

## **Pharmacological and signaling properties of endogenous P2Y1 receptors in CFTR-expressing CHO cells**

BRICE MARCET<sup>1†</sup>, VALERIE CHAPPE<sup>2</sup>, PATRICK DELMAS<sup>3</sup> and BERNARD VERRIER<sup>1</sup>

<sup>1</sup>Institut de Neurosciences Physiologiques et Cognitives, CNRS-INPC, 13402 Marseille (France).

<sup>2</sup>McGill University, Department of Physiology, Montreal (Canada).

<sup>3</sup>Intégration des Informations Sensorielles, CNRS-UMR 6150, Faculté de Médecine, IFR Jean Roche, Bd pierre Dramard, 13916 Marseille (France).

This work was supported by institutional grants from the Centre National de la Recherche Scientifique (CNRS) and the Institut National de la Santé Et de la Recherche Médicale (INSERM), and by the French association “Vaincre la Mucoviscidose”. B.M. was supported by the French association “Vaincre la Mucoviscidose”.

**Running title : CFTR expression and P2Y1 receptor signaling pathway**

†To whom correspondence should be addressed:

BRICE MARCET, INPC-CNRS, 31 Chemin Joseph Aiguier 13402 Marseille cedex 20,  
France.

Phone: +33491164521, Fax: +33491775084

E-mail: [marcet@dpm.cnrs-mrs.fr](mailto:marcet@dpm.cnrs-mrs.fr)

Number of text pages : 26

Number of figures : 6

Number of references : 40

Number of words in Abstract : 249

Number of words in Introduction : 442

Number of words in Discussion : 978

Section assignment : Cellular & Molecular

Non-standard abbreviations used : adenylate cyclase, AC; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CHO, Chinese Hamster Ovary; FSK, forskolin;  $[Ca^{2+}]_i$ , intracellular calcium concentration; MRS2179, N6-methyl 2'-deoxyadenosine 3',5'-bisphosphate; P2YR, P2Y receptor; PLC $\beta$ , phospholipase C $\beta$ ; PPADS, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid; PTX, *pertussis* toxin; RT-PCR, reverse transcriptase-polymerase chain reactions.

## Abstract

The cystic fibrosis (CF) transmembrane conductance regulator (CFTR) is a cAMP-dependent Cl<sup>-</sup> channel that is defective in CF disease. CFTR activity has been shown to be regulated by the G<sub>q</sub>PLC-linked P2Y<sub>2</sub> subtype of P2Y nucleotide receptors (P2YR) in various systems. Here, we tested whether other P2YR may exert a regulation on CFTR activity and whether CFTR may in turn exert a regulation on P2YR signaling. Using reverse transcriptase-polymerase chain reactions (RT-PCR), antisense oligodeoxynucleotide knock-down and measurements of intracellular calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>), we showed that besides P2Y<sub>2</sub>R, Chinese Hamster Ovary (CHO) cells also express functional P2Y<sub>1</sub>R. P2Y<sub>1</sub>R were activated by 2-MeSADP>2-MeSATP>ADP with EC<sub>50</sub> of 30 nM, 0.2 μM and 0.8 μM, respectively. Activation of P2Y<sub>1</sub>R increased [Ca<sup>2+</sup>]<sub>i</sub>, which was prevented by the P2Y<sub>1</sub>R antagonists, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS, 10 μM) and N6-methyl 2'-deoxyadenosine 3',5'-bisphosphate (MRS2179, 10 μM) and by pre-treatment with P2Y<sub>1</sub>R antisense oligodeoxynucleotides. In CHO-K1 and CHO-KNUT (mock-transfected) cells lacking CFTR, both P2Y<sub>1</sub>R and P2Y<sub>2</sub>R caused [Ca<sup>2+</sup>]<sub>i</sub> mobilization via *pertussis* toxin (PTX)-insensitive G<sub>q/11</sub>-proteins. In contrast, in CFTR-expressing CHO cells (CHO-BQ1), the P2Y<sub>1</sub>R response was completely PTX-sensitive, indicating that P2Y<sub>1</sub>R couples to G<sub>i/o</sub>-proteins, whereas the P2Y<sub>2</sub>R response remained PTX-insensitive. In CHO-BQ1 cells, P2Y<sub>1</sub>R activation by ADP (100 μM) failed to inhibit both forskolin (1 μM)-induced CFTR activation, measured using iodide (<sup>125</sup>I) efflux, and forskolin (0.1 to 10 μM)-evoked cAMP increase. Taken together, our results indicate that, in contrast to P2Y<sub>2</sub>R, P2Y<sub>1</sub>R does not modulate CFTR activity in CHO cells and that CFTR expression may alter the G-protein coupling selectivity of P2Y<sub>1</sub>R.

## Introduction

Cystic fibrosis (CF), one of the most common lethal autosomal recessive genetic disease, is due to mutations in CFTR (Riordan et al., 1989), a cAMP-regulated Cl<sup>-</sup> channel that is localized in apical membranes of epithelia (Cheng et al., 1991). However, the pleiotropic effects due to CFTR dysfunctions and observed on epithelial ion transport in CF largely exceed the impairment of the Cl<sup>-</sup> channel function and may be related to other regulatory properties of CFTR. Indeed, in addition to functioning as a cAMP-regulated Cl<sup>-</sup> channel, CFTR has been shown to regulate several ion channels notably by activating P2YR subsequent to ATP release (Schwiebert et al., 1995; Urbach and Harvey, 1999).

Extracellular nucleotides are important signaling molecules that mediate diverse biological effects, including ion transport regulation, by acting on nucleotide-gated ion channel P2X(1-7) and G-protein linked P2YR(1-14) (see for reviews Ralevic and Burnstock, 1998; Abbracchio et al., 2003; Leipziger, 2003). Several studies emphasized the role of the P2Y2R subtype as key control for epithelial Cl<sup>-</sup> secretion by activating Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels that may constitute a potential substitute to Cl<sup>-</sup> secretion defect in CF (Knowles et al., 1991; Weisman et al., 1998). Moreover, P2Y2R has been shown to regulate CFTR activation (Paradiso et al., 2001; Marcet et al., 2003). For example, we have previously shown that, in CHO cells, the endogenous G<sub>q</sub>-coupled P2Y2R, which is equipotently activated by ATP and UTP, inhibited the CFTR activation in a cAMP independent manner (Marcet et al., 2003). Furthermore, a former work by Iredale and Hill (1993) has shown that CHO cells also respond to ADP by increasing [Ca<sup>2+</sup>]<sub>i</sub> though the P2YR subtype involved was not identified. Thus, it could be hypothesized that, like P2Y2R, other endogenous P2YR signaling can functionally modulate the CFTR channel. For example, the P2Y1R subtype, which is activated by ADP, is often colocalized with P2Y2R and CFTR in epithelial plasma

membrane (Homolya et al., 1999). The P2Y1R has been described to primarily couple to PTX-insensitive  $G_{q/11}$ -proteins and to promote  $[Ca^{2+}]_i$  increase subsequent to phospholipase  $C\beta$  (PLC $\beta$ ) stimulation (Ralevic and Burnstock, 1998).

In the present study, we have molecularly and functionally characterized the P2Y1R that is endogenously expressed in the CFTR-expressing CHO-BQ1 cells (Tabcharani et al., 1991; Chang et al., 1998). We found that the native P2Y1R, in contrast to the P2Y2R (Marcet et al., 2003), does not regulate the CFTR activity. However, we observed that, in the presence of CFTR, the G-protein coupling selectivity of native P2Y1R shifted from a  $G_{q/11}$ -protein coupling to a  $G_{i/o}$ -protein one, whereas that of P2Y2R remained unaltered. This suggests that the presence of CFTR may influence G-protein coupling selectivity of P2Y nucleotide receptors, a previously unsuspected aspect of CFTR regulatory function.

