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An inwardly rectifying whole cell current induced by G_q-coupled receptors ☆

Björn Buchholz[1], Robert Tauber[1], Daniel Steffl, Gerd Walz, Michael Köttgen*

Renal Division and Center for Clinical Research, University Hospital Freiburg, Breisacherstr. 66, 79106 Freiburg, Germany

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Abstract

Ca^{2+} influx across the plasma membrane after stimulation of G protein-coupled receptors is important for many physiological functions. Here we studied the regulation of an inwardly rectifying whole cell current and its putative role in Ca^{2+} entry in *Xenopus* oocytes. Expression of $P2Y_1$ or M1 receptors in *Xenopus* oocytes elicited a characteristic inwardly rectifying current without receptor stimulation. This current displayed distinct activation and inactivation kinetics and was highly Ca^{2+}-dependent. After stimulation of endogenous G_q-coupled receptors in water-injected cells similar currents were observed. We therefore speculated that the current could be activated via Ca^{2+} store depletion induced by constitutive stimulation of the IP_3 cascade in cells overexpressing G_q-coupled receptors. Receptor-independent Ca^{2+} store depletion also induced the current. In conclusion, this current is activated after store depletion suggesting a role in Ca^{2+} entry after stimulation of G_q-coupled receptors. Finally, our data do not support the proposed ionotropic properties of the $P2Y_1$ receptor.

Keywords: G protein-coupled receptor; P2Y receptor; M1 receptor; *Xenopus* oocyte; Store depletion; Ca^{2+}signaling; IP_3; Cascade; Capacitative Ca^{2+} entry

A rise in cytosolic Ca^{2+} is an important trigger for various cellular processes such as secretion, growth, and differentiation. Stimulation of G_q-coupled receptors leads to a characteristic biphasic increase in the cytosolic Ca^{2+} concentration. The first, rapid phase reflects Ca^{2+} release from intracellular IP_3-sensitive stores, whereas the second, sustained phase reflects Ca^{2+} influx into the cell. In several cell types this Ca^{2+} influx pathway is regulated by the Ca^{2+} content of the internal stores, a process termed capacitative Ca^{2+} entry (CCE) [1]. The second phase of Ca^{2+} mobilization is required for refilling of intracellular stores and for cellular responses that depend on a sustained increase in cytosolic Ca^{2+}, such as the transcriptional regulation in lymphocytes [1,2].

Xenopus oocytes are widely used as a model system to study Ca^{2+} signaling [3–6]. These cells endogenously express Ca^{2+}-activated Cl^- channels which have been used as an amplification system to detect Ca^{2+} signals. The Ca^{2+}-activated Cl^- current is very sensitive to changes in Ca^{2+} concentration near the plasma membrane and in previous studies it was shown that the peak amplitude of such currents has a linear relationship to the Ca^{2+} concentration [5,7].

Ca^{2+} signaling has pivotal functions in oocyte physiology. Differentiation of Ca^{2+} signaling pathways during

☆ *Abbreviations:* Ach, Acetylcholine; CCE, capacitative Ca^{2+} entry; EGTA, ethylene glycol-bis (2-aminoethylether)-tetraacetic acid; GPCR, G protein-coupled receptor; I_{crac}, calcium release-activated current; I_{inward}, inwardly rectifying whole cell current; IP_3, inositol-trisphosphate; LPA, lysophosphatidic acid; P2Y-R and M1-R, P2Y and M1 receptor; SERCA, sarco-endoplasmic reticulum ATP-ase; V_c, voltage clamp.

* Corresponding author. Fax: +49 761 270 6324.
E-mail address: koettgen@med1.ukl.uni-freiburg.de (M. Köttgen).
[1] These authors contributed equally to this work.

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oocyte maturation is a prerequisite for proper oocyte activation by inducing the block to polyspermy and releasing the arrest at metaphase of the second meiotic division [8]. Ca^{2+} entry from the extracellular space is sufficient to stimulate oocyte activation [9], further demonstrating the importance of tightly regulated Ca^{2+} entry pathways in these cells.

Here we describe an inwardly rectifying whole cell current that is induced upon overexpression of G_q-coupled receptors in *Xenopus* oocytes and address its putative role in Ca^{2+} influx after receptor stimulation. In the following this current will be termed I_{inward}. This study began as a reexamination of the hypothesis that the $P2Y_1$ receptor could function as an ionotropic receptor [10].

P2Y receptors are activated by extracellular nucleotides and belong to the superfamily of G protein-coupled receptors [11]. They are found in a variety of tissues and are known to function as important regulators of a wide spectrum of physiological processes [11,12]. To date the mammalian P2Y family includes the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptor [11,13–15].

In order to study the ionotropic properties of the $P2Y_1$ receptor that were proposed to be specific for this P2Y receptor subtype, we expressed different G_q-coupled receptors in *Xenopus* oocytes ($P2Y_1$, $P2Y_6$, and acetylcholine M1 receptor). Interestingly, we found that all receptors induced an inwardly rectifying whole cell current similar to the one previously described [10]. This suggested that I_{inward} is carried by endogenous ion channels in *Xenopus* oocytes that are activated by G_q-coupled receptors.

To investigate the possible regulation of I_{inward} in *Xenopus* oocytes, we studied the regulation of the current by using receptor-dependent and -independent store depletion.

