Nucleotide $P2Y_1$ receptor regulates EGF receptor mitogenic signaling and expression in epithelial cells

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Summary

Epidermal growth factor receptor (EGFR) function is transregulated by a variety of stimuli, including agonists of certain G-protein-coupled receptors (GPCRs). One of the most ubiquitous GPCRs is the $P2Y_1$ receptor (P2RY1, hereafter referred to as $P2Y_1R$) for extracellular nucleotides, mainly ADP. Here, we show in tumoral HeLa cells and normal FRT epithelial cells that $P2Y_1R$ broadcasts mitogenic signals by transactivating the EGFR. The pathway involves PKC, Src and cell surface metalloproteases. Stimulation of $P2Y_1R$ for as little as 15-60 minutes triggers mitogenesis, mirroring the half-life of extracellular ADP. Apyrase degradation of extracellular nucleotides and drug inhibition of $P2Y_1R$, both reduced basal cell proliferation of HeLa and FRT cells, but not MDCK cells, which do not express $P2Y_1R$. Thus, cell-released nucleotides constitute strong mitogenic stimuli, which act via $P2Y_1R$. Strikingly, MDCK cells ectopically expressing $P2Y_1R$ display a highly proliferative phenotype that depends on EGFR activity associated with an increased level of EGFR, thus disclosing a novel aspect of GPCR-mediated regulation of EGFR function. These results highlight a role of $P2Y_1R$ in EGFR-dependent epithelial cell proliferation. $P2Y_1R$ could potentially mediate both trophic stimuli of basally released nucleotides and first-line mitogenic stimulation upon tissue damage. It could also contribute to carcinogenesis and serve as target for antitumor therapies.

Key words: Nucleotides, GPCR, EGFR, Transactivation, Epithelia, Proliferation

Introduction

The epidermal growth factor (EGF) receptor (EGFR) and its complex network of protein interactions form one of the most widely distributed control systems of cell proliferation and differentiation, and represent a locus that is frequently mutated in cancer (Carpenter, 1999; Gschwind et al., 2001; Yarden and Sliwkowski, 2001). The large body of knowledge regarding EGFR function includes detailed data on the receptor structure, signaling and trafficking pathways, regulatory mechanisms and cancer therapy (Carpenter, 1999; Gschwind et al., 2004; Gschwind et al., 2001; Hackel et al., 1999; Jorissen et al., 2003; Polo and Di Fiore, 2006; Schlessinger, 2000; Schlessinger, 2002; Wiley, 2003; Yarden and Sliwkowski, 2001). However, the network of activating stimuli remains incompletely understood. The EGFR is not only activated by direct binding of its several ligands (Dong et al., 2005; Yarden and Sliwkowski, 2001), but also by indirect signals emerging from G-protein-coupled receptors (GPCRs) (Carpenter, 1999; Carpenter, 2000; Gschwind et al., 2001; Wetzker and Bohmer, 2003). This opens the possibility that stimuli as ubiquitous as extracellular nucleotides, such as ATP and ADP, acting through certain members of the P2Y receptor (P2YR) family constitute generalized regulators of EGFR function, under both physiological and pathological conditions.

The EGFR is a transmembrane protein that possesses an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain (Schlessinger, 2000). Upon ligand binding, the receptor dimerizes and its intracellular tyrosine kinase domain becomes activated, resulting in the phosphorylation of several tyrosines on the receptor tail that serve as recruitment sites for signaling elements (Schlessinger, 2000; Schlessinger, 2002). Activated EGFR conveys mitogenic signals mainly through the Ras-Raf-MEK-MAPK pathway (Marmor et al., 2004; Schlessinger, 2002). An important aspect in the regulation of EGFR function is the control of receptor activation by the availability of suitable stimuli (Dong et al., 2005). Different soluble ligands can bind and activate the EGFR, including the most characterized EGF, transforming growth factor α (TGFα) and heparin-binding EGF-like growth factor (HB-EGF) (Yarden and Sliwkowski, 2001). EGFR ligands are synthesized as integral membrane protein precursors (Massague and Pandiella, 1993), which, once integrated into the cell surface, can act as juxtacrine stimuli or as soluble stimuli released by cell surface metalloproteases (Dong et al., 2005; Dong et al., 1999; Izumi et al., 1998; Prenzel et al., 1999; Singh et al., 2004; Yan et al., 2002). The releasing process can be regulated by signaling elements such as Src, calcium influx and protein

kinase C (PKC) (Goishi et al., 1995; Gschwind et al., 2001; Izumi et al., 1998; Soltoff, 1998; Tsai et al., 1997; Zwick et al., 1999). This property places the EGFR as a downstream element in the signaling pathways of a variety of cell-surface receptors (Carpenter, 1999; Guerrero et al., 2004), most notably GPCRs coupled to Gi or Gq (Carpenter, 1999; Daub et al., 1997; Daub et al., 1996; Gschwind et al., 2001; Hackel et al., 1999; Wetzker and Bohmer, 2003). Abundant evidence suggests that GPCRs could be important regulators of cell growth and under certain circumstances can even act as oncoproteins (Marin and Chen, 2004; Marinissen and Gutkind, 2001; Parnot et al., 2002).

GPCRs of the P2YR family constitute generalized autocrine and paracrine control systems. To date, eight mammalian P2YR subtypes ($P2Y_{1,2,4,6}$ and $P2Y_{11\text{-}14}$) have been cloned and functionally characterized (von Kugelgen, 2006). Two of them – $P2Y_1$ and $P2Y_2$ – are widely expressed in a variety of cells (Janssens et al., 1996; Ralevic and Burnstock, 1998; Rice et al., 1995). P2YRs are activated by the extracellular nucleotides ATP, ADP, UTP and UDP, which are produced by all cells. Several P2YR subtypes are activated by the same nucleotides, but each receptor subtype displays a unique order of potency for specific nucleotides. Subsets of P2YR share the signal transduction pathway involving the Gq protein ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$), phospholipase Cβ and intracellular calcium mobilization, whereas others are Gi-coupled receptors ($P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) that modulate cAMP levels (Ralevic and Burnstock, 1998; Schwiebert and Zsembery, 2003; von Kugelgen, 2006).

Nucleotides are released into the medium in large quantities upon cell injury. Cells also have mechanisms, which are as yet incompletely understood, to continuously transport nucleotides down a steep gradient into the medium, either constitutively or enhanced by a variety of stress conditions, including hypoxia and cell swelling (Lazarowski et al., 2003; Schwiebert and Zsembery, 2003). Owing to the ubiquitous presence of ectonucleotidases, extracellular nucleotides have relatively short life spans, and are therefore specially suited for autocrine and paracrine control (Burrell et al., 2005).

Recent evidence suggests that extracellular nucleotides can exert mitogenic roles by activating certain P2YRs. Addition of ATP to the medium provides synergistic stimuli mediated by $P2Y_2R$ upon growth factor mitogenic effects in both normal and tumorigenic cells (Erlinge, 1998; Neary et al., 1999; Schafer et al., 2003; Tornquist et al., 1996; Tu et al., 2000; Wagstaff et al., 2000; Wang et al., 1992; Yu et al., 1996). $P2Y_6R$ (Schafer et al., 2003) and $P2Y_{12}R$ (Van Kolen et al., 2006) are also involved in mitogenesis. To date, $P2Y_2R$ is the only receptor subtype to transactivate the EGFR (Liu et al., 2004; Morris et al., 2004; Schafer et al., 2003; Soltoff et al., 1998; Wagstaff et al., 2000). The short life span of extracellular nucleotides raises concern about their actual mitogenic potential, because proliferative responses can require long exposure periods to mitogenic stimuli, including EGF (Jones and Kazlauskas, 2001a; Shechter et al., 1978). Most studies on cell proliferation have used high-dose, long-term incubation and repeated application of exogenously added nucleotides. Thus, it is important to better define the mitogenic potential of cell-released nucleotides and the contribution of the EGFR to the effects of other P2YR subtypes.