## Methods

### Chemicals and solutions

Fura2-AM was purchased from Molecular Probes (Netherlands). ATP, ADP, 2-methylthio-ATP (2-MeSATP), 2-methylthio-ADP (2-MeSADP), PPADS, PTX, fetal calf serum, antibiotics, forskolin (FSK) and other chemicals were purchased from Sigma (St Louis, MI).

### Cell culture

CHO cells, stably transfected with pNUT vector containing wild-type CFTR (CHO-BQ1), and KNUT cells, transfected with pNUT vector alone (mock-transfected cells) (Chang et al., 1998), were maintained in  $\alpha$ MEM and supplemented with methotrexate (100  $\mu$ M). CHO-K1 cells (parental) were maintained in DMEM/F12. Culture mediums were supplemented with 7.5% fetal calf serum, 2mM glutamine, penicillin (50 IU/ml), streptomycin (50  $\mu$ g/ml), and cells were grown in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

### Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to detect and identify P2Y<sub>1</sub>R mRNA in CHO cells, as previously described (Marcet et al., 2003). Total RNA was extracted from CHO cells using TriReagent (Euromedex, France) according to the manufacturer's instructions. First-strand cDNA synthesis was performed with 4  $\mu$ g of total RNA using the SuperScript preamplification system (Life Technologies, France). To rule out any genomic DNA contamination, total RNA was incubated with RQ1 DNase-RNase-free (Promega, France), and experiments were also performed without RT. To amplify the P2Y<sub>1</sub>R cDNA, a set of specific primers (sense primer, 5'-ATG-TTC-AAT-TTG-GCT-CTG-GC-3', antisense primer, 5'-CTG-TTG-AGA-CTT-GCT-AGA-CCT-CTT-GT-3') was designated from highly conserved regions between human

and mouse P2Y1 gene sequences (Tokuyama et al., 1995; Ayyanathan et al., 1995; Janssens et al., 1996) and synthesized (Eurogentec, Belgium). cDNAs were amplified by PCR (Robocycler, Stratagene, Europe) for 40 cycles at an annealing temperature of 50°C using P2Y1 specific primers (500 pM each) with 2.5U of the Taq DNA polymerase (Promega, France) in 1.5 mM MgCl<sub>2</sub>. PCR products were analyzed and sequenced (Eurogentec, Belgium). As a negative control, PCR was carried out as before, except that water was added instead of DNA template.

### **Sequence analysis**

DNA sequences obtained were identified, analyzed and compared with sequences compiled in different banks using computer programs BLAST, FASTA and ClustalW (version 1.8) on the infobiogen site (<http://www.infobiogen.fr>).

### **Measurement of Intracellular Free Calcium**

Fura-2 fluorescence Ca<sup>2+</sup> imaging was used to measure [Ca<sup>2+</sup>]<sub>i</sub> as previously described (Marcet et al., 2003). Briefly, cells were cultured four days on glass coverslips, and then loaded with 2.5 μM fura-2/AM for 1h at 37°C in serum-free DMEM/F12 medium. After rinsing with modified Earle's salt solution (B medium) containing (in mM): 137 NaCl, 5.36 KCl, 0.8 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 5.5 glucose, 10 HEPES-NaOH, pH 7.4, cells were placed into an open-topped microperfusion chamber and superfused with test solutions at 1ml/min. Ratiometric fluorescence (340/380) was used to calculate [Ca<sup>2+</sup>]<sub>i</sub> according to Grynkiewicz et al. (Grynkiewicz et al., 1985). For each experiment, fifty cells were individually analyzed and [Ca<sup>2+</sup>]<sub>i</sub> was averaged and plotted versus time. Agonists concentration-response curves were fitted using the Hill equation and the half maximal effect (EC<sub>50</sub>) values were calculated using GraphPad Prism v3.0 (GraphPad Software).

### **Antisense knockdown experiments**

We designated 19-mer phosphorothioate oligodeoxynucleotides (PTO-ODNs) according to the cDNA sequence for P2Y1R cloned in this study: P2Y1 antisense oligodeoxynucleotide (5'-GAA-GAT-CCA-GTC-AGT-CTT-G-3') and P2Y1 scrambled oligodeoxynucleotide (5'-AGA-TCG-CTT-GGA-CAT-ATG-C-3') as a negative control. PTO-ODNs, labeled with the fluorescent compound Cy3 (Indocarbocyanin) at the 5'-site in order to visualize and enhance cell penetration, were commercially synthesized and purified by HPLC (Eurogentec, Belgium).

Cells were plated onto Corning 35-mm dish (Dutscher, France) on microscope slide. The third day of culture, PTO-ODNs were directly added to subconfluent cells in culture medium at the optimal concentration of 2.5  $\mu$ M for 24 h.

### **Measurement of intracellular cAMP**

CHO cells grown four days in 12-well plates were washed twice with 2 ml of B medium, then 0.5 ml of this buffer containing the drug to be tested were added to each well. After a 5 min incubation period at 37°C, the cells were permeabilized, and intracellular cAMP content was measured using the cAMP Biotrack Enzymeimmunoassay (EIA) system according to the manufacturer's instructions (Amersham Biosciences, UK). cAMP levels were expressed as pmol / well  $\pm$  S.D.

### **Determination of iodide efflux**

<sup>125</sup>I efflux technique was used to monitor CFTR activation as previously described (Marcet et al., 2003). At the fourth day of culture, cells were loaded in B medium containing 1 $\mu$ M KI and 0.5  $\mu$ Ci of <sup>125</sup>INa/ml for 30 min, then washed. 500  $\mu$ l of B medium containing or not

agonists or antagonists to be tested were added and removed sequentially every 30 s for 2.5 min. At the end of the efflux, intracellular ions were extracted by the addition of 1 ml trichloroacetic acid (7.5%) to the cell layer. All samples were counted using a  $\gamma$  counter (Kontron). Tracer contained in the cell layer at the onset of the efflux was calculated as the sum of samples and extract count. Efflux curves were constructed by plotting the percent of  $^{125}\text{I}$  accumulated in the medium versus time and fitting as mono-exponential function using GraphPad Prism v3.0 (GraphPad Software). Data are expressed as means  $\pm$  S.D.

## Results

### P2Y1R is constitutively expressed in CHO cells

Previously, we have cloned and characterized P2Y2R natively expressed in CHO cells (Marcet et al., 2003). To test whether CHO cells also constitutively express P2Y1R, we designed specific primers from highly conserved regions of mouse and human P2Y1R sequences. A product of 665 base pairs (bp) was amplified by RT-PCR from cDNA isolated from both CHO-K1 (data not shown) and CFTR-expressing CHO-BQ1 cells (Fig. 1). The amplified sequence (GenBank™ accession number AY049063) shared 94% of nucleotide identity with both mouse and rat P2Y1R (data not shown). To functionally characterize P2Y1R, we used fura-2  $\text{Ca}^{2+}$  imaging (see Methods). The resting  $[\text{Ca}^{2+}]_i$  was determined over a period of 3 min in the presence of normal extracellular  $\text{Ca}^{2+}$  and averaged ( $108.1 \pm 10.8$  nM;  $n = 10$ ). Addition of ADP (10  $\mu\text{M}$ ) the natural agonist of P2Y1R evoked a sharp and monophasic  $\text{Ca}^{2+}$  response in CHO-BQ1 cells (Fig. 2A) as well as in the mock-transfected CHO-KNUT cell line or in the parental CHO-K1 cells (data not shown). The amplitude of the peak over basal level ( $[\Delta\text{Ca}^{2+}]_i$ ) reached  $47.5 \pm 13$  nM ( $n = 7$ ) (Fig. 2C). The same amplitude of ADP-induced  $\text{Ca}^{2+}$  response was obtained in  $\text{Ca}^{2+}$ -free solution ( $n = 3$ ) (Figure 2B), indicating that the rise in  $[\text{Ca}^{2+}]_i$  caused by ADP is mainly due to  $\text{Ca}^{2+}$  release from intracellular stores.