Experimental procedures

Preparation and injection of oocytes. *Xenopus* oocytes were isolated and injected as described previously [16]. Briefly, 12–24 h following surgical isolation and defolliculation stage V–VI oocytes were injected with 30 nl of water containing either 10 ng cRNA of $P2Y_1$, $P2Y_6$ or acetylcholine M1 receptor. Voltage clamp experiments were performed 2–5 days after injection.

Preparation of cRNA for injection. cRNA was synthesized from linear cDNA encoding different G protein-coupled receptors using the Ambion Message Machine kit. The rat $P2Y_1$ and $P2Y_6$ receptors were cloned from cDNA isolated from rat colonic crypts as described previously [16]. The human muscarinic M1 receptor cDNA was a kind gift of T. Jentsch (Hamburg, Germany).

Electrophysiological measurements. Whole cell currents of oocytes were recorded using standard two electrode voltage clamp (Turbo TEC 03X voltage/current clamp amplifier, npi electronic, Tamm, Germany). Microelectrodes were pulled on a vertical puller (Physiologisches Institut, Universität Freiburg, Germany) from borosilicate glass capillaries (Clark Instruments, Reading, GB) and had resistances of 0.5–2 MΩ when filled with 2 M KCl solution. All experiments were performed at 20–22 °C. Voltage clamp experiments were conducted in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, and 2.5 mM Na-pyruvate, pH 7.4). Equimolar substitution of Cl^- by gluconate and Na^+ by *N*-methyl-D-glucamine (NMDG) was done as indicated. Reduction of extracellular Ca^{2+} to 10 μM was achieved by adding 1 mM nitrilotriacetic acid (NTA) to ND96 solution. In this solution, 0.093 mM calcium gluconate was added instead of 1.8 mM $CaCl_2$. All chemicals were obtained from Sigma–Aldrich (Deisenhofen, Germany). Two alternative voltage clamp protocols were used as indicated: (i) oocytes were clamped at 0 mV, and repetitive voltage ramps from −100 to +50 mV were applied every second and (ii) starting from a holding potential of 0 mV oocytes were clamped from −100 mV to +60 mV in 20 mV steps for 3 or 5 s. Due to its characteristic kinetic properties, I_{inward} can only be detected using the second voltage clamp protocol. Whole cell conductance was calculated according to Ohm's law.

Statistics. Data are presented as original recordings or as mean values ± SEM (n = number of experiments). Unpaired and paired Student's t test was used for statistical analysis. A p value of <0.05 was accepted to indicate statistical significance. The experiments were performed with at least three different batches of oocytes.

Results

Overexpression of G_q-coupled receptors activates an inwardly rectifying current in Xenopus oocytes

Recently, it was suggested that the $P2Y_1$ receptor could function as an ionotropic nucleotide receptor [10]. This hypothesis was based on the observation that overexpression of the $P2Y_1$ receptor gave rise to an inwardly rectifying whole cell current in *Xenopus* oocytes when the receptor was stimulated by agonists. Our initial observation was that overexpression of the $P2Y_1$ receptor in *Xenopus* oocytes induced an inwardly rectifying whole cell current with characteristic kinetic properties (I_{inward}) (Figs. 1A and C). I_{inward} was invariably observed without receptor stimulation. To determine whether the inwardly rectifying current we observed was uniquely associated with the $P2Y_1$ receptor, two additional G_q-coupled receptors were expressed in oocytes. Interestingly, expression of the M1 muscarinic and the $P2Y_6$ receptor induced similar currents (Fig. 1A, data not shown for the $P2Y_6$ receptor). The current peaks of I_{inward} with their slow activation and inactivation kinetics were significantly higher than the currents measured in control oocytes (Fig. 1B, control oocytes: -0.41 ± 0.05 μA, $n = 31$; $P2Y_1$-R: -1.41 ± 0.16 μA, $n = 46$; and M1-R: -1.36 ± 0.14 μA, $n = 47$). It is important to mention that I_{inward} can only be detected by the use of hyperpolarizing voltage steps lasting at least 2 s, since the activation and inactivation kinetics are too slow to be detected via fast voltage ramps (Figs. 1A, D, and E). Fig. 1C shows the strong inward rectification of I_{inward} in an oocyte expressing the $P2Y_1$ receptor. Successful expression of the G_q-coupled receptors could be detected by the use of voltage ramps as depicted in Figs. 1D and F. Upon receptor stimulation the conductance

