to 50 pmol/oocyte (Fig. 5C). Since the AdA-induced response was evaluated 10 min after the injection, the magnitude of the AdA-induced response was smaller than that of the ACh-induced response. The partial activation by AdA may be due to the limited diffusion of AdA within 10 min after the injection, as revealed by the AdA-evoked increase in the Cl^- conductance, resulting in an incomplete stimulation of IP_3 receptors entirely distributed in the large oocytes (Kume et al., 1993). However, longer time after the AdA injection tended to cause an undesirable increase in the Cl^- leakage conductance in oocytes. Therefore, in the following experiments, an apparent maximal dose of 50 pmol of AdA was used for eliciting constant responses of mTRP5 channels within 10 min of incubation.

We also tested the effects of IP_3 and OAG in evoking a $I_{Cl(Ca)}$ response in mTRP5-expressing oocytes. The second Ca^{2+} perfusion 5 min after an injection of IP_3 (50 pmol) elicited an average response of 1.38 ± 0.19 μA in only 6 of 38 tested oocytes. In the remaining 32 cells, no mTRP5-mediated response was observed after injection of IP_3, but the subsequent ACh-induced $I_{Cl(Ca)}$ to the third Ca^{2+} perfusion was constantly observed. In addition, injection of OAG (50 pmol) evoked only a small $I_{Cl(Ca)}$ (0.082 ± 0.042 μA, $n = 4$) when Ca^{2+}-containing FR was perfused 2 min after the injection of OAG (traces not shown).

Antisense ODNs to *X. laevis* G_q and G_{11} Did Not Affect the Opening of mTRP5 Channels by Activation of IP_3 Receptors. Since the above findings strongly indicated that direct activation of IP_3 receptors with AdA evoked the opening of mTRP5 channels, we further tested whether the reduction in the amount of *X. laevis* G proteins by antisense ODNs might affect the AdA-induced opening of mTRP5 channels. As shown in Fig. 6, preloading with XAG_{i1} showed no inhibitory effect on the mTRP5-mediated $I_{Cl(Ca)}$ responses evoked after AdA or ACh compared with the control group. In oocytes preloaded with XAG_q, XAG_{11}, or both at the same total amount, a significant reduction in the ACh-induced mTRP5 response was observed, as previously shown in Fig. 4. However, unlike GTPγS, the AdA-evoked mTRP5 opening was not affected by the pretreatments of oocytes with XAG_q, XAG_{11}, or both. These findings demonstrated that direct activation of mTRP5 channels by an activation of IP_3 receptors was not modified by G proteins and also disprove the