### Pharmacological characterization of P2Y1R

Transient  $\text{Ca}^{2+}$  responses were also evoked by the P2Y1R agonists 2-MeSATP (10  $\mu\text{M}$ ) and 2-MeSADP (10  $\mu\text{M}$ ) in CHO-BQ1 cells (Figs. 2C,D,E). Hechler et al. (1998) reported that the apparent agonist activity of 2-MeSATP was actually due to contamination of 2-MeSATP from commercial sources with 2-MeSADP. To eliminate the possibility that the

response to 2-MeSATP was due to the presence of 2-MeSADP, cells were stimulated with 2-MeSATP which had been pre-treated 90 min at room temperature with 20 unit/ml creatine phosphate kinase (CPK) and 10 mM creatine phosphate (CP) to regenerate ATP derivatives from potential ADP derivative contaminants. This treatment did not abolish the agonist activity of 2-MeSATP ( $n = 3$ ) on the  $\text{Ca}^{2+}$  response, indicating that 2-MeSATP promoted the  $\text{Ca}^{2+}$  response (Fig. 2C). Application of 2-MeSADP (10  $\mu\text{M}$ ) prior to ADP strongly desensitized the ADP-induced  $\text{Ca}^{2+}$  response, indicating a cross-desensitization between these two agonists (Fig. 2F). Moreover, pre-addition of PPADS (10  $\mu\text{M}$ ) or MRS2179 (10  $\mu\text{M}$ ), both used to preferentially antagonize P2Y1R (Brown et al., 1995; Moro et al., 1998), abolished ADP-induced  $\text{Ca}^{2+}$  response (Fig. 2F). Figure 2G shows the effect of increasing concentrations of 2-MeSADP, 2-MeSATP and ADP on  $[\text{Ca}^{2+}]_i$ . The rank order of potency was 2-MeSADP > 2-MeSATP > ADP with  $\text{EC}_{50}$  values of 30 nM, 0.2  $\mu\text{M}$  and 0.8  $\mu\text{M}$ , respectively.

Next, we examined the putative protein kinase C (PKC)-dependent desensitization of the ADP response using the PKC activator-TPA (10 nM for 30 min;  $n = 3$ ). ADP-induced  $\text{Ca}^{2+}$  response was abolished by TPA pre-treatment (Fig. 3). Moreover, a treatment with GF109203X (10 nM for 30 min;  $n = 3$ ), used to block specifically PKC, had no effect on the time course of the  $\text{Ca}^{2+}$  signal induced by ADP (Fig. 3), indicating that the rapid decay of  $\text{Ca}^{2+}$  responses induced by ADP was not due to rapid desensitization by PKC.

### **Inhibition of ADP-induced $\text{Ca}^{2+}$ response with specific antisense oligodeoxynucleotides**

Because antagonists capable of discriminating among the different subtypes of P2Y receptors are not currently available, we have developed specific P2Y1 antisense knockdown strategy (see Methods) to further demonstrate that ADP-induced  $\text{Ca}^{2+}$  response was specifically mediated by P2Y1R in CHO-BQ1 cells. The ADP-induced  $\text{Ca}^{2+}$  response was

abolished by specific P2Y1 antisense treatment (2.5  $\mu$ M for 24 h) ( $P < 0.001$ , *t-test*;  $n = 6$ ) but was not significantly changed by scrambled antisense treatment ( $P > 0.05$ , *t-test*;  $n = 5$ ) (Fig. 4). We have previously shown that ATP and UTP activate endogenous P2Y2R in CHO cells and that ATP-induced  $\text{Ca}^{2+}$  response was not due to P2X7R (Marcet et al., 2003), the sole P2X receptor expressed in CHO cells, and shown to be insensitive to ADP and 2-MeSATP (Michel et al., 1998). Figure 4 shows that the ATP-induced  $\text{Ca}^{2+}$  response, mediated by P2Y2R (Marcet et al., 2003), was not significantly modified by P2Y1 antisense treatment ( $P > 0.05$ , *t-test*;  $n = 6$ ). These results indicate that ADP-induced  $\text{Ca}^{2+}$  response is mediated by P2Y1R, thus excluding in this effect an involvement of other putative ADP receptors like P2Y12R or P2Y13R, which have amino acid sequence poorly related to P2Y1R (Communi et al., 2001; Hollopeter et al., 2001).

#### **Effect of P2Y1R stimulation on intracellular cAMP level**

Recently, two ADP receptors distinct from P2Y1R have been cloned and characterized: P2Y12R and P2Y13R (Hollopeter et al., 2001; Communi et al., 2001; Simon et al., 2002; Marteau et al., 2003). Since both P2Y12R and P2Y13R are known to negatively couple to adenylyate cyclase pathway (Simon et al., 2002; Marteau et al., 2003), we studied the modulation of the cAMP level by saturating concentration of ADP (100  $\mu$ M). We measured cAMP content in response to increasing concentrations of FSK (from 0.1 to 10  $\mu$ M;  $n = 8$ ) with or without ADP (100  $\mu$ M). No inhibition of the FSK-stimulated cAMP level was observed in response to ADP (Fig. 5A). These data, together with the pharmacological and antisense experiments, strongly suggest that the endogenous P2Y1R is the only ADP receptor subtype expressed in CHO cells.

### **Effect of P2Y1R stimulation on cAMP-induced CFTR activation**

Next, we tested whether P2Y1R activation could inhibit CFTR activation, as previously reported for the endogenous P2Y2R subtype (Marcet et al., 2003). We previously showed that FSK-stimulated  $^{125}\text{I}$  efflux was specific of CFTR activation in CHO-BQ1 cells (Chappe et al., 1998; Marcet et al., 2003). Here, we monitored FSK-induced CFTR activation in the presence or absence of ADP using the same  $^{125}\text{I}$  efflux technique. We tested the effect of a saturating concentration of ADP (100  $\mu\text{M}$ ) on FSK (1  $\mu\text{M}$ )-induced CFTR activation, concentration of FSK which evoked ~60% of maximal effect (Marcet et al., 2003). Activation of P2Y1R by ADP failed to produce any significant inhibition of FSK (1  $\mu\text{M}$ )-stimulated  $^{125}\text{I}$  efflux ( $n = 12$ ) (Fig. 5B). We confirmed that the lack of effect of P2Y1R on CFTR was not due to PKA-induced desensitization of the receptor, since FSK (10  $\mu\text{M}$ ) failed to alter the ADP-induced  $\text{Ca}^{2+}$  response ( $n = 3$ ) (Fig. 5C).

### **G-protein coupling selectivity exhibited by P2Y1R**

P2Y1R was often described to promote a rise in  $[\text{Ca}^{2+}]_i$  by mainly coupling to  $\text{G}_{q/11}$  proteins (see for review Ralevic and Burnstock, 1998). In order to determine the nature of the G-protein coupling of P2Y1R, we examined, using the fura2 fluorescence  $\text{Ca}^{2+}$  imaging, the G-protein coupling selectivity of P2Y1R in the absence or presence of CFTR. CHO-K1 (parental), CHO-KNUT (mock-transfected) and CHO-BQ1 cells were incubated with PTX (100 ng/ml) for 24 h. PTX treatment failed to inhibit  $\text{Ca}^{2+}$  responses to ADP and ATP in both CHO-K1 and KNUT cells (Figs. 6A,B,E,F) ( $P > 0.05$ , *t-test*;  $n = 3-6$ ). In striking contrast, PTX treatment in CHO-BQ1 cells (Figs. 6C,D,G) fully prevented ADP-induced  $\text{Ca}^{2+}$  response ( $P < 0.001$ , *t-test*;  $n=8$ ) without altering ATP response ( $P > 0.05$ , *t-test*;  $n = 8$ ). Consistently, UTP, another P2Y2R agonist, induced PTX-insensitive  $[\text{Ca}^{2+}]_i$  rise in CHO-K1, CHO-KNUT (not shown) and CHO-BQ1 cells (Marcet et al., 2003). Collectively, these data indicate that

P2Y1R and P2Y2R couple to PTX-insensitive  $G_{q/11}$ -proteins to mobilize  $[Ca^{2+}]_i$  in the absence of CFTR whereas, in CFTR-expressing cells, P2Y1R but not P2Y2R couples to PTX-sensitive  $G_{i/o}$ -proteins.