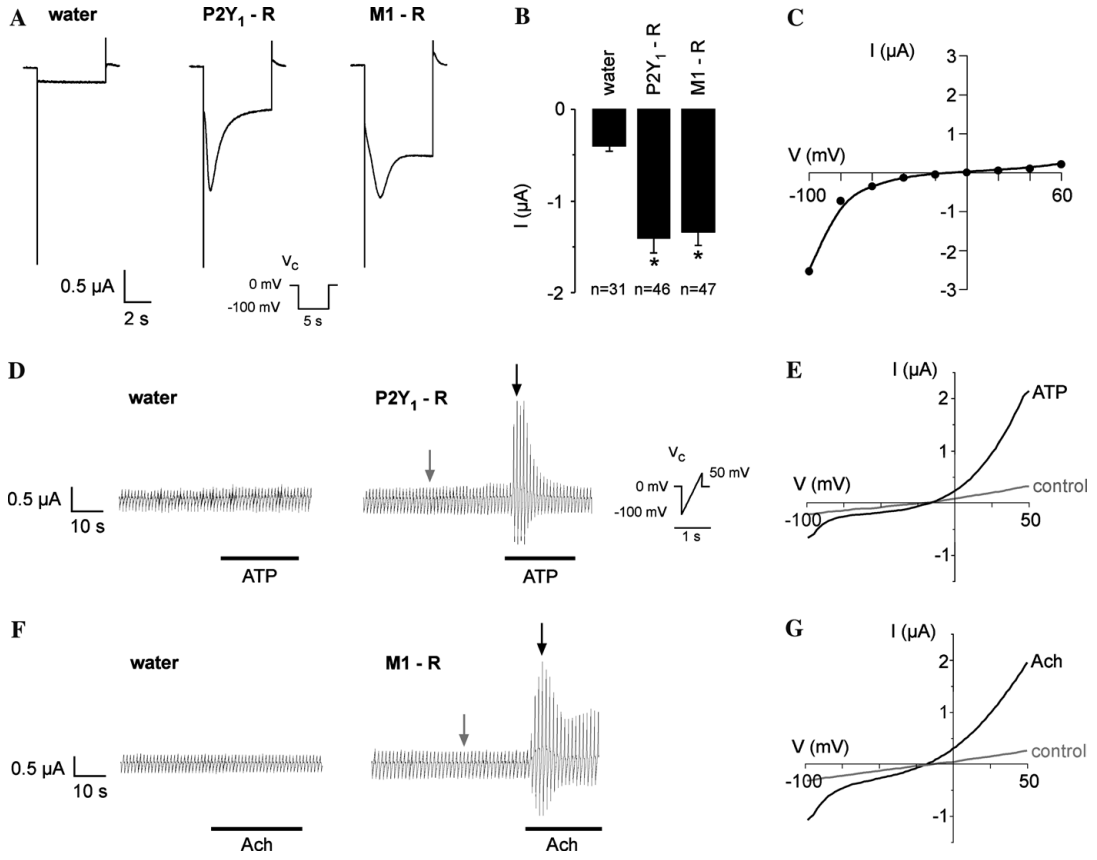

Fig. 1. Whole cell currents in oocytes overexpressing G_q-coupled receptors. (A) Representative traces of two electrode voltage clamp experiments (oocytes were injected with 10 ng $P2Y_1$-R, M1-R cRNA or water as control). Voltage clamp protocol as depicted. (B) Statistical analysis of the current peaks. Asterisks indicate significant differences ($p < 0.05$). (C) Current–voltage relationship of a $P2Y_1$-R-expressing oocyte. Analysis of peak currents of an oocyte clamped from −100 to +60 mV in 20 mV steps for 3 s (compare Fig. 2F). (D) Original experiment showing the effect of ATP (10 µM) in oocytes injected with water or $P2Y_1$-R (voltage ramps every second as depicted). (E) Current–voltage relationship of a $P2Y_1$-R-expressing oocyte [data taken from (D) as indicated by arrows]. (F,G) Analogous to (D,E) in M1-R-expressing oocytes (acetylcholine 10 µM).

increased due to activation of endogenous Ca^{2+}-activated Cl^- channels. The current–voltage relationship of the Ca^{2+}-activated Cl^- channel that is activated by Ca^{2+} release from intracellular stores is shown in Figs. 1E and G. Due to the slow activation kinetics the inwardly rectifying component of the current was usually not detected under control conditions using fast voltage ramps. It was proposed in two recent studies that there are two different Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes [4,17], namely $I_{Cl\text{-}1}$ activated by mobilization of Ca^{2+} from intracellular stores and $I_{Cl\text{-}2}$ activated by capacitative Ca^{2+} entry. Our data shown in Figs. 1C, E, and G are consistent with this model.

I_{inward} is highly dependent on extracellular Ca^{2+}

Increasing the extracellular Ca^{2+} concentration from 1.8 to 18 mM significantly raised the conductance in oocytes expressing G_q-coupled receptors (Figs. 2A and B; control oocytes: $\Delta G = 1.33 \pm 0.22$ µS, $n = 18$; M1-R: $\Delta G = 7.51 \pm 2.35$ µS, $n = 13$; and $P2Y_1$-R: $\Delta G = 24.55 \pm 4.73$ µS, $n = 54$). The conductance at a physiological Ca^{2+} concentration (1.8 mM Ca^{2+}) was also significantly increased in oocytes expressing G_q-coupled receptors (Figs. 2A and B; control oocytes: $G = 3.59 \pm 0.47$ µS, $n = 18$; M1-R: $G = 6.08 \pm 0.64$ µS, $n = 13$; and $P2Y_1$-R: $G = 6.77 \pm 0.47$ µS, $n = 54$). Lowering or increasing extracellular Ca^{2+} elicited only marginal changes of whole cell currents in control oocytes when measured using a voltage clamp protocol as depicted in Fig. 2C (Figs. 2C and E). In contrast, the same maneuvers induced marked changes in oocytes expressing the $P2Y_1$ receptor. Increasing the extracellular Ca^{2+} concentration induced a potentiation of the current amplitude (Fig. 2D). Furthermore, increased Ca^{2+} concentrations led to a significant depolarization of the membrane potential in $P2Y_1$ expressing oocytes (from -31.9 ± 3.2 to -19.3 ± 2.5 mV, $n = 21$). Lowering of extracellular Ca^{2+} significantly decreased I_{inward} (Fig. 2F). The increased current in the presence of 10 µM Ca^{2+} at positive clamp voltages is due to well-described Ca^{2+}-inactivated currents in *Xenopus* oocytes [18]. Reduction of extracellular Na^+ (10 mM) by NMDG resulted in a significant decrease in whole cell conductance