It is also important to assess the mitogenic role of extracellular nucleotides in epithelial cells, in which the EGFR is a crucial regulator of proliferation and differentiation (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995) and from which most human cancers derive (Peto, 2001). In epithelial cells, the role of extracellular nucleotides has been mainly characterized in short-term responses associated with ion transport, including airway mucus fluidity, cell volume regulation and ischemic protection (Leipziger, 2003; Schwiebert and Zsembery, 2003). Mitogenic responses to exogenously added P2YR agonists have been described in endothelial cells (Erlinge, 1998), but other normal epithelial cells remain largely unexplored.

Here, we studied the P2RY1 (hereafter referred to as $P2Y_1R$), which is as ubiquitous as $P2Y_2R$ but has different properties. $P2Y_1R$ and $P2Y_2R$ share only 31% amino acid similarity (Sak and Webb, 2002), have different preferences for nucleotides (von Kugelgen, 2006) and distribute with opposite polarity in epithelial cells (Wolff et al., 2005). Although $P2Y_2R$ is better activated by ATP and UTP, and resides in the apical domain of epithelial cells, $P2Y_1R$ is more sensitive to ADP, and is basolaterally distributed in epithelial cells (Wolff et al., 2005), sharing this polarity with the EGFR (He et al., 2002). We provide evidence that $P2Y_1R$ can mediate mitogenic signals of endogenously released nucleotides in normal epithelial cells, Madin-Darby canine kidney (MDCK) and Fisher rat thyroid (FRT) cells, as well as in tumoral HeLa cells, by transactivating the EGFR pathway. The results indicate that nucleotides constitute strong mitogenic signals, even for short stimulation time periods. Strikingly, we also found that ectopic expression of $P2Y_1R$ in epithelial MDCK cells leads to a state of increased EGFR-dependent cell proliferation, accompanied by increased expression of EGFR. These results show a prominent role of the $P2Y_1R$ in the regulation of epithelial cell proliferation. Abnormalities in the functional relationship between $P2Y_1R$ and the EGFR may contribute to cancerogenesis.

Results

$P2Y_1R$ and EGFR in FRT, MDCK and HeLa cell lines

To study the role of $P2Y_1R$ in cell proliferation and its dependency on EGFR we used normal epithelial FRT and MDCK cells and epithelial-derived tumoral HeLa cells as model systems. Immunoblot analysis showed that HeLa cells and FRT cells have readily detectable expression levels of both receptors (Fig. 1; lanes 1 and 2). Densitometric scanning indicated that HeLa cells express higher levels of $P2Y_1R$ (~8.5-fold) and EGFR (~29-fold) compared with FRT cells. Thus, these cells provide model systems to study the functional relationships of endogenously expressed $P2Y_1R$ and EGFR in normal and tumoral cell contexts. Instead, in MDCK cells neither $P2Y_1R$ nor EGFR could be detected under the immunoblot conditions of the other cells (lane 3). However, the EGFR but not $P2Y_1R$ became apparent by increasing the exposure time (lane 4). Functional assays further indicated that MDCK cells do not express $P2Y_1R$ (see later). Thus, MDCK cells provide a suitable model system to study the mitogenic effect of ectopically expressed $P2Y_1R$ in the context of low EGFR expression levels.

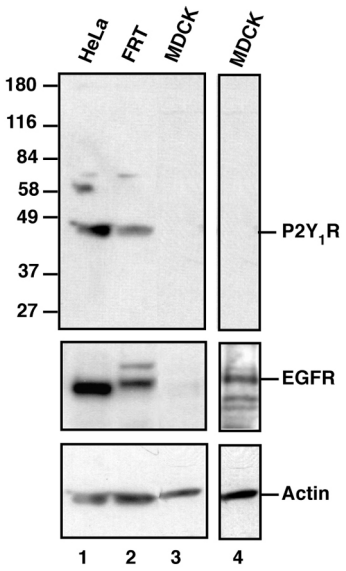

Fig. 1. P2Y$_1$R and EGFR expression in non-tumoral epithelial FRT and MDCK cells and in tumoral HeLa cells. Cell extracts analyzed by immunoblot and ECL with polyclonal antibodies against P2Y$_1$R and the EGFR show readily detectable expression levels of both receptors in HeLa and FRT cells (lanes 1 and 2) but not in MDCK cells (lane 3). The EGFR, but not the P2Y$_1$R, becomes apparent in MDCK extracts when the film is exposed for a longer time (lane 4). Molecular size markers (in kDa) are indicated on the left.

P2Y$_1$R mediates cell proliferation induced by extracellular nucleotides

Before testing the effects of different P2Y$_1$R agonists on cell proliferation, we determined the doubling-time periods of HeLa (18.2±0.7 hours) and FRT (20.3±1.3 hours) cells. With these results, we performed [^{3}H]thymidine incorporation assays over 16-18 hours to include almost the total cell cycle. The cells were previously synchronized by 24 hours of serum starvation. Under these conditions the P2Y$_1$R synthetic agonist 2-methylthioadenosine 5′-diphosphate (2-MeSADP) (Fig. 2A) and its natural ligand ADP (Fig. 2B), increased FRT and HeLa cell proliferation in a concentration-dependent manner, with similar effective concentration 50 (EC_{50}), in the range of 25 to 75 nM. Both HeLa and FRT cells also increased their proliferation rate in response to ATP, but not UTP, which is a P2Y$_2$R and P2Y$_4$R agonist. UDP, a P2Y$_6$R agonist, provoked mitogenic effects only in FRT cells (Fig. 2C).

The agonist 2-MeSADP can potentially activate other nucleotide receptors, such as P2Y$_{12}$R, P2Y$_{13}$R and P2X.