## Discussion

Growing evidence suggest functional interaction between CFTR and P2Y<sub>2</sub>R. P2Y<sub>2</sub>R can regulate CFTR activity in different systems (Paradiso et al., 2001; Marcet et al., 2003). Here, we extend these findings by showing that CFTR modulates P2Y<sub>1</sub>R signaling pathway. We report that CHO cells express, besides P2Y<sub>2</sub>R (Marcet et al., 2003), the P2Y<sub>1</sub>R subtype, which does not regulate CFTR activity in CHO-BQ1 cells. Moreover, we show that, in CHO cells, the expression of the recombinant CFTR (CHO-BQ1), but not the expression of the vector alone (CHO-KNUT), causes an apparent switch in the G-protein coupling of P2Y<sub>1</sub>R from a G<sub>q/11</sub>- to G<sub>i/o</sub>-type. This change in G-protein selectivity was specific of P2Y<sub>1</sub>R since P2Y<sub>2</sub>R G-protein coupling remained unaffected by CFTR.

Our findings that the P2Y<sub>1</sub>R antagonist MRS2179 and the P2Y<sub>1</sub>R knock-down antisense experiments abolished the ADP-induced Ca<sup>2+</sup> response, strongly suggest that putative hamster homologous of P2Y<sub>12</sub>- or P2Y<sub>13</sub>-G<sub>i</sub> protein-coupled receptors are not involved in the ADP-induced Ca<sup>2+</sup> response in CHO cells. P2Y<sub>1</sub>R has been shown to be activated by 2-MeSADP, 2-MeSATP, ADP and ATP (Palmer et al., 1998), although ATP or 2-MeSATP have been reported in some studies to be competitive antagonists of P2Y<sub>1</sub>R (Léon et al., 1997; Hechler et al., 1998). Here, we provide evidence that 2-MeSATP activated P2Y<sub>1</sub>R in CHO cells, and that this activation was not due to degradation of 2-MeSATP in 2-MeSADP derivative.

We have previously suggested an involvement of the plateau phase of the Ca<sup>2+</sup> response in the inhibition of CFTR by the G<sub>q/11</sub>-coupled P2Y<sub>2</sub>R (Marcet et al., 2003). On the contrary of that we previously observed for P2Y<sub>2</sub>R signaling (Marcet et al., 2003), P2Y<sub>1</sub>R activation evoked in CHO-BQ1, CHO-KNUT or CHO-K1 cells a relatively rapid and monophasic increase in [Ca<sup>2+</sup>]<sub>i</sub> mainly by releasing Ca<sup>2+</sup> from internal stores. Therefore, it can

be hypothesized that the lack of inhibitory effect of P2Y1R on CFTR activity was due in some way to the rapid and monophasic increase in  $[Ca^{2+}]_i$  produced by P2Y1R. Because kinetically similar P2Y1R-induced  $Ca^{2+}$  responses were observed in CHO-BQ1, CHO-KNUT and CHO-K1 cells, it is unlikely that the nature of the G-protein coupled to P2Y1R was responsible for the sharp P2Y1R  $Ca^{2+}$  response. Instead, the difference of  $Ca^{2+}$  signal between P2Y1R and P2Y2R may arise from a weak number of P2Y1R expressed in CHO cells which may not be sufficient to trigger  $Ca^{2+}$ -induced  $Ca^{2+}$  release and  $Ca^{2+}$  wave propagations, both phenomena known to be involved in generation of  $Ca^{2+}$  plateau phase (see for review Røttingen and Iversen, 2000). Furthermore, the short response promoted by P2Y1R activation was neither due to ADP degradation since the cells were continuously superfused with fresh ADP solution during the  $Ca^{2+}$  response nor to PKC-induced desensitization because inhibition of PKC by GF109203X failed to promote longer  $Ca^{2+}$  response.

Previous data have shown that P2Y2R coupled to PLC $\beta$  *via* PTX-insensitive  $G_{q/11}$  proteins in CHO cells (Strasheim and Williams, 2000; Marcet et al., 2003). Here, we show that, in the absence of CFTR, P2Y1R promotes  $[Ca^{2+}]_i$  rise in a PTX-insensitive manner, whereas P2Y1R mobilizes  $Ca^{2+}$  from intracellular stores in a PTX-sensitive manner in the presence of CFTR. This suggests that in CFTR-expressing CHO cells P2Y1R activates PLC- $\beta$  either *via*  $G\alpha_{i/o}$ -associated  $G\beta\gamma$  dimers, or *via* a cooperative process between  $G_q$ - and  $G_{i/o}$ -proteins, as suggested previously for P2Y2R and P2Y13R in other systems (Baltensperger and Porzig, 1997; Communi et al., 2001).

Recently, both P2Y1R and CFTR have been shown to interact with the  $Na^+/H^+$  exchanger regulatory factor (NHERF), a PDZ-domain-containing protein that plays a scaffold role in many transduction systems (Hall et al., 1998), including PLC $\beta$ -mediated signaling (Hwang et al., 2000). Interestingly, P2Y2R exhibited poor NHERF binding (Hall et al., 1998). Thus, one possible mechanism may be that in CHO-BQ1 cells P2Y1R and CFTR interact

with NHERF-like proteins. This interaction might provoke a reorganization of the signaling molecules associated with P2Y1R. Alternatively, CFTR expression may cause P2Y1R to assemble with other G-protein-coupled receptors to form heteromers, which would result in change in P2Y1R signaling, as previously suggested in other systems (Yoshioka et al., 2001). Since P2Y1R couples to  $G\alpha_{i/o}$ -proteins in CHO-BQ1 cells, one may have expected an inhibition of CFTR activity either by decreasing cAMP level or by inhibiting vesicle trafficking and exocytosis (Schwiebert et al., 1994). We did not observe such an inhibition of CFTR by P2Y1R. Moreover, we showed that the stimulation of the natively expressed P2Y1R did not inhibit adenylate cyclase (AC), since no reduction of the cAMP level was observed in response to ADP. In agreement with previous studies performed in CHO cells (Communi et al., 2001; Marteau et al., 2003), this suggests that putative P2Y12- or P2Y13- $G_i$  protein-coupled receptors are not functionally expressed in CHO cells. There are precedents showing that activation of  $G_i$  protein-coupled receptors failed to cause an AC pathway inhibition. For example, P2Y2R has been shown to couple to  $G_{i/o}$ -proteins without inhibiting AC pathway (Janssens et al., 1999). Although the reasons for this remain uncertain in our preparation, two main hypotheses can be put forward. One possibility may be that P2Y1R couples differentially to  $G\alpha_i$  subunits and that appropriate combination of G-proteins is required to inhibit the AC pathway, a proposal that has been substantiated previously (Liu et al., 1999). Alternatively, the absence of apparent inhibition on cAMP levels may result from a dual effect on overall AC activity by P2Y1R stimulation, for example by concordantly activating ACVII and inhibiting ACVI, two AC endogenously expressed in CHO cells and that are modulated by distinct signaling routes (Sunahara et al., 1996; Varga et al., 1998).