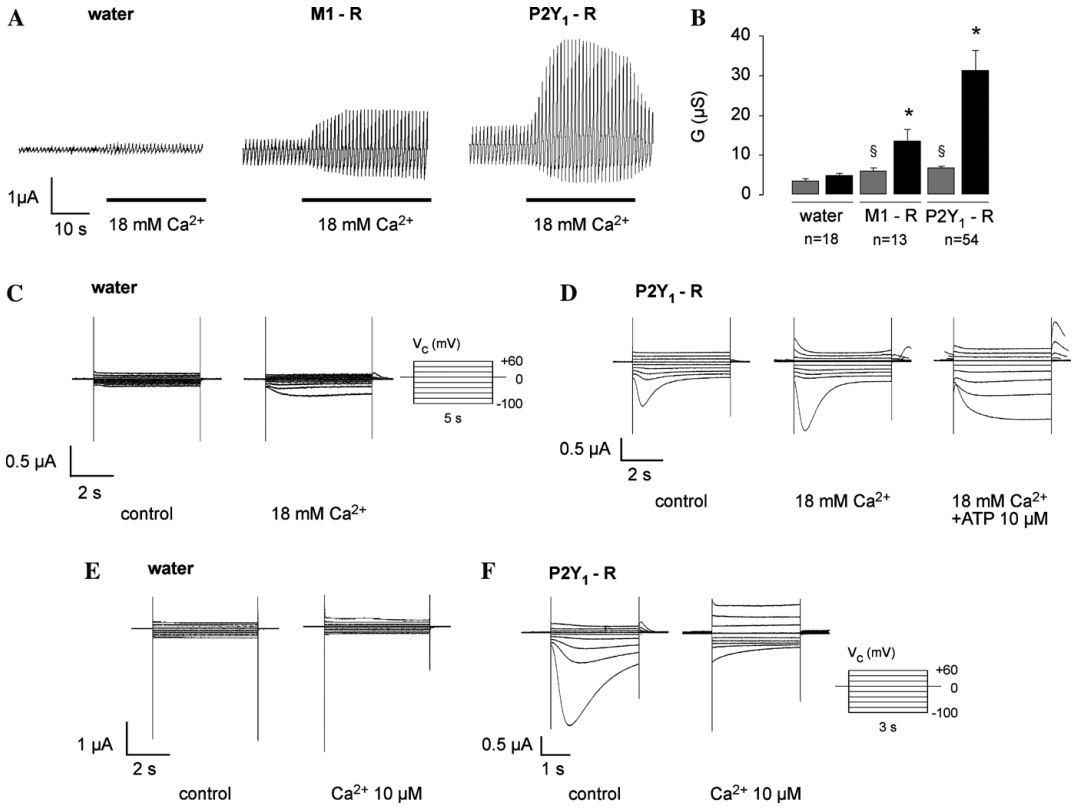

Fig. 2. Dependence of I_{inward} on extracellular Ca^{2+}. (A) Original experiments showing the effects of increasing the extracellular Ca^{2+} concentration from 1.8 to 18 mM (voltage ramps as depicted in Fig. 1D). (B) Statistical analysis of the whole cell conductance (G) of the experiments shown in (A) (grey bars, control solution; black bars, Ca^{2+} 18 mM; paragraph symbols indicate statistical significance comparing grey bars and asterisks comparing black bars). (C) Effect of increasing extracellular Ca^{2+} to 18 mM in control oocytes and (D) $P2Y_1$-R-expressing oocytes. (E) Effect of lowering extracellular Ca^{2+} to 10 µM in control oocytes and (F) $P2Y_1$-R-expressing oocytes.

by 0.26 ± 0.1, $n = 10$. Substitution of extracellular Cl^- by gluconate largely reduced I_{inward}, showing that we monitored Ca^{2+} entry by means of the previously described Ca^{2+}-activated Cl^- channel (data not shown) [4,5].

Stimulation of endogenous G_q-coupled receptors also activates I_{inward}

To investigate whether I_{inward} was only activated upon overexpression of G_q-coupled receptors or due to effects evoked downstream of the receptor's signaling cascade, we stimulated endogenous G protein-coupled receptors in water-injected oocytes. Trypsin and LPA are described as potent agonists of endogenous G_q-coupled receptors in *Xenopus* oocytes [19,20]. Application of LPA markedly increased the conductance via activation of Ca^{2+}-activated Cl^- channels induced by Ca^{2+} release from intracellular stores (Fig. 3A). When the peak of the conductance increase was reached, we started the voltage clamp protocol as depicted in Fig. 2C in order to detect the typical kinetics of I_{inward}. Immediately after receptor stimulation I_{inward} was not detectable (the outwardly rectifying Ca^{2+}-activated Cl^- current activated by store release was seen instead) (Fig. 3A). However, 2 min after receptor stimulation I_{inward} was observed invariably (Fig. 3A). In order to study whether this delayed activation of I_{inward} was dependent on store depletion we stimulated the LPA receptor 2 min after application of trypsin (Fig. 3B). In fact, no conductance increase could be observed, consistent with depleted Ca^{2+} stores (Fig. 3B). Interestingly, I_{inward} was detectable again when applying the voltage clamp protocol shown in Fig. 2C. We used two different agonists to exclude effects due to receptor downregulation. The same effects could be observed when changing the sequence of agonist stimulation (Fig. 3C).