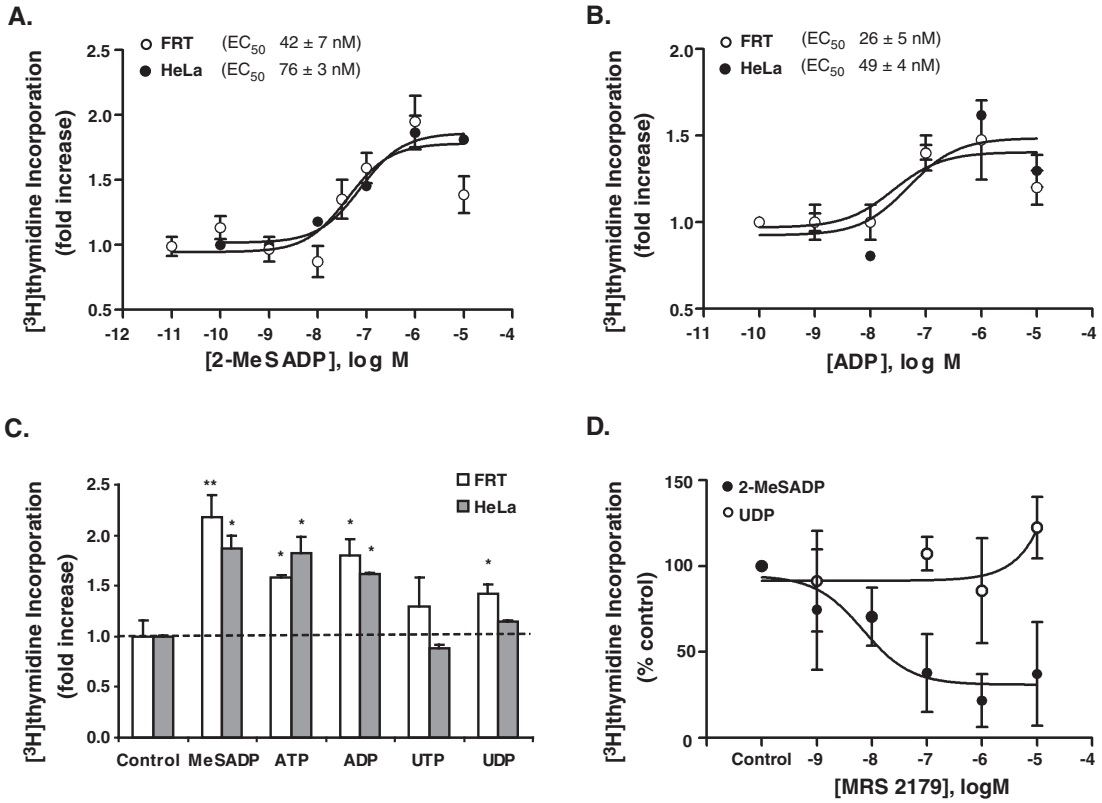

Fig. 2. Nucleotides stimulate cell proliferation through P2Y$_1$R. P2Y$_1$R agonists, 2-MeSADP (A) and ADP (B), increase cell proliferation in a concentration-dependent manner (n=4-8). [^{3}H]thymidine incorporation assays show that HeLa and FRT cells pretreated for 1 hour with different concentrations of the agonists increase their proliferation rate. The EC_{50} of the agonists is indicated. (C) [^{3}H]thymidine incorporation after 1 hour of stimulation with 1 μM P2YR agonist. Compared with P2Y$_1$R agonists 2-MeSADP, ATP and ADP, which increase cell proliferation, the P2Y$_2$R agonist UTP shows no effect. The P2Y$_6$R agonist UDP induces cell proliferation only in FRT cells. Results are normalized against control cells. Values are the means ± s.e.m. *P<0.05, **P<0.01, Dunnett's test compared with controls (n=5). (D) Cell proliferation induced by 1 μM 2-MeSADP is selectively mediated by P2Y$_1$R, since it was abrogated by the selective P2Y$_1$R antagonist MRS2179, which in turn does not affect the proliferative response to 10 μM UDP, as shown for FRT cells (n=3-4). Only the carrier culture medium was added for controls.

However, the $P2Y_1R$-specific antagonist 2′-deoxy-N6-methyladenosine 3′, 5′-biphosphate (MRS2179) blocked the 2-MeSADP induced cell proliferation in a concentration-dependent manner (Fig. 2D). As control of specificity, MRS2179 did not affect the mitogenic effect of UDP (Fig. 2D). Therefore, $P2Y_1R$ conveys mitogenic signals in response to activation by both synthetic agonist and natural nucleotides.

The mitogenic effect elicited by a 1-hour incubation with nucleotides was surprising. Cells usually require several hours of exposure to growth factors, covering two distinct phases of signaling to orchestrate a proliferative response: one that triggers entering into G1 and the other to surpass the restriction point of the cell cycle 7-9 hours later (Jones and Kazlauskas, 2001a; Jones and Kazlauskas, 2001b; Pennock and Wang, 2003; Shechter et al., 1978). Therefore, we assessed the effect of incubating the cells for 1 hour with different concentrations of either EGF or fetal bovine serum (FBS). Although FRT cells responded to EGF in a concentration-dependent manner, HeLa cells showed no response to a wide range of EGF concentrations (Fig. 3A). Neither FRT nor HeLa cells responded to a 1-hour incubation with 10% FBS, but were rescued from the proliferative arrest by longer incubation periods, as shown for a 16-hour stimulation (Fig. 3B). These results illustrate the effectiveness of the nucleotides as mitogenic signals, suggesting that they trigger a cascade of events encompassing later processes required for surpassing the restriction point of the cell cycle.

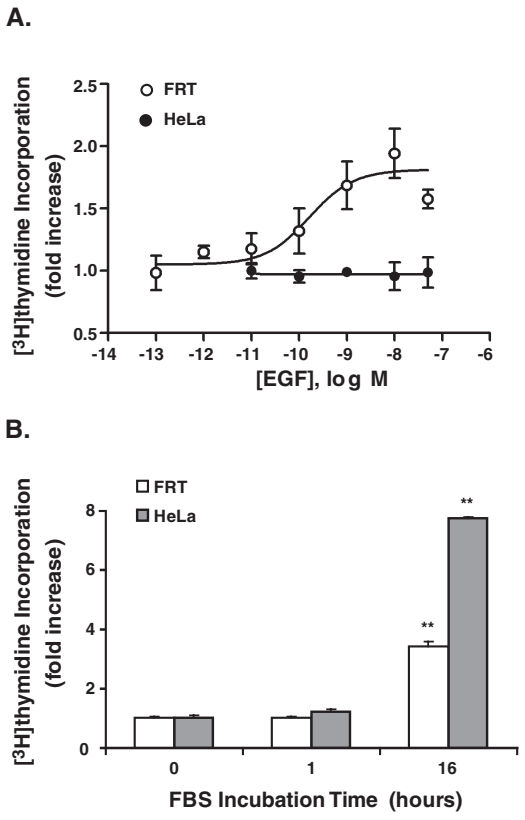

Fig. 3. Effect of EGF and FBS on FRT and HeLa cell proliferation. Serum-arrested cells were incubated with different concentrations of EGF for 1 hour (A) or with 10% FBS for either 1 hour before, or 16 hours during [^{3}H]thymidine incubation (B). Only FRT cells show a proliferative response to EGF, indicating concentration dependency. None of the cells responded to just 1 hour of FBS, contrasting with the effect of a long incubation period, which could override the proliferative arrest (n=4-6).

Cell-released nucleotides constitute $P2Y_1R$-mediated mitogenic signals

An important unsolved question is whether and to what extent nucleotides endogenously released by cells regulate basal cell proliferation rates. We approached this problem by analyzing the extracellular concentrations of nucleotides and the effects of both the enzyme apyrase, which degrades cell-released nucleotides, and the antagonist MRS2179, which selectively blocks the interaction of released nucleotides with $P2Y_1R$. As nucleotides are rather transient stimuli, because of their short half-lives (Lazarowski et al., 2003), we also determined the time dependency of selective $P2Y_1R$ stimulation to evoke proliferative responses, as well as the lifetime of ATP and ADP in the media.