In conclusion, our data suggest that G-protein selectivity of P2Y1R may be dependent on CFTR, outlining further the multifunctional roles of CFTR. It will be therefore interesting to investigate P2YR coupling pathways in CF airway epithelial cells and to compare them

with those occurring in wild-type cells. This may be helpful for a better understanding of the multiple defects caused by CFTR dysfunctions.

### *Acknowledgments*

Authors would like to thank J.W. Hanrahan, J.R. Riordan and X.B. Chang for the gift of CHO cell lines. This work was supported by institutional grants from the Centre National de la Recherche Scientifique (CNRS) and the Institut National de la Santé Et de la Recherche Médicale (INSERM), and by the French association “Vaincre la Mucoviscidose”. B.M. was supported by a doctoral fellowship from “Vaincre la Mucoviscidose”.

## References

Abbracchio MP, Boeynaems J-M, Barnard EA, Boyer JL, Kennedy C, Miras-Portugal MT, King BF, Gachet C, Jacobson KA, Weisman GA and Burnstock G (2003) Characterization of the UDP-glucose receptor (re-named here the P2Y<sub>14</sub> receptor) adds diversity to the P2Y receptor family. *Trends Pharmacol Sci* **24**:52-55.

Ayyanathan K, Webbs TE, Sandhu AK, Athwal RS, Barnard EA and Kunapuli SP (1996) Cloning and chromosomal localization of the human P2Y<sub>1</sub> purinoceptor. *Biochem Biophys Res Commun* **218**:783-788.

Baltensperger K and Porzig H (1997) The P2U purinoceptor obligatorily engages the heterotrimeric G protein G16 to mobilize intracellular Ca<sup>2+</sup> in human erythroleukemia cells. *J Biol Chem* **272**:10151-10159.

Brown C, Tanna B and Boarder MR (1995) PPADS: an antagonist at endothelial P<sub>2Y</sub>-purinoceptors but not P<sub>2U</sub>-purinoceptors. *Br J Pharmacol* **116**:2413-2416.

Chang XB, Kartner N, Seibert FS, Aleksandrov AA, Kloser AW, Kiser GL and Riordan JR (1998) Heterologous expression systems for study of cystic fibrosis transmembrane conductance regulator. *Methods Enzymol* **92**:616-629.

Chappe V, Mettey Y, Vierfond JM, Hanrahan JW, Gola M, Verrier B and Becq F (1998) Structural basis for specificity and potency of xanthine derivatives as activators of the CFTR chloride channel. *Br J Pharmacol* **123**:683-693.

Cheng SH, Rich DP, Marshall J, Gregory RJ, Welsh MJ and Smith AE (1991) Phosphorylation of the R domain by cAMP-dependant protein kinase regulates the CFTR chloride channel. *Cell* **66**:1027-1036.

Communi D, Suarez-Gonzalez N, Detheux M, Brézillon S, Lannoy V, Parmentier M and Boeynaems J-M (2001) Identification of a novel human ADP receptor coupled to G<sub>i</sub>. *J Biol Chem* **276**:41479-41485.

Grynkiewicz G, Poenie M and Tsien RY (1985) A New generation of Ca<sup>2+</sup> indicators with greatly improved fluorescence properties. *J Biol Chem* **260**:3440-3450.

Hall RA, Ostedgaard LS, Premont RT, Blitzer JT, Rahman N, Welsh MJ and Lefkowitz RJ (1998) A C-terminal motif found in the β<sub>2</sub>-adrenergic receptor, P2Y<sub>1</sub> receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor family of PDZ proteins. *Proc Natl Acad Sci USA* **95**:8496-8501.

Hechler B, Vigne P, Léon C, Breittmayer JP, Gachet C and Frelin C (1998) ATP derivatives are antagonists of the P2Y<sub>1</sub> receptor: similarities to the platelet ADP receptor. *Mol Pharmacol* **53**:727-733.

Hollopeter G, Jantzen H-M, Vincent D, Li G, England L, Ramakrishnan V, Yang R-B, Nurden P, Nurden A, Julius D and Conley PB (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* **409**:202-207.

Homolya L, Watt WC, Lazarowski ER, Koller BH and Boucher RC (1999) Nucleotide-regulated calcium signaling in lung fibroblasts and epithelial cells from normal and P2Y<sub>2</sub> receptor (-/-) mice. *J Biol Chem* **274**:26454-26460.

Hwang JI, Heo K, Shin KJ, Kim E, Yun C, Ryu SH, Shin HS and Suh PG (2000) Regulation of phospholipase C-β<sub>3</sub> activity by Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor 2. *J Biol Chem* **275**:16632-16637.

Iredale PA and Hill SJ (1993) Increases in intracellular calcium via activation of an endogenous P<sub>2</sub>-purinoceptors in cultured CHO-K1 cells. *Br J Pharmacol* **110**: 1305-1310.

Janssens R, Communi D, Piroton S, Samson M, Parmentier M and Boeynaems J-M (1996) Cloning and tissue distribution of the human P2Y<sub>1</sub> receptor. *Biochem Biophys Res Commun* **221**:588-593.

Janssens R, Paindavoine P, Parmentier M and Boeynaems J-M (1999) Human P2Y<sub>2</sub> receptor polymorphism : identification and pharmacological characterization of two allelic variants. *Br J Pharmacol* **126**:709-716.

Knowles MR, Clarke LL and Boucher RC (1991) Activation by extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis. *N Engl J Med* **325**:533-538.

Leipzig J (2003) Control of epithelial transport via luminal P2 receptors. *Am J Physiol Renal Physiol* **284**:F419-F432.

Léon C, Hechler B, Vial C, Leray C, Cazenave JP and Gachet C (1997) The P2Y<sub>1</sub> receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. *FEBS Lett* **403**:26-30.

Liu YF, Ghahremani MH, Rasenick MM, Jakobs KH, and Albert PR (1999) Stimulation of cAMP synthesis by Gi-coupled receptors upon ablation of distinct Galphai protein expression. Gi subtype specificity of the 5-HT<sub>1A</sub> receptor. *J Biol Chem* **274**:16444-16450.

Marcet B, Chappe V, Delmas P, Gola M and Verrier B (2003) Negative regulation of CFTR activity by extracellular ATP involves P2Y<sub>2</sub> receptors in CFTR-expressing CHO cells. *J Membrane Biol* **194**:21-32.

Marteau F, Le Poul E, Communi D, Communi D, Labouret C, Savi P, Boeynaems JM and Gonzalez NS (2003) Pharmacological characterization of the human P2Y<sub>13</sub> receptor. *Mol Pharmacol* **64**:104-112.

Michel AD, Chessell IP, Hibell AD, Simon J and Humphrey PP (1998) Identification and characterization of an endogenous P2X7 (P2Z) receptor in CHO-K1 cells. *Br J Pharmacol* **125**:1194-1201.

Moro S, Guo D, Camaioni E, Boyer JL, Harden TK and Jacobson KA (1998) Human P2Y1 receptor: molecular modeling and site-directed mutagenesis as tools to identify agonist and antagonist recognition sites. *J Med Chem* **41**:1456-1466.

Palmer RK, Boyer JL, Schachter JB, Nicholas RA and Harden TK (1998) Agonist action of adenosine triphosphates at the human P2Y1 receptor. *Mol Pharmacol* **54**:1118-1123.

Paradiso AM, Ribeiro CM and Boucher RC (2001) Polarized signaling via purinoceptors in normal and cystic fibrosis airway epithelia. *J Gen Physiol* **117**:53-67.

Ralevic V and Burnstock G (1998) Receptors for Purines and Pyrimidines. *Pharmacol Rev* **50**:413-492.