Receptor-independent Ca^{2+} store depletion

Next we studied receptor-independent Ca^{2+} store depletion with thapsigargin, a potent inhibitor of the endoplasmic reticulum Ca^{2+} ATP-ase (SERCA). After preincubation of oocytes in control solution containing 1 µM thapsigargin for 2 h we could measure a slightly increased steady state current and I_{inward} (Fig. 4A). In order to deplete the Ca^{2+} stores more effectively we preincubated oocytes in 1 µM thapsigargin with a

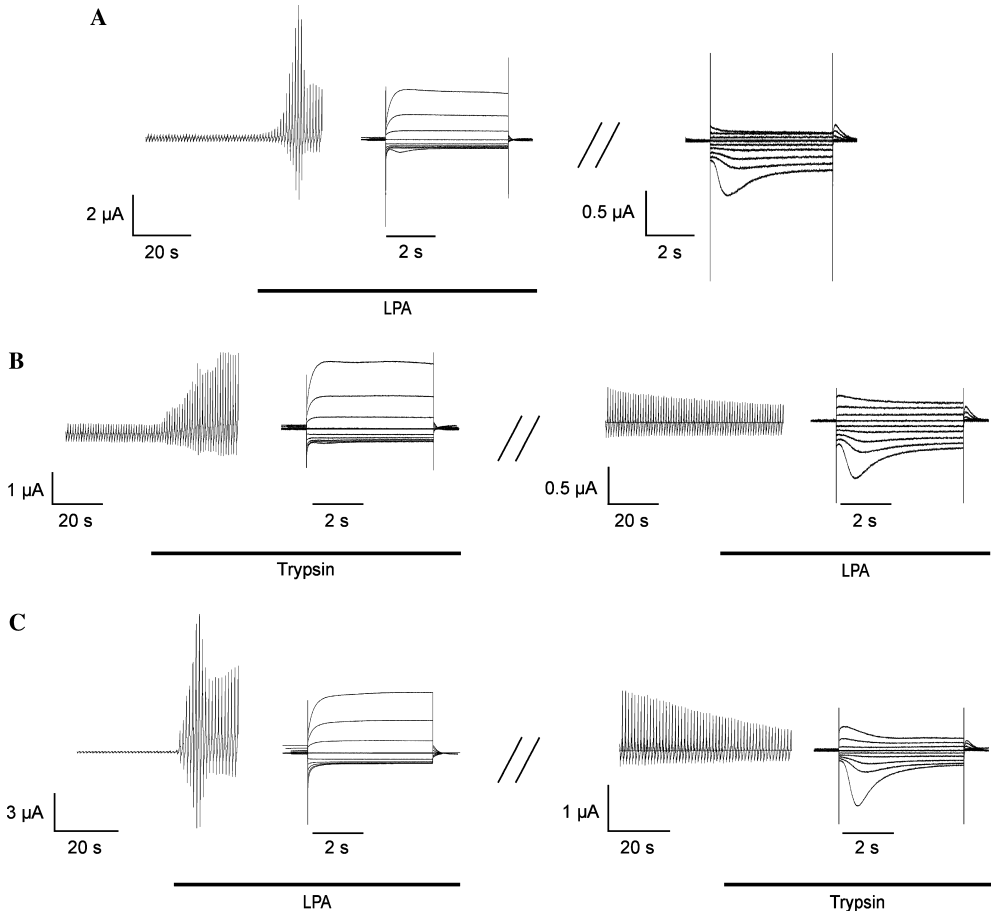

Fig. 3. Activation of I_{inward} after stimulation of endogenous G_q-coupled receptors in oocytes. (A) Stimulation of endogenous LPA receptors (LPA 2 μM) resulted in an increase of the intracellular Ca^{2+} concentration, which led to activation of endogenous Ca^{2+}-dependent Cl^- channels. I_{inward} was not detected immediately after receptor stimulation. Approximately 2 min after application of LPA I_{inward} was detected (voltage ramps and steps as described in Figs. 1D and 2C, respectively). (B) Application of trypsin (10 μg/ml) similarly increased the whole cell current. Subsequent stimulation of LPA receptors did not increase whole cell currents demonstrating store depletion. After 2 min I_{inward} was activated. (C) The same effects as shown in (B) could be observed when the sequence of agonist stimulation was reversed (representative of 20 experiments).

decreased extracellular Ca^{2+} concentration (10 μM). This led to a strong increase of I_{inward} measured in control solution (Figs. 4A and B; oocytes preincubated in control solution: $I = -0.41 \pm 0.05$ μA, $n = 31$; thapsigargin: $I = -0.54 \pm 0.07$ μA, $n = 9$; and thapsigargin in 10 μM Ca^{2+}: $I = -3.95 \pm 0.6$ μA, $n = 8$). The current detected in oocytes preincubated in control solution containing thapsigargin also showed a high dependence on extracellular Ca^{2+} comparable to the experiments shown in Fig. 2A (Fig. 4C; $\Delta I = 1.0 \pm 0.29$ μA, $n = 9$). Like thapsigargin, application of the Ca^{2+} ionophore ionomycin (1 μM) activated I_{inward} ($n = 4$, data not shown).