Extracellular ATP can be metabolized to ADP and AMP, which, depending on the presence of externally exposed nucleotidases, was subsequently transformed to adenosine. In basal conditions, the media of FRT and HeLa cells showed similar levels of ATP (30-40 nM). However, the FRT cell medium showed higher levels of ADP (sixfold) and adenosine (threefold) (Fig. 4A), whereas AMP was undetectable. At the concentration of ADP detected in the volume of media currently used for growing the cells, this nucleotide can certainly contribute to basal mitogenesis of both cells, because it is within the EC_{50} range. The effect on basal proliferation of apyrase and the $P2Y_1R$ antagonist MRS2179 supports this possibility more directly. Apyrase added to the medium for 1 hour led to complete degradation of ATP and ADP, accompanied by accumulation of AMP in FRT cells and adenosine in HeLa cells (Fig. 4B). Under these conditions, FRT and HeLa cells reduced their basal proliferation rates by 15-25%. Cells incubated with MRS2179 showed a similar inhibitory effect (Fig. 4C).

We next studied how long $P2Y_1R$ needs to be stimulated in FRT cells to elicit a proliferative response. The result was striking. An incubation period as short as 10-15 minutes with 2-MeSADP was enough to provoke an increased proliferation activity, as revealed by both [^{3}H]thymidine incorporation (Fig. 5A) and induction of cyclin D expression (Fig. 5B). Maximal proliferative responses occurred after 2 hours of incubation with 1 μM 2-MeSADP, decreasing thereafter. With such time dependency, cell-released nucleotides do have a mitogenic role. Furthermore, after cell injury by a cross-scratch, which disrupted about 15% of the cell monolayer, the extracellular concentration of ATP and ADP increased dramatically and rapidly, reaching levels of about 500 nM within the first minute. The half-life of ATP was about 5-10 minutes and that of ADP extended to about 45-60 minutes (Fig. 5C) – long enough to exert $P2Y_1R$-mediated mitogenic effects.

Taken together, these results reveal that endogenously released nucleotides can act as strong trophic factors. The evidence also highlights the relevance of ADP, the preferred ligand of $P2Y_1R$. Endogenous nucleotides are therefore first-line mitogens during tissue-repair processes.

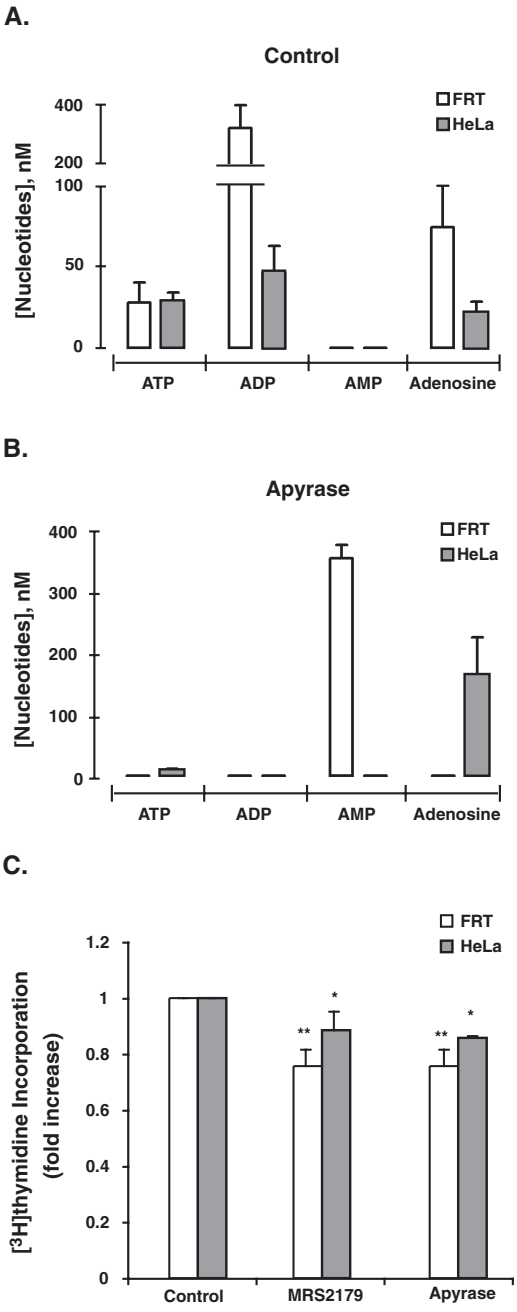

Fig. 4. Cell-released nucleotides contribute to basal cell proliferation. Steady-state concentration of extracellular ATP and its metabolites measured in the medium of FRT and HeLa cells in control conditions (A; *n*=10) and after a 1-hour incubation with 2 U/ml apyrase (B; *n*=5). ATP and ADP induce cell proliferation and both become undetectable after apyrase treatment. (C) Endogenous nucleotides acting through P2Y$_1$Rs participate in the regulation of basal cell proliferation (*n*=5). Basal [^{3}H]thymidine incorporation in FRT and HeLa cells measured after 16 hours of incubation decreased by about 15-25% in the presence of 1 μM MRS2179 (P2Y$_1$R antagonist) or 2 U/ml apyrase. All results were normalized against the control. Values are the means ± s.e.m. **P*<0.05, ***P*<0.01, Dunnett's test compared with control. Carrier culture medium only was added for the control.

Cell proliferation induced by extracellular nucleotides requires EGFR transactivation

The mitogenic effects of other GPCRs require transactivation of the EGFR through pathways involving Src and cell-surface metalloproteases (Carpenter, 1999; Daub et al., 1997; Gschwind et al., 2001; Prenzel et al., 1999; Schafer et al., 2004). PKC has also been implicated (Soltoff et al., 1998; Tsai et al., 1997). Immunoblot assessment of tyrosine phosphorylation measures the activation status of both the EGFR and the downstream extracellular-regulated kinase (ERK). Incubation of HeLa and FRT cells for just 5 minutes with 2-MeSADP (1 or 10 nM) activated the EGFR in a concentration-dependent manner (Fig. 6A). ERK1/2 also became activated (Fig. 6B), thus mimicking the effect of EGF. Inhibition of EGFR tyrosine kinase activity with AG1478 in FRT cells reduced the mitogenic effect of both 2-MeSADP and EGF in a concentration-dependent manner, with inhibitory concentration 50 (IC_{50}) values in the range of 1 to 3 nM (Fig. 7A). Similarly, inhibitors of PKC (Ro 318220), Src (PP2) and metalloproteases (Ilomastat), all prevented 2-MeSADP-induced proliferation, as shown for HeLa cells (Fig. 7B). The AG1478 inhibitor of EGFR tyrosine kinase activity completely abrogated the ERK1/2 activation induced by 2-MeSADP, indicating that it was dependent on EGFR transactivation (Fig. 7C).

These results indicate that the mitogenic signals conveyed by P2Y$_1$R stimulation require transactivation of the EGFR through pathways involving Src, PKC and metalloprotease-mediated release of EGFR ligands from the cell surface.

Ectopic expression of P2Y$_1$R leads to increased EGFR-mediated cell proliferation and higher EGFR expression

As we could not detect P2Y$_1$R by immunoblotting in MDCK cells (see Fig. 1), we tried the more sensitive method of measuring nucleotide-induced intracellular Ca^{2+} changes. MDCK cells did not respond to the selective P2Y$_1$R agonists ADP and 2-MeSADP, but did increase their intracellular Ca^{2+} when stimulated with the P2Y$_2$R agonists ATP and UTP (Fig. 8A). The low agonist efficacy of ATP might be expected considering that this nucleotide is a poor agonist upon canine P2Y$_2$R (von Kugelgen, 2006). An action of ATP through ionic P2X receptors that usually require higher nucleotide concentrations cannot be discarded.