Riordan JR, Rommens JM, Kerem B-S, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou J-L, Drumm ML, Iannuzzi MC, Collins FS and Tsui L-C (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245**:1066-1072.

Røttingen J and Iversen JG (2000) Ruled by waves? Intracellular and intercellular calcium signalling. *Acta Physiol Scand* **169**:203-219.

Schwiebert EM, Egan ME, Hwang TH, Fulmer S, Allen SS, Cutting GR and Guggino WB (1995) CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. *Cell* **81**:1063-1073.

Schwiebert EM, Gesek F, Ercolani L, Wjasow C, Gruenert DC, Karlson K and Stanton BA (1994) Heterotrimeric G proteins, vesicle trafficking, and CFTR Cl<sup>-</sup> channels. *Am J Physiol* **267**:C272-C281.

Simon J, Filippov AK, Goransson S, Wong YH, Frelin C, Michel AD, Brown DA and Barnard EA (2002) Characterization and channel coupling of the P2Y<sub>12</sub> nucleotide receptor of brain capillary endothelial cells. *J Biol Chem* **277**:31390-31400.

Strasheim D and Williams CL (2000) P2Y<sub>2</sub> purinergic and M3 muscarinic acetylcholine receptors activate different phospholipase C-beta isoforms that are uniquely susceptible to protein kinase C-dependent phosphorylation and inactivation. *J Biol Chem* **275**:39767-39772.

Sunahara RK, Dessauer CD and Gilman A (1996) Complexity and diversity of mammalian adenylyl cyclases. *Annual Rev Pharmacol Toxicol* **36**:461-480.

Tabcharani JA, Chang XB, Riordan JR and Hanrahan JW (1991) Phosphorylation-regulated Cl<sup>-</sup> channel in CHO cells stably expressing the cystic fibrosis gene. *Nature* **352**:628-631.

Tokuyama Y, Hara M, Jones EM, Fan Z and Bell GI (1995) Cloning of rat and mouse P2Y purinoceptors. *Biochem Biophys Res Commun* **211**:211-218.

Urbach V and Harvey BJ (1999) Regulation of intracellular Ca<sup>2+</sup> by CFTR in Chinese Hamster Ovary Cells. *J Membrane Biol* **171**:255-265.

Varga EV, Stropova D, Rubenzik M, Wang M, Landsman RS, Roeske WR and Yamamura HI (1998) Identification of adenylyl cyclase isoenzyme in CHO and B82 cell. *Eur J Pharmacol* **348**:R1-R2.

Weisman GA, Garrad RC, Erb LJ, Otero M, Gonzalez FA and Clarke LL (1998) Structure and function of P2Y<sub>2</sub> nucleotide receptors in cystic fibrosis (CF) epithelium. *Adv Exp Med Biol* **431**:417-424.

Yoshioka K, Saitoh O and Nakata H (2001) Heteromeric association creates a P2Y-like adenosine receptor. *Proc Natl Acad Sci USA* **98**:7617-7622.

## Figure legends

**Fig. 1. Detection of P2Y1 mRNAs in CHO-BQ1 cells by RT-PCR.** The extraction of total RNA and the amplification by RT-PCR were performed as described in Methods. Amplification with P2Y1 specific primers resulted in a single band of a 665-bp product (lane 3). Negative controls were carried out for RT (lane 1) and PCR (lane 2) experiments.

**Fig. 2. Effects of P2Y1R agonists on Ca<sup>2+</sup> responses.** (A, C, D and E) show the Ca<sup>2+</sup> responses for ADP, 2-MeSATP with or without CP (10 mM)/CPK (20 unit/ml) treatment as indicated on bars of the figure, and 2-MeSADP (10 μM each) in the presence of extracellular Ca<sup>2+</sup>. (B) shows the ADP-induced Ca<sup>2+</sup> response in the absence of extracellular Ca<sup>2+</sup>. Traces shown are representative of n independent experiments (n = 3-8). (F) Cross-desensitisation between ADP- and 2-MeSADP-stimulated Ca<sup>2+</sup> responses, and inhibition of ADP-stimulated Ca<sup>2+</sup> responses by PPADS (10 μM) or MRS2179 (10 μM). ADP-induced Ca<sup>2+</sup> responses were measured following no pre-addition (*None*) or pre-addition of 10 μM of 2-MeSADP, PPADS (10 μM) or MRS2179 (10 μM) (n = 3-8). (G) Concentration-response relationship for ADP, 2-MeSATP and 2-MeSADP expressed as percent of maximal response. Data are means ± S.E.M., for n independent experiments (n = 3-8) (\*\*\*, P < 0.001; \*, P < 0.05; *t*-test).

**Fig. 3. PKC-dependent desensitization of the P2Y1R-induced Ca<sup>2+</sup> response.** (A, B and C) show the Ca<sup>2+</sup> responses for ADP (10 μM) represent typical Ca<sup>2+</sup> responses induced by ADP (10 μM; n = 3) in the presence of TPA (10 nM for 30 min; n = 3) or GF109203X (10 nM for 30 min; n = 3), respectively.

**Fig. 4.** Effects of P2Y1 antisense oligodeoxynucleotides treatment (2.5  $\mu\text{M}$  for 24 h) on ADP- and ATP-induced  $\text{Ca}^{2+}$  response. Scrambled antisense oligodeoxynucleotides were used as negative controls. ADP- and ATP-induced  $\text{Ca}^{2+}$  responses were measured in untreated cells as control. Data are means  $\pm$  S.E.M., for n independent experiments (n = 3-8) (\*\*\*,  $P < 0.001$ ; ns, not significant,  $P > 0.05$ ; *t*-test).

**Fig. 5.** (A) Effect of P2Y1R activation on intracellular cAMP level. Concentration-dependent increase of cAMP content for FSK alone (black square) or in the presence (black triangle) of ADP (100  $\mu\text{M}$ ). Data are expressed as pmol of cAMP per well and represent means  $\pm$  S.D for n = 8 experiments. (B) Effect of P2Y1R activation on cAMP-induced CFTR activation.  $^{125}\text{I}$  efflux kinetics were measured in the absence or presence of FSK (1  $\mu\text{M}$ ) with or without ADP (100  $\mu\text{M}$ ). Results are expressed as percent of intracellular  $^{125}\text{I}$  released in the medium. Data are means  $\pm$  S.D. for (n = 12). (C) Effect of FSK (10  $\mu\text{M}$ )-induced CFTR activation on ADP (10  $\mu\text{M}$ )-induced  $\text{Ca}^{2+}$  responses. Trace is representative on three independent experiments.

**Fig. 6.** Effect of PTX treatment (100 ng/ml, 24 h) on P2Y1 and P2Y2 receptor-induced  $\text{Ca}^{2+}$  responses in CHO-K1 (A), CHO-KNUT (B) and CHO-BQ1 (C) cells. (D,E) represent typical  $\text{Ca}^{2+}$  responses induced by ADP (10  $\mu\text{M}$ ) and ATP (10  $\mu\text{M}$ ) in the absence or presence of PTX, respectively. Data are means  $\pm$  S.E.M., for n independent experiments (n = 3-8) (\*\*\*,  $P < 0.001$ ; ns, not significant,  $P > 0.05$ ; *t*-test).