In order to compare the biophysical properties of I_{inward} induced by over-expression versus stimulation of endogenous receptors or store depletion by thapsigargin we studied the anion selectivities of the Cl^- currents. The anion selectivity for $P2Y_1$-R and M1-R expressing oocytes compared to trypsin or thapsigargin stimulated oocytes was invariably $I^- > Br^- > Cl^-$, respectively (Fig. 4D). This observation is in agreement with the selectivity that was reported for the endogenous Ca^{2+}-activated Cl^- current in *Xenopus* oocytes [21].

I_{inward} is active at physiological membrane potentials

There are many reports on hyperpolarization-activated cation currents in *Xenopus* oocytes. Most of these currents are activated at membrane potentials more negative than −150 mV [18,22]. However, these membrane potentials do not occur under physiological conditions. We therefore tested the activity of the channel at a holding potential of −50 mV which is close to the resting membrane potential of *Xenopus* oocytes. Oocytes were held at −50 mV for 30 s in a bath solution containing 10 μM Ca^{2+}. Then the bath solution was changed to a solution containing 18 mM Ca^{2+}. Oocytes expressing G_q-coupled receptors ($P2Y_1$-R, M1-R) and oocytes preincubated in thapsigargin (10 μM Ca^{2+}) showed significant inward currents (Figs. 5A and B; control oocytes: $I = 0.01 \pm 0.03$ μA, $n = 24$; M1-R:

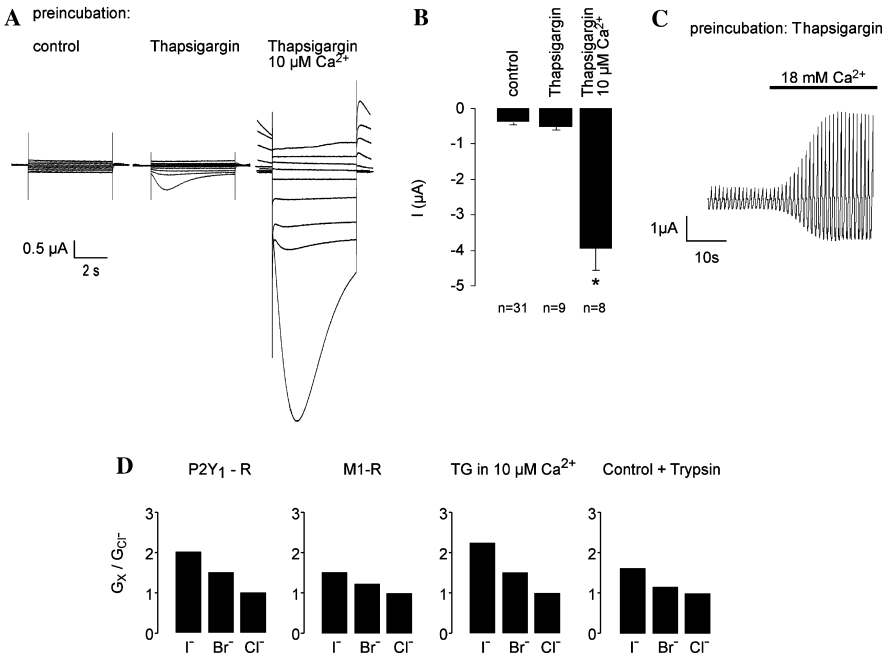

Fig. 4. Activation of I_{inward} by receptor-independent Ca^{2+} store depletion. (A) Representative traces of oocytes preincubated with thapsigargin. Control oocytes were preincubated with thapsigargin (1 μM for at least 2 h) in solutions containing physiological or low (10 μM) Ca^{2+} concentrations. (B) Statistical analysis of the current peaks measured at a holding potential of −100 mV. Asterisks indicate statistical significance. (C) Representative experiment of an oocyte preincubated with thapsigargin. Voltage ramps as depicted in Fig. 1D. (D) Anion conductance sequences of the currents induced by different protocols (normalized to Cl^-: G_x/G_{Cl}) ($n = 4$–5).

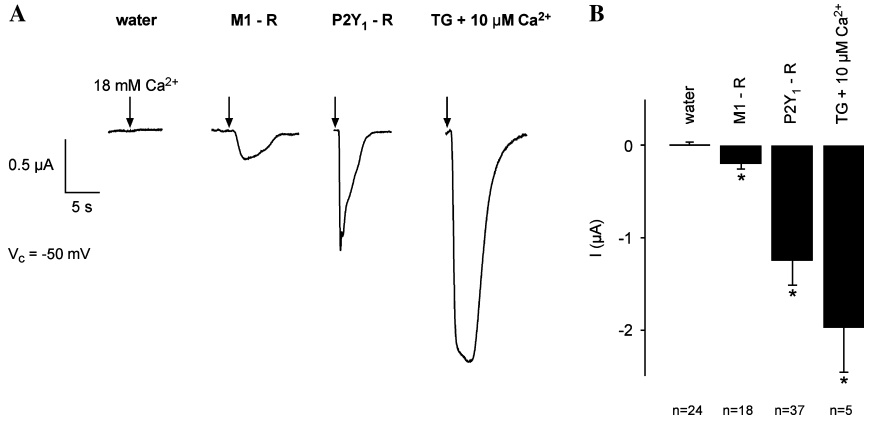

Fig. 5. Ca^{2+} influx after store depletion. (A) Oocytes were held at a potential of −50 mV for 30 s in a solution containing a low Ca^{2+} concentration (10 μM). Then they were exposed to a high extracellular Ca^{2+} concentration (18 mM, arrows). (B) Statistical analysis of the peak currents. Asterisks indicate statistical significance.