Because our immunoblot and functional assays rule out P2Y$_1$R expression in MDCK cells, we used these cells as a model system to study the effect of ectopically expressed P2Y$_1$R. This is interesting because gain-of-function phenotypes can be produced by overexpression of wild-type GPCRs (Parnot et al., 2002). In addition, ectopic expression of a particular GPCR in a cell context that normally lacks the receptor can disrupt cell proliferation control and produce cell transformation in the presence of the corresponding ligand (Marin and Chen, 2004).

We transfected MDCK cells permanently and selected colonies that expressed P2Y$_1$R at similar or even lower levels than the FRT endogenous receptor (Fig. 8B; lanes 2 and 3). MDCK cells ectopically expressing P2Y$_1$R showed an increased viability, reflected by the higher slope and maximal number of living cells reached in culture (Fig. 9A). These MDCK P2Y$_1$R cells reduced their doubling time to 13.4±0.4 hours, compared with 21.8±0.9 hours in wt MDCK cells (Fig.

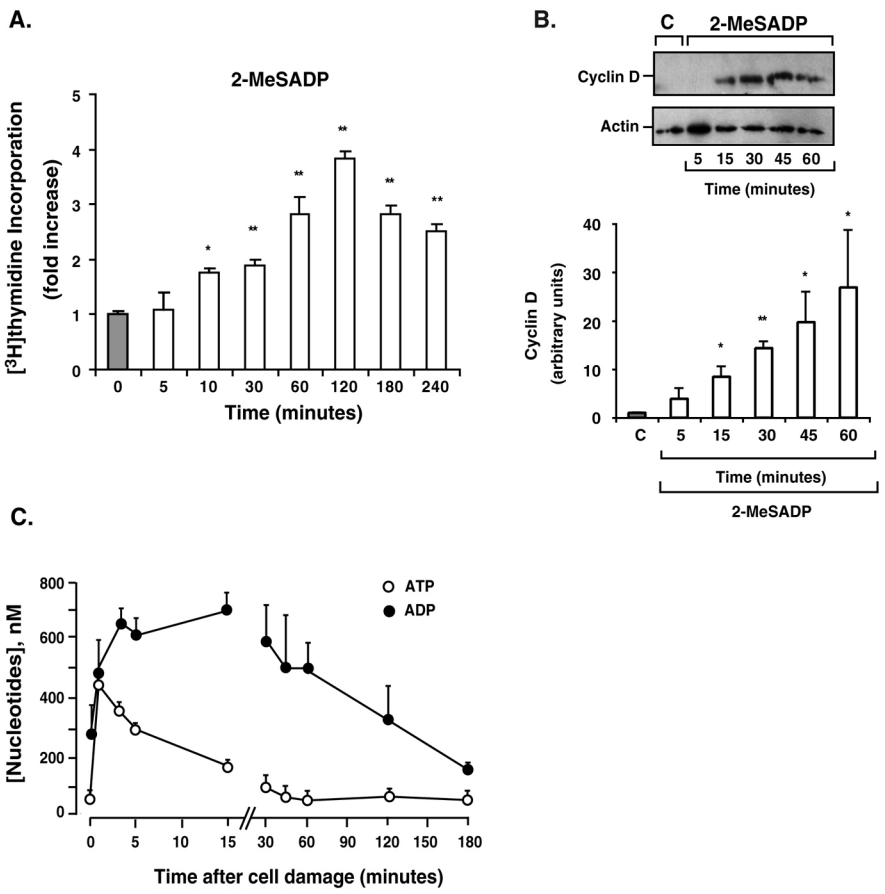

Fig. 5. Congruence between time dependency of $P2Y_1R$ stimulation for cell proliferation and half-life of injury-released nucleotides. (A) Stimulation time-period necessary for $P2Y_1R$-induced cell proliferation. FRT cells were incubated with 1 μM 2-MeSADP for the indicated time periods and then the ligand was removed and the cells incubated for 16 hours with [^{3}H]thymidine. The proliferative response requires a minimal stimulation time of 10 minutes, reaching a maximum by 120 minutes and decreases thereafter (n=4); (B) Changes in cyclin D levels detected by immunoblot. FRT cells were incubated either with carrier culture medium alone (Control, C) or with 1 μM 2-MeSADP for the indicated times. Stimulation for as little as 15 minutes leads to increased levels of cyclin D, analyzed 16 hours later. The quantification of three separate assays normalized with respect to actin is shown in the graph. (C) Half-life of nucleotides released from wounded cultured cells. Extracellular ATP and ADP concentrations show a dramatic increase after cell injury and then gradually decrease. ADP maintained higher levels for longer periods – enough to induce $P2Y_1R$-mediated cell proliferation (n=4). Values are the means ± s.e.m. *P<0.05, **P<0.01; Dunnett's test against control conditions without agonist.

9A insert). By contrast, MDCK cells stably transfected with the δ-opioid receptor, another GPCR, did not show these differences, indicating a selective effect.

MDCK cells permanently expressing $P2Y_1R$ also displayed a higher proliferation rate than wild-type cells, as indicated by a twofold increase in [^{3}H]thymidine incorporation (Fig. 9B). MDCK cells expressed relatively low levels of the EGFR, compared with HeLa and FRT cells (see Fig. 1). However, the EGFR kinase inhibitor AG1478 (100 nM) abrogated the high proliferation rate of MDCK $P2Y_1R$ (Fig. 9B). MDCK $P2Y_1R$ cells respond to stimulation with $P2Y_1R$ exogenous agonist 2-MeSADP increasing the [^{3}H]thymidine incorporation (Fig. 9C) and transactivating the EGFR (Fig. 9D). All these results suggest that ectopic expression of $P2Y_1R$ promotes a highly proliferative phenotype in MDCK cells through a mechanism involving transactivation of the EGFR.

Interestingly, the MDCK/$P2Y_1R$ cells showed threefold increased levels of EGFR mass, as detected by immunoblotting (Fig. 10A) and twofold higher expression of newly synthesized EGFR, as revealed by immunoprecipitation from metabolically labeled cells (Fig. 10B). EGFR expression is under the control of the EGFR itself and PKC activities. Thus, we treated the cells with EGFR and PKC blockers and found a decreased EGFR expression (Fig. 10C). These results suggest that ectopic expression of $P2Y_1R$ leads to an increased EGFR expression through a mechanism involving activation of the EGFR and PKC.

All the results illustrated in Figures 9 and 10 correspond to the same MDCK $P2Y_1R$ clone. However, the analysis of several clones of stably transfected MDCK cells showed similar results. All the clones displayed increased cell proliferation and EGFR expression with respect to wild-type cells.

Discussion

We found that $P2Y_1R$ activation with extracellular nucleotides acting upon the EGFR constitutes a mitogenic stimulating system in normal epithelial and tumoral cells. The evidence indicates that $P2Y_1R$ contributes to maintenance of basal proliferation rates and has the potential to rapidly initiate proliferative responses after tissue damage. Because both extracellular nucleotides and $P2Y_1R$ are ubiquitous, they are probably generalized trophic systems and first-line 'sentinels' of proliferative requirements in the tissue-repair processes. In addition, we found that ectopic expression of $P2Y_1R$ promotes the development of a highly proliferative EGFR-mediated state, associated with an increased expression level of the EGFR. Therefore, $P2Y_1R$ can modulate the mitogenic function of the EGFR through pathways leading first to its acute transactivation and later to a huge increase in its expression levels and signaling activity. The recognized importance of the EGFR in tumorigenesis suggests the possibility that abnormally increased $P2Y_1R$ signaling contributes to carcinogenesis and may serve as a target for antitumor therapies.