Fig. 1

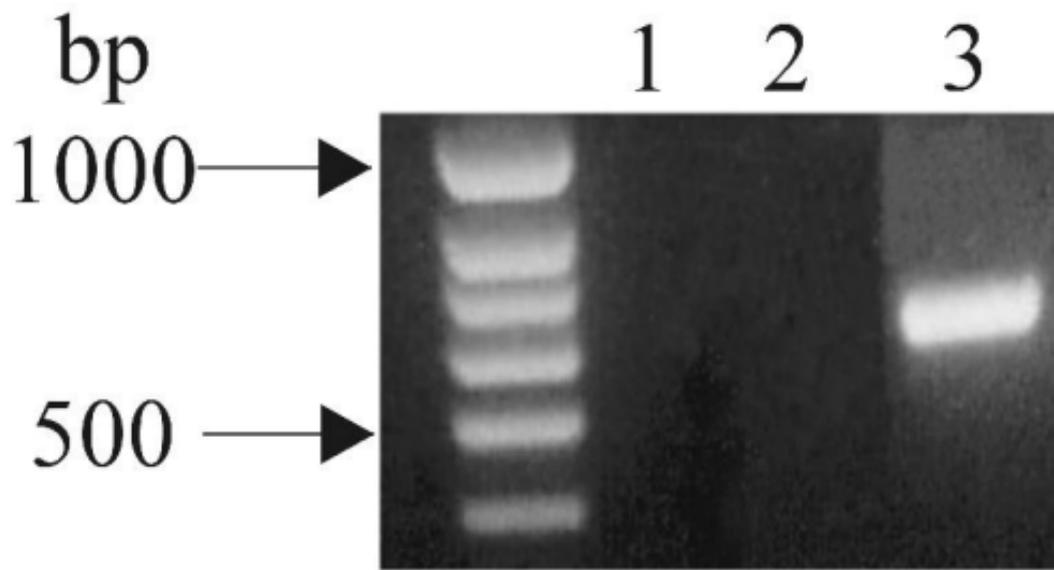


Fig. 2

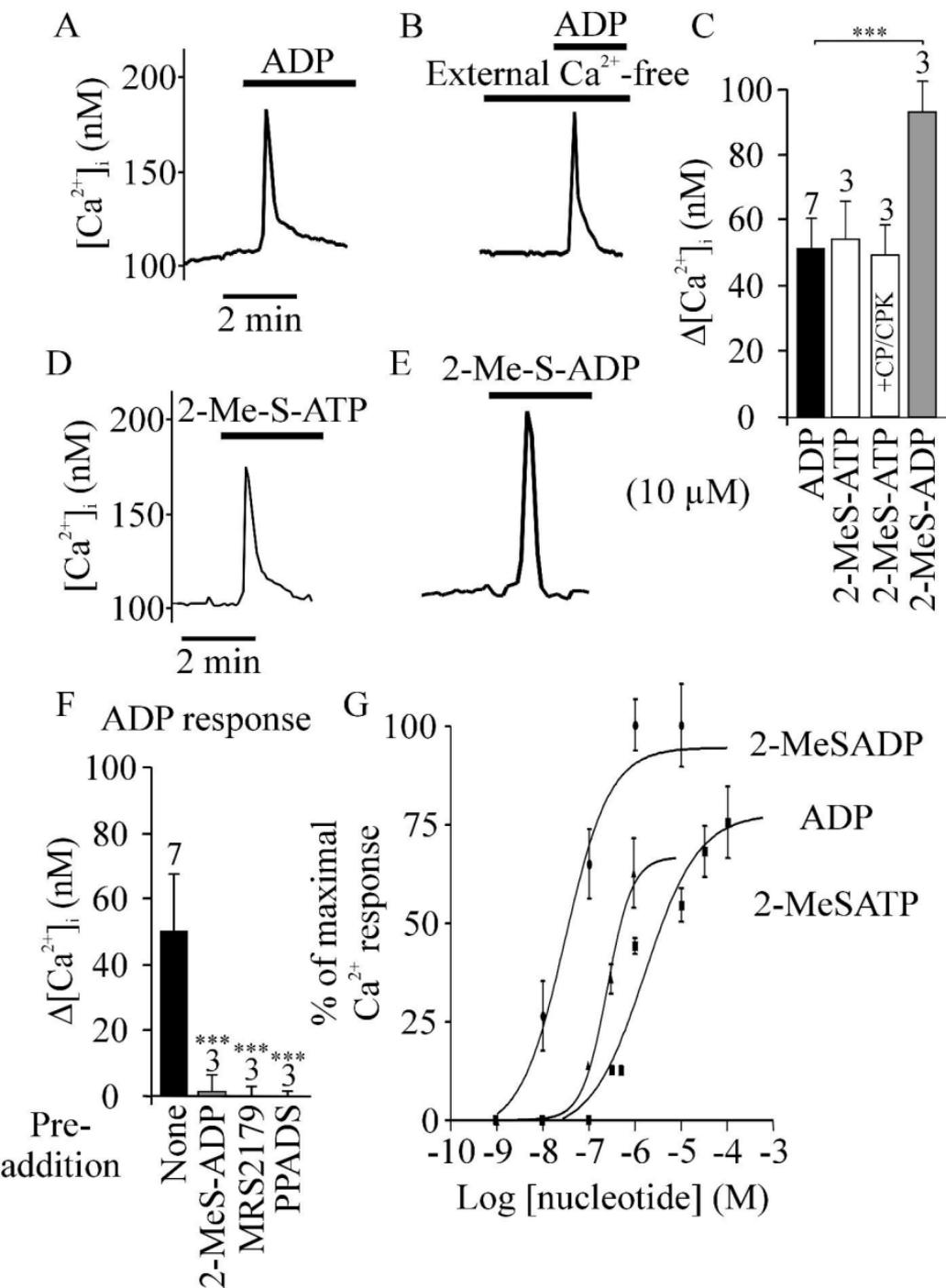


Fig. 3

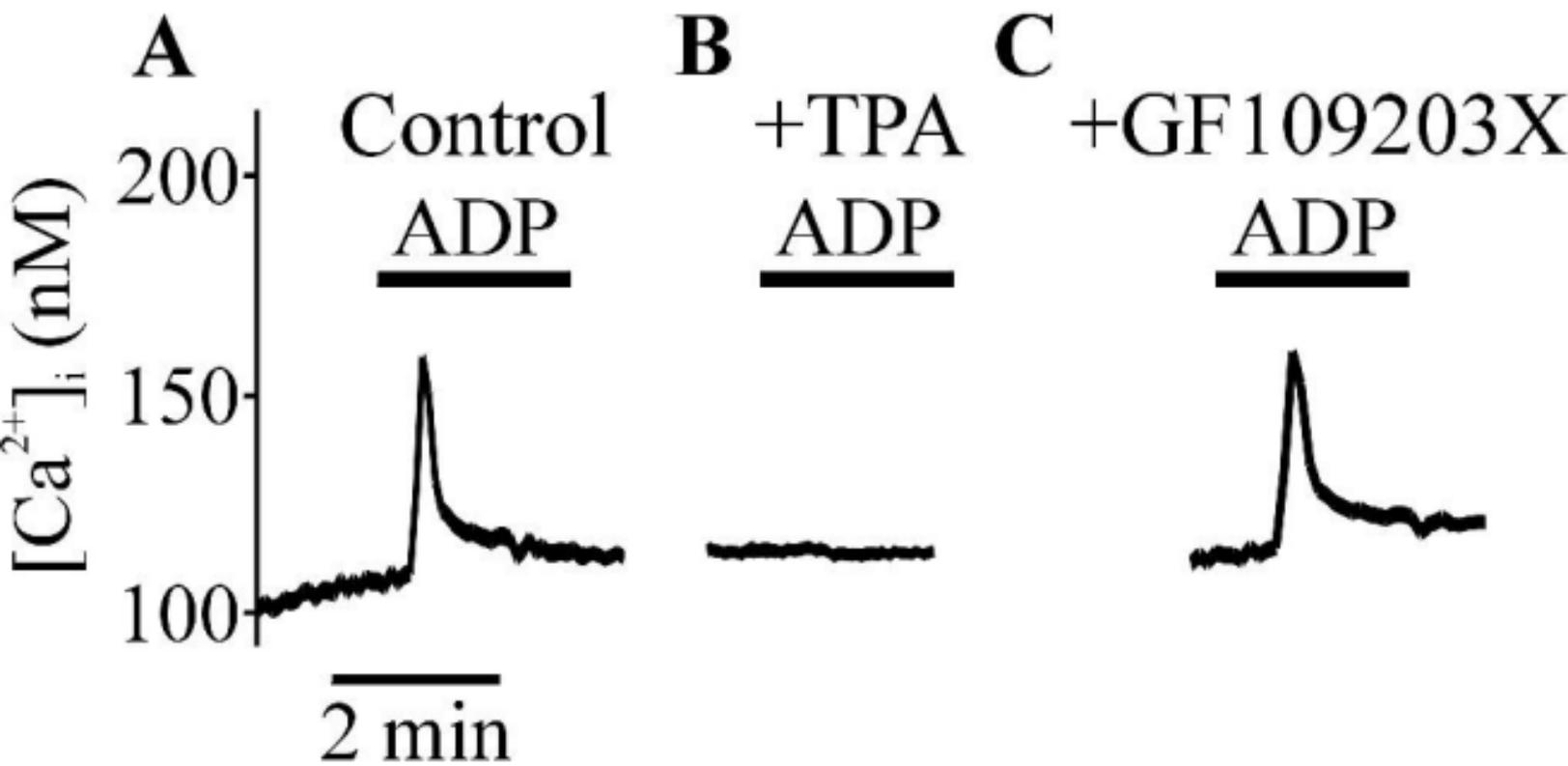


Fig. 4

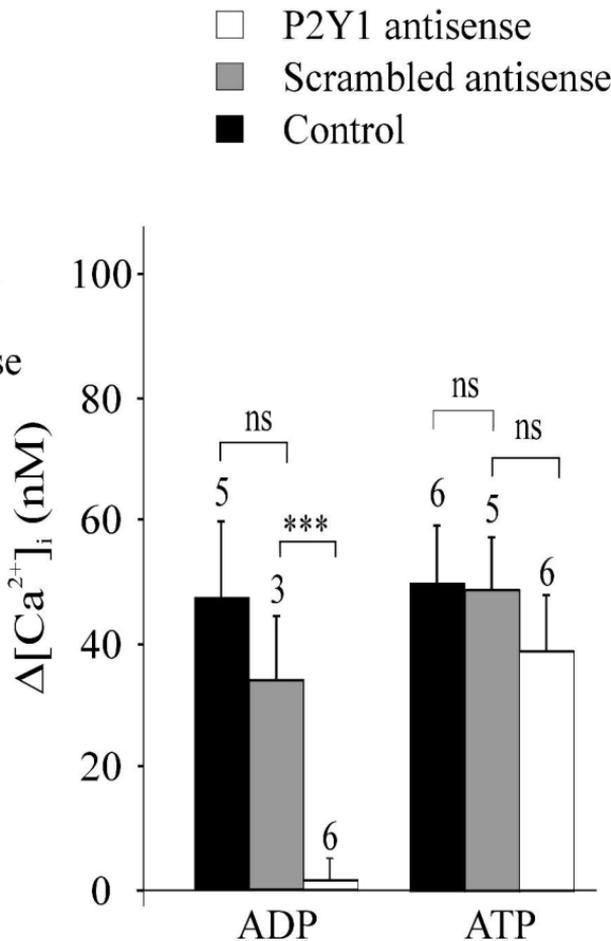