$I = -0.21 \pm 0.06$ μA, $n = 18$; $P2Y_1$-R: $I = -1.26 \pm 0.22$ μA, $n = 37$; and oocytes preincubated in 1 μM thapsigargin in 10 μM Ca^{2+}: $I = -1.99 \pm 0.49$ μA, $n = 5$). This indicates that I_{inward} mediates Ca^{2+} entry in *Xenopus* oocytes in the physiological voltage range.

La^{3+} and Niflumate inhibit I_{inward} at high concentrations

La^{3+} and Niflumate are two inhibitors of non-selective cation channels and capacitative Ca^{2+} entry [23]. Both were used in a concentration of 500 μM and inhibited I_{inward} significantly in oocytes expressing M1 receptors after stimulation with 10 μM acetylcholine (Figs. 6A and B; $G_{control} = 18.51 \pm 3.03$ μS, $G_{La^{3+}} = 9.99 \pm 1.6$ μS, $n = 17$; Figs. 6C and D; $G_{control} = 27.2 \pm 5.27$ μS, and $G_{Niflumate} = 13.0 \pm 5.4$ μS, $n = 5$).

Agonist stimulation of the $P2Y_1$ receptor slows the activation and inactivation kinetics of I_{inward}

As we observed I_{inward} in oocytes expressing G_q-coupled receptors without agonist stimulation, we wondered whether stimulation of the receptors might modulate I_{inward}. Hence, we stimulated $P2Y_1$ receptor-

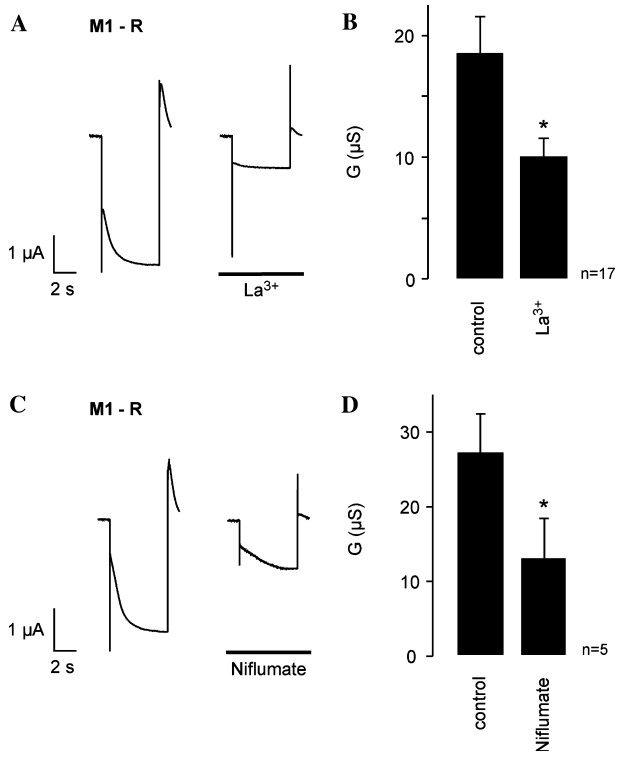

Fig. 6. Inhibition of I_{inward} by La^{3+} or Niflumate. Whole cell conductance of M1 receptor-overexpressing cells could be reduced significantly by La^{3+} (A,B) or Niflumate (C,D) (both 500 µM). Asterisks indicate statistical significance.

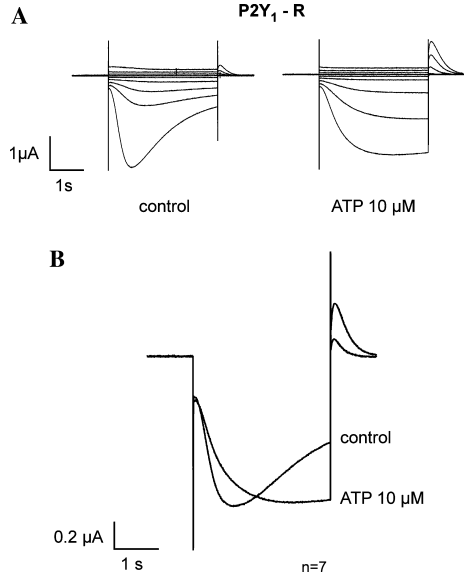

Fig. 7. Agonist stimulation of the $P2Y_1$ receptor slowed the activation and inactivation kinetics of I_{inward}. (A) $P2Y_1$-R-expressing oocytes were clamped from −100 to +60 mV in 20 mV steps for 3 s. Paired experiments with and without 10 µM ATP. (B) Mean currents at −100 mV of seven experiments. Agonist stimulation with ATP significantly slowed the activation and inactivation kinetics measured 20 s after adding the agonist ($p < 0.05$).

expressing oocytes with 10 µM ATP which resulted in a slowing of the activation and inactivation kinetics (Figs. 7A and B). The analysis of the kinetic properties in the presence of ATP was performed 20 s after agonist application. This change of kinetics after receptor stimulation was also observed in the presence of elevated extracellular Ca^{2+} (Fig. 2D).