We first showed that the $P2Y_1R$ agonists 2-MeSADP and ADP stimulate proliferation of epithelial FRT cells and cancerous HeLa cells. Although 2-MeSADP can also activate

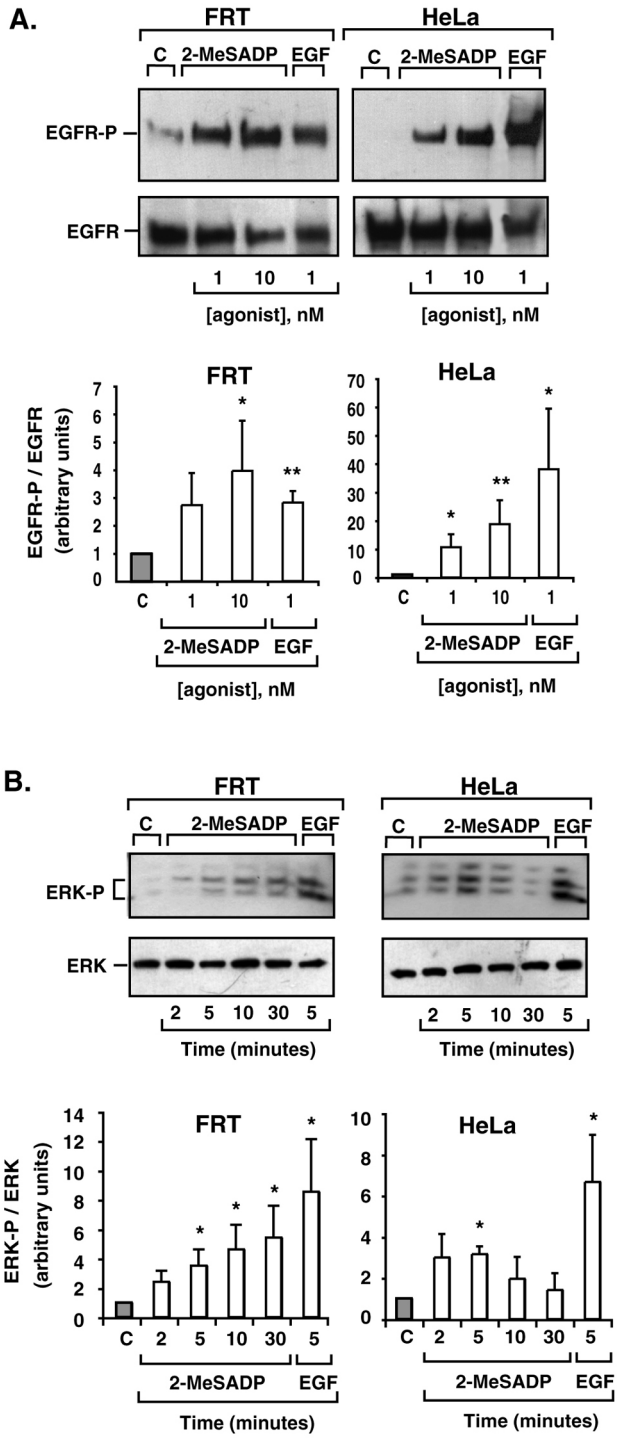

Fig. 6. Stimulation of the P2Y$_1$R transactivates the EGFR pathway. (A) 2-MeSADP increases the EGFR tyrosine phosphorylation in a concentration-dependent manner. Quiescent FRT and HeLa cells were treated for 5 minutes with either 1 or 10 nM 2-MeSADP or 1 nM EGF, as indicated. The EGFR was then immunoprecipitated, resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine monoclonal antibody 4G10 to detect the tyrosine phosphorylated EGFR (EGFR-P). After stripping, blotted membranes were incubated with polyclonal antibody EGFR984 for total EGFR detection. EGFR-P is clearly more intense than in control cells ('C') without agonist stimulation. (B) 2-MeSADP increases ERK1/2 activation. Quiescent cells treated for the indicated time periods either with carrier culture medium alone (Control, 'C') or with 10 nM 2-MeSADP or 1 nM EGF were analyzed for ERK activation by immunoblot against total ERK or phosphorylated ERK (ERK-P). Each graph corresponds to quantification of three independent experiments, normalized with respect to the corresponding total EGFR or ERK protein in each lane. Values are the means ± s.e.m. *P<0.05, **P<0.01; Dunnett's test compared with control conditions without agonist.

the P2Y$_{12}$R and P2Y$_{13}$R subtypes, there is no evidence that these receptors are expressed in epithelial cells and the P2Y$_1$R specific antagonist MRS2179 totally blocked the 2-MeSADP-promoted proliferation. Therefore, the proliferative response to 2-MeSADP selectively involves stimulation of P2Y$_1$R. As described for other GPCRs (Carpenter, 1999; Gschwind et al., 2001; Wetzker and Bohmer, 2003), the P2Y$_1$R mitogenic response requires transactivation of the EGFR. The blocking effects of several inhibitors indicate that P2Y$_1$R-mediated cell proliferation requires Src, PKC and metalloprotease activities. This is congruent with the proposed pathway involving the release of an EGFR ligand, such as HB-EGF or TGFα from a cell-surface precursor (Gschwind et al., 2001). Because P2Y$_1$R is widely distributed in different cell types, including endothelium, smooth muscle, neurons, glia and epithelial cells (Buvinic et al., 2006; Janssens et al., 1996; Ralevic and Burnstock, 1998; Schwiebert and Zsembery, 2003), our results reveal one of the most ubiquitous EGFR-mediated control systems of cell proliferation.

Whether cell-released nucleotides have a functional significance in cell proliferation remains an unresolved question. All kinds of cells permanently release nucleotides to the medium. The extracellular resting concentration of nucleotides is in the nanomolar range and can increase after hypoxia or cell injury up to the millimolar range (Lazarowski et al., 2003). However, ectonucleotidase activity rapidly degrades ATP consecutively to ADP, AMP and adenosine (Burrell et al., 2005; Lazarowski et al., 2003; Schwiebert and Zsembery, 2003). We found basal concentrations of ATP and ADP in the medium surpassing the EC_{50} for P2Y$_1$R and showed that nucleotide degradation by apyrase and P2Y$_1$R inhibition by the antagonist MRS2179 both decreased the basal proliferation rate of FRT and HeLa cells. Recently, similar studies in adult neural stem cells (Mishra et al., 2006) and in prostate cancer cells (Nandigama et al., 2006) show evidence of mitogenic potential of cell-released nucleotides, and at least in neurospheres, mediated by P2Y$_1$R (Mishra et al., 2006). We also show that upon cell injury, ATP and ADP increased several fold and then decreased with a half-life of 10-15 minutes and 45-60 minutes, respectively. Finally, we determined that a period as short as 15 minutes of selective P2Y$_1$R stimulation can elicit proliferative responses. Therefore, nucleotides released from cells provide P2Y$_1$R-mediated mitogenic signals. Under basal conditions, extracellular nucleotides constitute the most ubiquitous trophic factors, whereas after cellular injury or other stress situations requiring tissue repair, they can act as first line of defense mitogens.