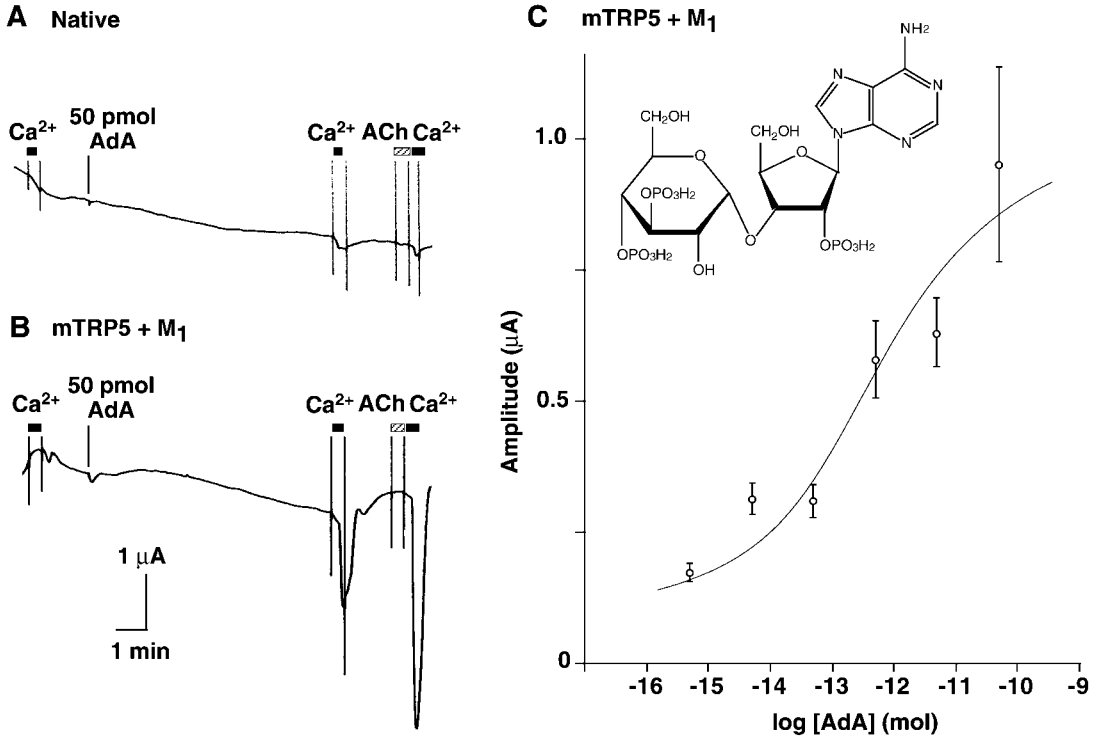

Fig. 5. Activation of mTRP5 channels after injection of AdA (chemical structure is indicated). Control, noninjected oocytes (A) and oocytes coexpressing mTRP5 and M_1 receptors (B) were voltage-clamped at −80 mV in Ca^{2+}-free FR and injected with AdA at a dose of 50 pmol/oocyte. Ten minutes after the injection of AdA, 2 mM Ca^{2+} was perfused externally. The functional expression of mTRP5 was confirmed by application of ACh (100 μM) and the subsequent perfusion with Ca^{2+}. C, dose-response effect of AdA on the amplitude of $I_{Cl(Ca)}$ response to the external perfusion with 2 mM Ca^{2+} after injection of AdA into oocytes coexpressing mTRP5 and M_1 receptors. $n = 6$.

direct activation of mTRP5 channels by activated G proteins, which was implicated by analogy to *Drosophila* TRPL channels (Obukhov et al., 1996).

The Opening of mTRP5 Channels by AdA Was Inhibited by an Allosteric Antagonist of IP_3 Receptor, Xestospongin C, but Not by a PLC Inhibitor, U73122. To characterize the differences in the opening of mTRP5 channels induced by ACh, GTPγS, and AdA, we utilized a potent membrane-permeable blocker of the IP_3-induced Ca^{2+} release, XeC (Gafni et al., 1997), and a potent PLC blocker, U73122 with its inactive analog U73343 (Thompson et al., 1991). When oocytes coexpressing mTRP5 and M_1 receptors were preincubated for 30 min in Ca^{2+}-free FR with 1 μM XeC (Fig. 7A), 10 μM U73122 (Fig. 7B), or 10 μM U73343 (Fig. 7C), both GTPγS- and ACh-induced mTRP5 responses of oocytes were almost completely abolished in XeC- and U73122-treated cells, but not in U73343-treated cells (Fig. 7D), indicating that the PLC-mediated PI turnover is required for the ACh- and GTPγS-induced opening of mTRP5 channels. In contrast, the opening of mTRP5 by injection of AdA was blocked by XeC, but not by U73122 or U73343 (Fig. 7E). These findings demonstrate that the allosteric antagonist of IP_3 receptor XeC is capable of blocking the stimulation with AdA, and that the upstream PLC activation is not involved in the activation of mTRP5 channels by direct stimulation of IP_3 receptors with AdA.