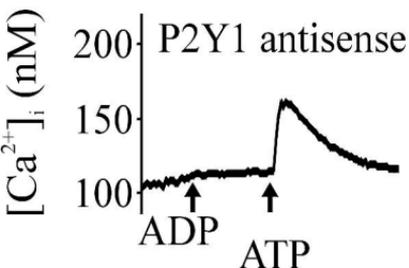
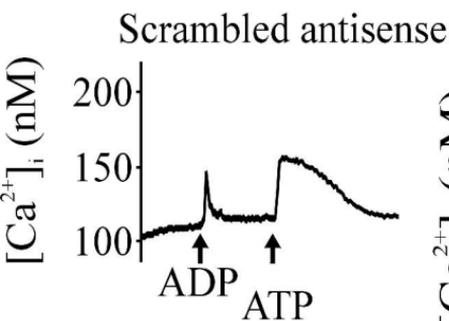
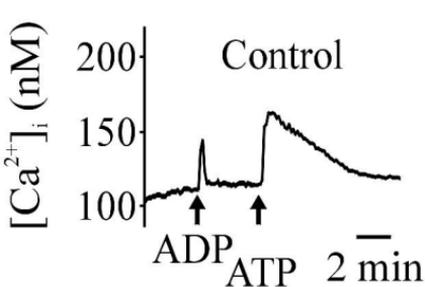


Fig. 5

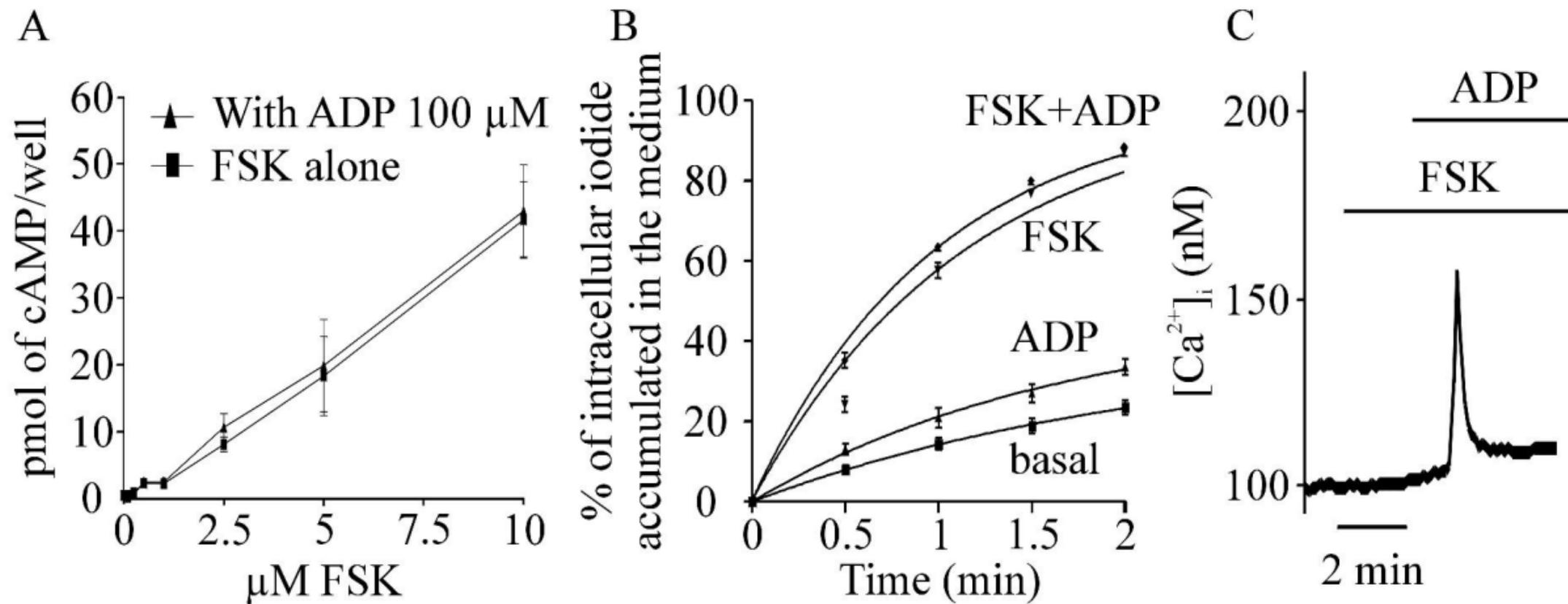


Fig. 6