The mechanism by which nucleotides are capable of eliciting mitogenic responses even after short periods of P2Y$_1$R stimulation remains to be elucidated. To enter into proliferation, serum-arrested epithelial cells, like other cells,

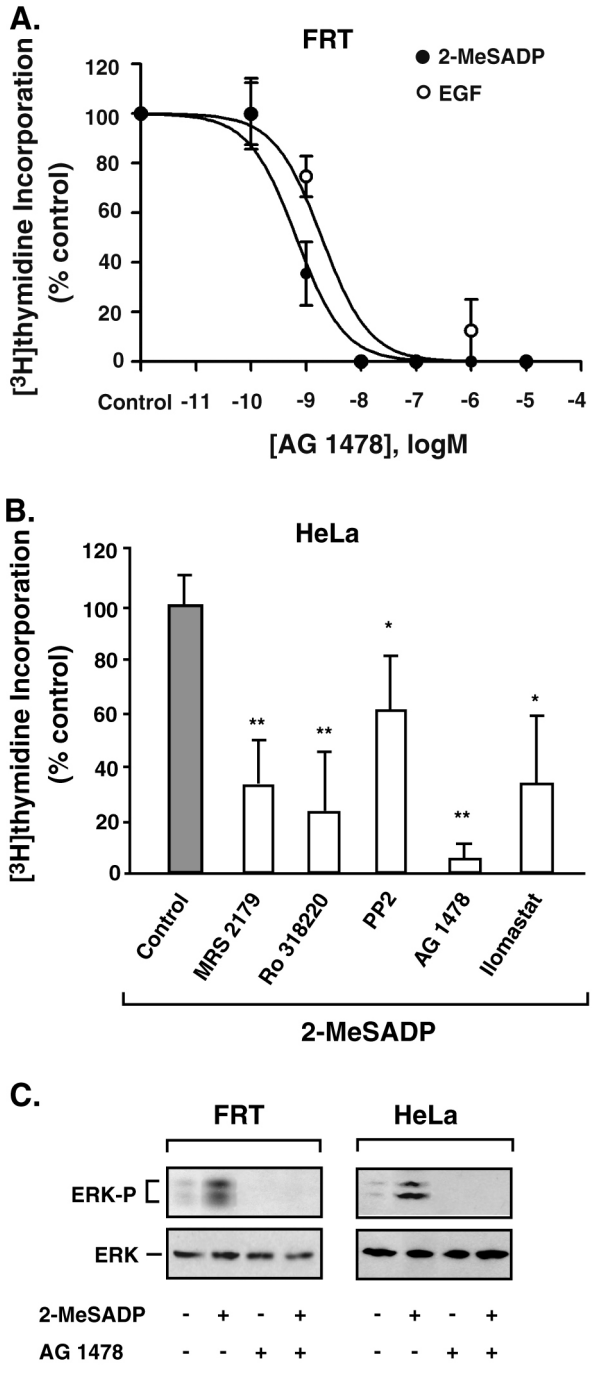

Fig. 7. Cell proliferation mediated by $P2Y_1R$ stimulation involves EGFR transactivation, PKC, Src and metalloprotease activity. (A) Induction of cell proliferation mediated by $P2Y_1R$ stimulation requires EGFR activation. The EGFR tyrosine kinase blocker AG1478 inhibits the proliferation of FRT cells induced by either 1 μM 2-MeSADP or 10 nM EGF in a concentration-dependent manner (n=4). (B) HeLa cells pre-incubated for 30 minutes in the absence or presence of 1 μM of MRS2179 ($P2Y_1R$ antagonist), Ro 318220 (non-selective PKC inhibitor), PP2 (Src kinase inhibitor), AG1478 (blocker of EGFR tyrosine-kinase activity) or Ilomastat (metalloprotease inhibitor), were then stimulated with 1 μM 2-MeSADP for 1 hour in the presence of each inhibitor. Results are normalized against control cells incubated only with the agonist. Values are the means ± s.e.m. *P<0.05, **P<0.01; Dunnett's test compared with control (n=3-6). (C) ERK1/2 activation in response to $P2Y_1R$ agonist depends on EGFR activity. The EGFR tyrosine kinase blocker AG1478 (100 nM) completely abrogates ERK1/2 activation induced by 2-MeSADP (10 nM, 5 minutes), in FRT and HeLa cells. The immunoblot is representative of three independent experiments.

usually require long periods of stimulation with growth factors, or two pulses of stimulation separated by several hours (Jones and Kazlauskas, 2001a; Jones and Kazlauskas, 2001b; Pennock and Wang, 2003; Shechter et al., 1978). This is explained by two different requirements: an initial stimuli-dependent signaling for driving resting cells into G1, and then a second stimuli-dependent process, which stabilizes components responsible for surpassing the restriction point that takes place 7-9 hours later (Jones and Kazlauskas, 2001a). Therefore, it is intriguing that a short stimulation (15-60 minutes) with nucleotide was enough to trigger mitogenesis, even in HeLa cells, in which 1 hour of EGF or serum stimulation did not result in proliferation. It seems that nucleotides are initiating a cascade of events that progress toward surpassing the restriction point of the cell cycle via unknown mechanisms.

It has been previously described that EGF can induce proliferation of unsynchronized HeLa cells (Hutchings and Sato, 1978), as we also observed (results not shown). However, EGF stimulation for either one (Fig. 3A) or 16 hours (results not shown) was unable to rescue growth-arrested HeLa cells and induce their proliferation. It is surprising therefore, that proliferation induced by nucleotides was completely abrogated by EGFR inhibitors, indicating its dependence on EGFR activation. How could this apparently contradictory result be explained? It is known that $P2Y_1R$ signaling involves an increase in intracellular calcium and PKC activation (Ralevic and Burnstock, 1998; von Kugelgen, 2006). Our results here provide evidence that $P2Y_1R$ signaling leads to a transactivation of the EGFR that triggers the ERK pathway. Therefore, nucleotide stimulation through the $P2Y_1R$ could potentially trigger at least two pathways, including the EGFR pathway, which are both required for proliferation. Inhibition of one might be enough to abrogate the proliferative response. A similar explanation, called the 'dual-signaling-track' model has been proposed for other ERK-dependent processes activated by certain GPCRs (Wetzker and Bohmer, 2003). Thus, considering that different signaling events seem to be required to push cells through the G0 to S interval (Jones and Kazlauskas, 2001b), nucleotides most probably fulfil this requirement by including the activation of the EGFR, which by itself would be insufficient to rescue arrested HeLa cells. Another possibility is that nucleotide-induced transactivation of the EGFR involves a different, more mitogenic ligand than EGF.