The Opening of mTRP5 Channels by AdA Was Inhibited by a Synthetic Peptide for the IP_3 Binding Site. To clarify the role of the IP_3 binding site for the activation of mTRP5 channels, we synthesized a polypeptide, IP_3RF, mimicking the IP_3 binding site of *X. laevis* IP_3 receptors and injected it into oocytes coexpressing mTRP5 and M_1 receptors. As shown in Fig. 8, injection of IP_3RF significantly inhibited both AdA- and ACh-induced openings of mTRP5 channels. The incomplete suppression of ACh-induced response may reflect that the quantitatively insufficient antagonism against massive amounts of IP_3 produced after stimulation of M_1 receptors with ACh. In the control oocytes injected with a peptide with the N-terminal sequence of *X. laevis* $G\alpha_o$, PGON1, which was effective for the ablation of oocyte-endogenous $G\alpha_o$ protein in the study of voltage-dependent Ca^{2+} channel regulation by $G\alpha_o$ (Kinoshita et al., 2001), there was no change in the amplitude of AdA- or ACh-induced mTRP5 responses compared with the noninjected oocytes coexpressing mTRP5 and M_1 receptors.

Discussion

With regard to the opening mechanisms of TRP5 channels, it is still controversial whether these channels are store-operated or dependent on other components of PLC pathway. Evidence supportive of the store-operated story has been provided by the potentiation of native CRAC-like responses of cells used in the expression study (Philipp et al., 1998). However, although independence of store depletion and the inhibitory effects of the PLC inhibitor are strongly suggestive of the direct involvement of the PLC pathway (Okada et al., 1998), no TRP5 channel opening was induced either by DAG