Discussion

Our findings demonstrate that overexpression of different G_q-coupled receptors activates an inwardly rectifying current (I_{inward}) in *Xenopus* oocytes. Previously, it has been suggested that the $P2Y_1$ receptor, in contrast to other G protein-coupled receptors (GPCR), might function as an ionotropic receptor [10]. This hypothesis was based on the observation that $P2Y_1$ receptor overexpression in *Xenopus* oocytes gave rise to an inwardly rectifying whole cell current with some characteristics resembling those of the current we describe here. Interestingly, we found that I_{inward} is also activated upon overexpression of other G_q-coupled receptors (M1 receptor and $P2Y_6$ receptor). Since I_{inward} was observed without stimulation of the receptors we wondered whether this current is induced by GPCR signaling or if it might occur simply due to overexpression of membrane proteins in *Xenopus* oocytes [24]. Hence, we stimulated two different endogenous G_q-coupled receptors in water-injected oocytes which are known to couple to IP_3-induced Ca^{2+} release from internal stores [19,20]. With a delay of about 2 min after stimulation of the endogenous oocyte receptors we observed I_{inward}, suggesting a role of this current in GPCR-induced Ca^{2+} signaling. In particular, we speculated that I_{inward} could be activated after store depletion. In *Xenopus* oocytes, like in many other non-excitable cells, increased IP_3 concentrations release Ca^{2+} from intracellular stores and subsequently evoke Ca^{2+} influx, a process called capacitative Ca^{2+} entry [1,3,5,7,23,25]. Fortunately, *Xenopus* oocytes offer a unique property to monitor transmembrane Ca^{2+} entry because the Ca^{2+}-activated Cl^- channels located in the plasma membrane can be used as sensitive Ca^{2+} detectors. In an earlier study it was shown that this Cl^- current behaves linearly with membrane potential and responds in a linear fashion to elevation of the cytosolic Ca^{2+} concentration [5]. Using the Ca^{2+}-activated channels to monitor Ca^{2+} influx we took advantage of the fact that these channels provide a robust measure of the submembranous Ca^{2+} concentration whereas it is almost impossible to directly measure capacitative Ca^{2+} currents in *Xenopus* oocytes under physiological conditions, i.e., without heavy buffering of cytosolic calcium [4,5,26].

The observation that stimulation of endogenous GPCRs also activated I_{inward} prompted us to investigate

whether this activation is caused by depletion of intracellular Ca^{2+} stores. Depletion of the Ca^{2+} stores in a receptor-independent manner using thapsigargin or ionomycin also resulted in activation of I_{inward}. Since both inhibition of SERCA by thapsigargin and membrane permeabilization with ionomycin are commonly used maneuvers to activate CCE, this may point to a role for I_{inward} in CCE in *Xenopus* oocytes [1]. Furthermore, it indicates that overexpression of G_q-coupled receptors might lead to store depletion without experimental stimulation of the receptors. What could be possible explanations for this phenomenon? First, it is known that overexpression of GPCRs induces constitutive activation of signaling cascades. This might result in constitutive activation of the IP_3 cascade in cells overexpressing G_q-coupled receptors leading to store depletion without receptor stimulation. Second, in cells expressing P2Y receptors, autocrine stimulation of the receptors by nucleotides secreted from oocytes might lead to store depletion [27]. Interestingly, about 50% of the oocytes expressing $P2Y_1$ and $P2Y_6$ receptors did not show any nucleotide-induced Ca^{2+} release from stores as depicted in Fig. 1C. However, in these cells I_{inward} was observed invariably and receptor stimulation led to the typical change of kinetics of the current (Fig. 7). This shows that overexpression of these receptors per se may lead to store depletion. We did no attempts to study the underlying mechanisms in more detail since the physiological significance of store depletion after overexpression of receptors is questionable.

Even though others found similarities of CCE in *Xenopus* with I_{crac} in mast and other cells, the current we describe here is clearly distinct from I_{crac} [5,6,25]. For instance, La^{3+} does not inhibit I_{inward} in the low micromolar range whereas it potently blocks I_{crac} [25]. Moreover, the inward rectification of I_{inward} is much stronger than that of I_{crac}.

It was an intriguing finding that receptor stimulation slowed the deactivation kinetics of I_{inward} even though the current could already be observed in $P2Y_1$-R-expressing oocytes without stimulation of the receptor (Fig. 7). We can only speculate on the putative reasons for the changes of the kinetic properties of the current. Among many possible explanations one might envision: (i) direct modulation of the channel by G proteins and (ii) modulation of the channel by protein kinase C [5]. The slowing of deactivation kinetics might modulate Ca^{2+} entry after receptor stimulation. The Ca^{2+}-activated Cl^- channel in *Xenopus* oocytes does not show inactivation [5]. Therefore, this regulation seems to be a property of I_{inward}. However, we cannot completely rule out that ATP modulates the Ca^{2+}-activated Cl^- channels, Ca^{2+} extrusion or sequestration in *Xenopus* oocytes.

The activation of I_{inward} by overexpression of different GPCRs should be kept in mind when coexpression studies of ion channels with GPCRs are performed. Under these conditions studies on channel regulation have to be carefully evaluated since I_{inward} might interfere with the analysis of the properties of the channel being studied. Finally, our data do not support the hypothesis that the $P2Y_1$ receptor might function as an ionotropic receptor.

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