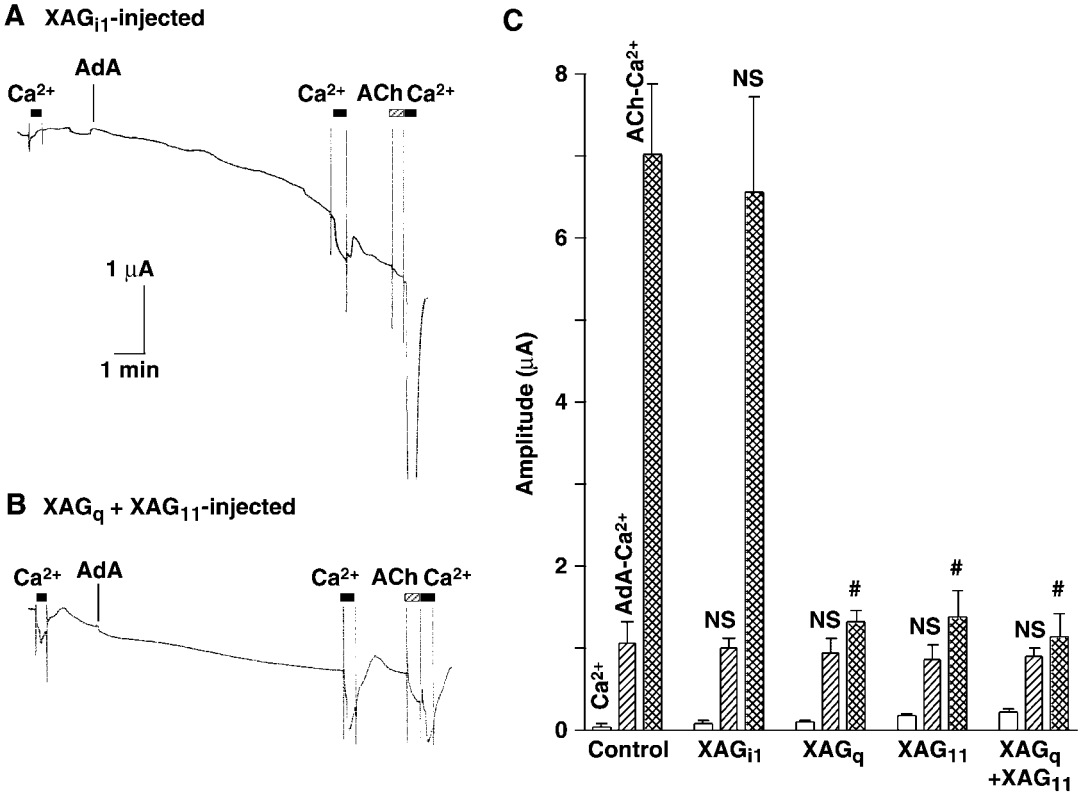

Fig. 6. Effects of the preinjection of antisense ODNs to *X. laevis* G proteins on AdA-induced activation of mTRP5 channels. Representative current traces were recorded from oocytes coexpressing mTRP5 and M_1 receptors and preinjected with 50 ng of anti-G_{i1} ODN, XAG_{i1} (A) or 25 ng + 25 ng of mixture of anti-G_q XAG_q and anti-G_{11} XAG_{11} (B). AdA (50 pmol) was injected and Ca^{2+}-containing FR was perfused 10 min after the injection. C, average ($n = 6$) amplitudes of $I_{Cl(Ca)}$ in response to the first application of Ca^{2+} (open column), the second application of Ca^{2+} 10 min after injection of AdA (hatched column), and the third application of Ca^{2+} after ACh stimulation (cross-hatched column). #, $P < 0.05$ versus control ACh-Ca^{2+}. NS, not significant.

or IP_3 (Schaefer et al., 2000), and thus mediators downstream from the activation of PLC remained unclear.

Three findings of the present study demonstrate that activation of IP_3 receptors triggers the opening of recombinant mTRP5 channels: 1) activation of the mTRP5 channel response by a potent IP_3 receptor agonist AdA, 2) inhibition of the mTRP5 channel response by an IP_3 receptor antagonist XeC, and 3) inhibition of the mTRP5 channel response by a peptide IP_3RF mimicking the IP_3 binding site of IP_3 receptor. The $I_{Cl(Ca)}$ responses of oocytes coexpressing G_q-couple muscarinic M_1 receptors and mTRP5 channels were evoked either by ACh, GTPγS, or AdA independently of Ca^{2+} store depletion and were clearly distinguishable from the oocyte-native CRAC channel response by the marked difference in their amplitudes. The upstream signals from muscarinic receptors or direct G protein stimulation with GTPγS were abolished either by knock down of endogenous $G\alpha_{q/11}$ with antisense ODNs or by PLC inhibitor. In contrast, the direct activation of IP_3 receptors with AdA was insensitive to these treatments, indicating that the activation of IP_3 receptor is essential for the opening of mTRP5 channels. The present findings also suggest that neither G proteins nor PI metabolism modifies the opening of mTRP5 channels evoked by activation of IP_3 receptors. However, we cannot rule out the possibility that several factors are needed to act in concert to maximally activate TRP channels, since the ACh-induced response was more resistant to the treatments disrupting the G protein-PLC pathway than the AdA-induced response.

Previously, activation of the TRP4/5 channel response was not observed by activation of IP_3 receptors by IP_3. In the present study using IP_3 itself, the potency of IP_3 in evoking an mTRP5 response was weak and inconsistent in oocytes from batch to batch. However, the potent IP_3 receptor agonist AdA could evoke a Ca^{2+} influx in mTRP5-expressing oocytes in a dose-dependent manner. The half-maximal dose of AdA (5×10^{-13} mol) corresponded to a putative cytosolic concentration of 1 μM in oocytes, which was comparable with the effective dose of AdA in evoking slow Ca^{2+} release from stores in *Xenopus* oocytes (DeLisle et al., 1997; Hartzell et al., 1997). Since AdA has a potency about 100-fold greater than that of IP_3 in receptor binding (Takahashi et al., 1994), AdA, but not IP_3, may evoke the opening of mTRP5 channels functionally coupling to IP_3 receptor activation. It is also likely that AdA overcomes the Ca^{2+}-dependent inactivation of IP_3 receptors. In type 1 and type 2 IP_3 receptors, IP_3-induced Ca^{2+} release from stores is biphasically dependent on the cytosolic Ca^{2+} concentration, of which the declining phase at a higher Ca^{2+} concentration is responsible for negative feedback regulation of Ca^{2+} release and causes cytosolic Ca^{2+} oscillation (Miyakawa et al., 1999). Because the primary amino acid sequence of IP_3 receptors expressed in *Xenopus* oocytes is equivalent to that of mammalian type 1 (Kume et al., 1993), IP_3 applied without $[Ca^{2+}]_i$ buffering only causes a transient Ca^{2+} release followed by quick inactivation of IP_3 receptors, which may not be sufficient to signal mTRP5 channel opening. In this context, a recent study using rat basophilic leukemia cells has shown the ability of AdA to activate the CRAC current under minimal $[Ca^{2+}]_i$ buffering conditions where inactivation of IP_3 receptor was

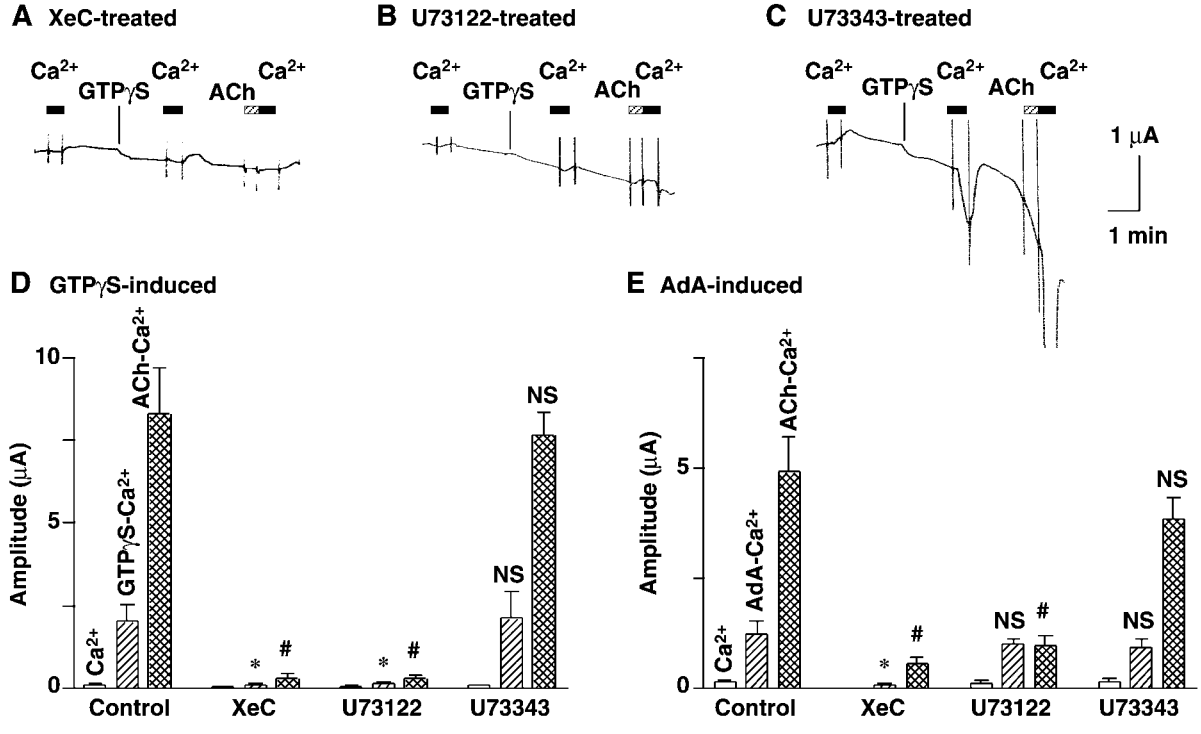

Fig. 7. Effects of oocyte pretreatments with intracellular signaling inhibitors on the activation of mTRP5 channels induced by GTPγS, AdA, and ACh. Current recordings were made from oocytes coexpressing mTRP5 and M_1 receptors after 30-min pretreatment with 1 μM XeC (A), 10 μM U73122 (B), or 10 μM U73343 (C) in Ca^{2+}-free FR. D, effects of the pretreatments on the average ($n = 6$) amplitudes of $I_{Cl(Ca)}$ in response to the first application of Ca^{2+} (open column), the second application of Ca^{2+} 2 min after injection of 250 pmol of GTPγS (hatched column), and the third application of Ca^{2+} after 100 μM ACh stimulation (cross-hatched column). *, $P < 0.05$ versus control GTPγS-Ca^{2+}. #, $P < 0.05$ versus control ACh-Ca^{2+}. NS, not significant. E, effects of the pretreatments on the average ($n = 8$) amplitudes of $I_{Cl(Ca)}$ in response to the first application of Ca^{2+} (open column), the second application of Ca^{2+} 10 min after injection of 50 pmol of AdA (hatched column), and the third application of Ca^{2+} after ACh (cross-hatched column). *, $P < 0.05$ versus control AdA-Ca^{2+}. #, $P < 0.05$ versus control ACh-Ca^{2+}. NS, not significant.

caused after the activation of receptors with IP_3 and the resulting Ca^{2+} influx (Broad et al., 1999).

XeC is a noncompetitive blocker of the IP_3 receptor which inhibits IP_3-induced Ca^{2+} release without interacting with the IP_3 binding site (Gafni et al., 1997). The fact that XeC was effective in inhibiting all the ACh-, GTPγS-, and AdA-evoked mTRP5 responses indicates that activation of IP_3 receptor is essential for the mTRP5 channel opening. However, XeC was shown to not only block the IP_3 receptors but also inhibit the endoplasmic reticulum Ca^{2+} pump at an equivalent concentration (De Smet et al., 1999). Although the mTRP5 response was not affected by the filling state of Ca^{2+} stores, we have synthesized a peptide mimicking the IP_3 binding site of the IP_3 receptor and shown that the peptide IP_3RF is effective in inhibiting the ACh- and AdA-evoked mTRP5 channel responses. In mammalian type 1 IP_3 receptor, the inner surface of the IP_3 binding domain was suggested to be lined with 10 basic amino acid residues for which substitutions to neutral amino acids cause significant reduction of the binding activity, and among them, three amino acids are critical residues for the specific IP_3 binding (Yoshikawa et al., 1996). Since these amino acids are well conserved in *X. laevis* IP_3 receptors (Kume et al., 1993), IP_3RF (N**R**ER**Q**K**K**LM**R**E**Q**) was designed to include 4 of 10 amino acids for the IP_3 binding pocket (underlined) and 2 of 4 critical amino acids (boldface). A decrease in the mTRP5 response as well as a decrease in the AdA-induced slow increase in the baseline current may reflect that IP_3RF acts as a "decoy" peptide whose basic amino acids interrupt IP_3 binding to the binding pocket.

Recent studies have shown the direct interactions of TRP proteins including TRP5 with mammalian IP_3 receptors, which indicate a direct coupling model in the activation of all TRP channels (Tang et al., 2001). Although the present findings do not demonstrate the direct interaction of mTRP5 channels with *X. laevis* IP_3 receptors, physical coupling of TRP5 with IP_3 receptor is likely in oocytes since *X. laevis* oocyte IP_3 receptors are present in the cortical layer as well as cytoplasm of the animal hemisphere and perinuclear layer (Kume et al., 1993). Coexpression of TRP1 and TRP5 forms a nonselective cation channel with different channel properties from the original TRP5 or TRP1 channels (Strübing et al., 2001). *X. laevis* TRP1 proteins are abundantly expressed in native oocytes (Bobanovic et al., 1999), which is, however, unlikely to form CRAC channels intrinsically present in oocytes (Brereton et al., 2000). Exogenously expressed mTRP5 proteins may form heteromeric channels with endogenous TRP1 proteins, which are functionally coupled to activated IP_3 receptors. In the brain, TRP5 is colocalized with TRP1 in hippocampal neurons (Strübing et al., 2001), and the heteromeric complex forms nonselective cation channels activated by G_q-couple receptors with similar cytosolic Ca^{2+} sensitivity to recombinant TRP1/5 channels (Congar et al., 1997). Since recombinant TRP5 channels also require cytosolic Ca^{2+} for

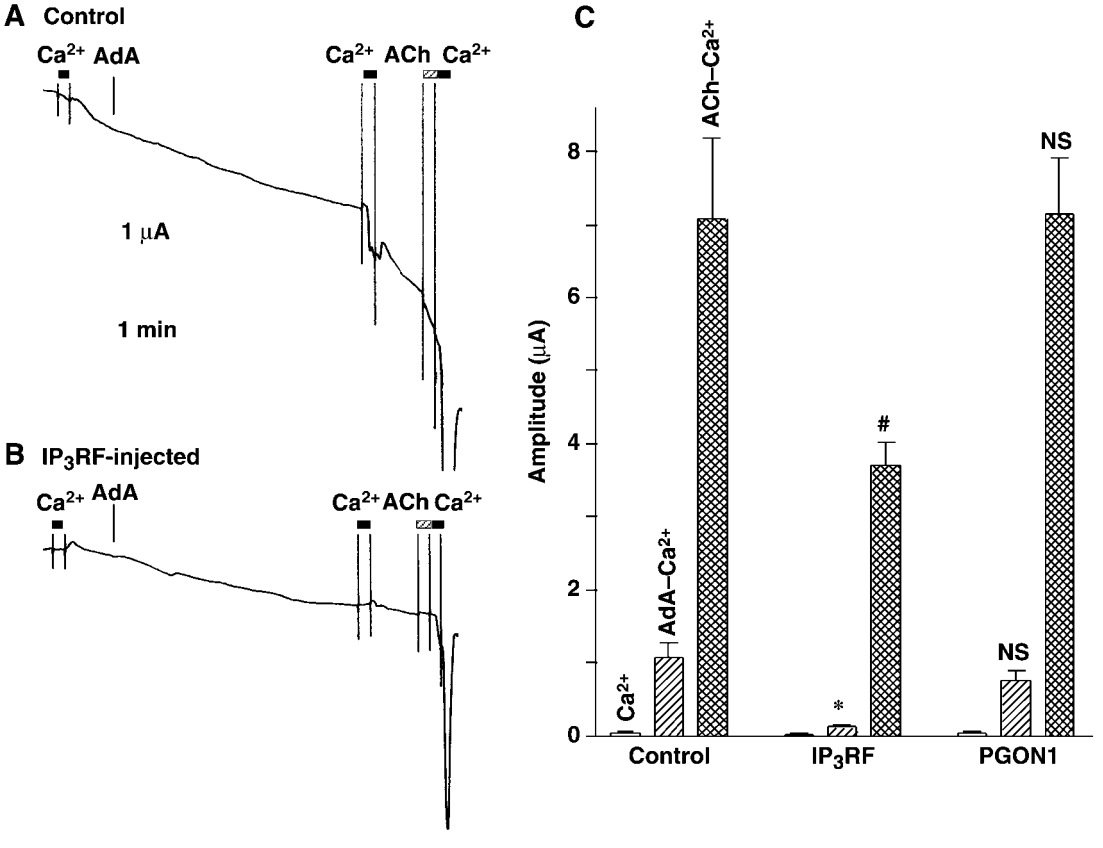

Fig. 8. Effects of the preinjection of decoy peptides IP_3RF and PGON1 on the AdA-induced activation of mTRP5 channels. Synthetic peptides IP_3RF and PGON1 were injected at a dose of 10 ng (putative cytosolic concentration of 10 μM) into oocytes coexpressing mTRP5 and M_1 receptors 1 day before the recording. Representative responses of oocytes to external perfusion with Ca^{2+} before and after injection with AdA (50 pmol) and perfusion with ACh (100 μM) are shown in the control, nonpeptide-injected (A) and IP_3RF-injected (B) cells. C, average ($n = 6$) amplitudes of $I_{Cl(Ca)}$ in response to the first application of Ca^{2+} (open column), the second application of Ca^{2+} 10 min after injection of AdA (hatched column), and the third application of Ca^{2+} after ACh (cross-hatched column). *, $P < 0.05$ versus control AdA-Ca^{2+}. #, $P < 0.05$ versus control ACh-Ca^{2+}. NS, not significant.

maintaining the activity (Okada et al., 1998; Philipp et al., 1998), Ca^{2+} may act as a coactivator for the activation of IP_3 receptors, which results in opening TRP5 channels.

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