The relative contribution of different P2YR subtypes to EGFR-mediated epithelial cell proliferation is not yet clear. $P2Y_2R$ is the only other P2YR subtype previously described to mediate cell proliferation by transactivating the EGFR (Liu et al., 2004; Morris et al., 2004; Schafer et al., 2003; Soltoff et al., 1998; Wagstaff et al., 2000). $P2Y_2R$ is mainly activated by ATP and UTP. Stimulation with these nucleotides leads to EGFR activation and an increased proliferation of a variety of

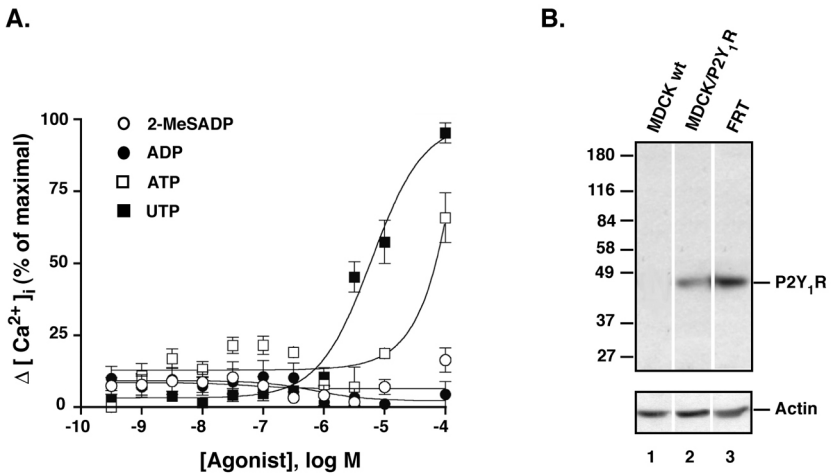

Fig. 8. P2Y$_1$R ectopically expressed in MDCK cells lacking this receptor. (A) Measures of intracellular calcium changes show that MDCK cells do not respond to P2Y$_1$R agonists (n=6-8). ATP and UTP, but neither ADP nor 2-MeSADP, provoke rises in intracellular Ca^{2+} levels in MDCK cells, further indicating that these cells do not express P2Y$_1$R. Data are means ± s.e.m. (B) MDCK cells permanently transfected to express the P2Y$_1$R (MDCK/P2Y$_1$R). Cells were selected for ectopic P2Y$_1$R expression levels lower than those of the endogenous receptor of FRT cells.

cells, including PC12 cells (Soltoff, 1998), MCF7 breast cancer cells (Wagstaff et al., 2000), astrocytoma cells transfected with P2Y$_2$R (Liu et al., 2004), cardiomyocytes (Morris et al., 2004) and A549 lung cancer cells (Schafer et al., 2003). However, these studies analyzed neither the effects of apyrase nor the congruency between the lifetime of P2Y$_2$R stimuli and proliferation requirements. We discarded the participation of P2Y$_2$R in the nucleotide-dependent proliferation of FRT and HeLa cells, since in these cells ATP, ADP and UDP, but not UTP, had mitogenic effects. Therefore, even though P2Y$_1$R and P2Y$_2$R are frequently co-expressed in the same cells, including epithelial cells (Schwiebert and

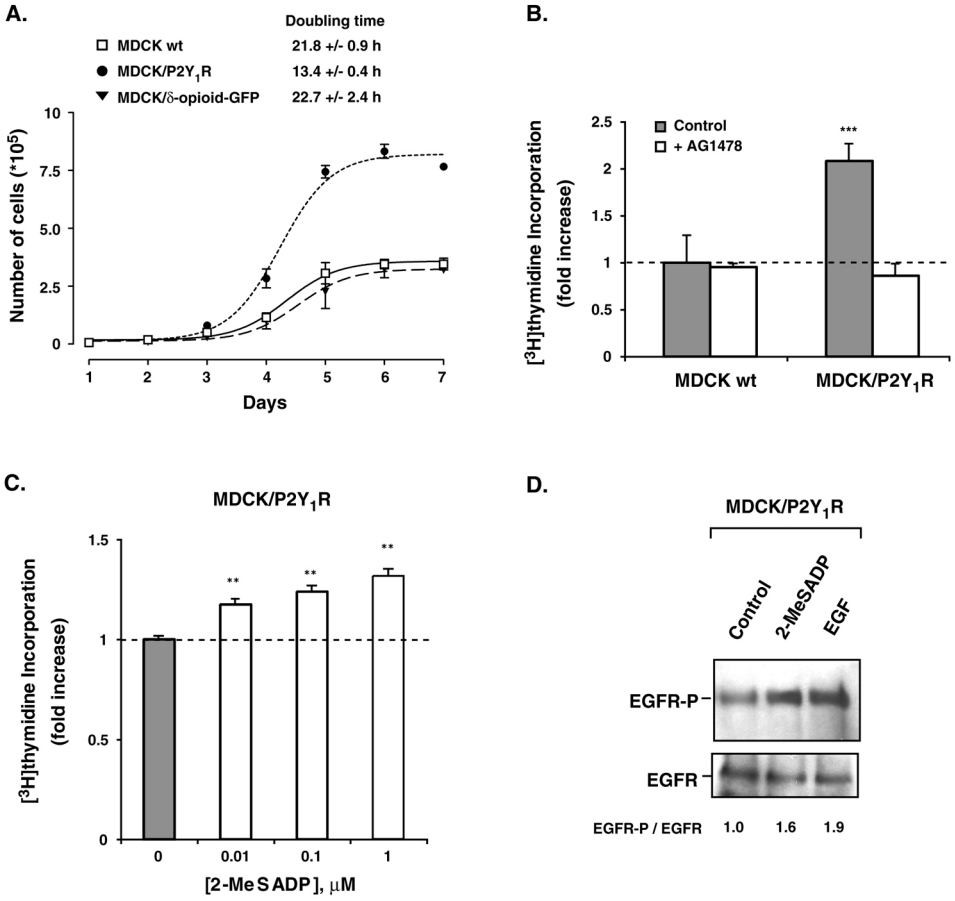

Fig. 9. MDCK cells ectopically expressing the P2Y$_1$R display lower doubling times and EGFR-dependent increased proliferation rates. (A) Viability curves for wild-type (wt) MDCK cells, or cells stably transfected with either the P2Y$_1$R (MDCK/P2Y$_1$R) or δ-opioid receptor (MDCK/δ-opioid). The number of cells increases with higher slope and reaches a higher maximum for MDCK/P2Y1R cells, whereas cells expressing ectopic δ-opioid receptor behave like wild-type MDCK cells. MDCK P2Y$_1$R cells also show a reduced doubling time. Values are the means ± s.e.m. (n=4-6). (B) Basal proliferation rate of MDCK P2Y$_1$R is higher than wild-type cells and sensitive to 0.1 μM AG1478, thus indicating a dependency on EGFR activity. In MDCK P2Y$_1$R cells, inhibition of EGFR activity significantly decreased the [^{3}H]thymidine incorporation. Cells were treated with 100 nM AG1478 during 16 hours of [^{3}H]thymidine incorporation. Values are the means ± s.e.m. *P<0.05, **P<0.01; Dunnett's test compared with control wild-type cells (n=6). (C) The selective P2Y$_1$R agonist 2-MeSADP increases proliferation of MDCK/P2Y$_1$R cells in a concentration-dependent manner. Cells were treated with exogenous 2-MeSADP for 1 hour before [^{3}H]thymidine incorporation. Values are the means ± s.e.m. **P<0.01; Dunnett's test compared with untreated cells (n=3). (D) P2Y$_1$R transactivates the EGFR in MDCK P2Y$_1$R cells. The immunoblot shows an increased EGFR tyrosine phosphorylation in cells treated with either 10 nM 2-MeSADP or 1 nM EGF for 5 minutes, quantified with respect to total EGFR mass and depicted below each lane. Only the carrier culture medium was added for controls.

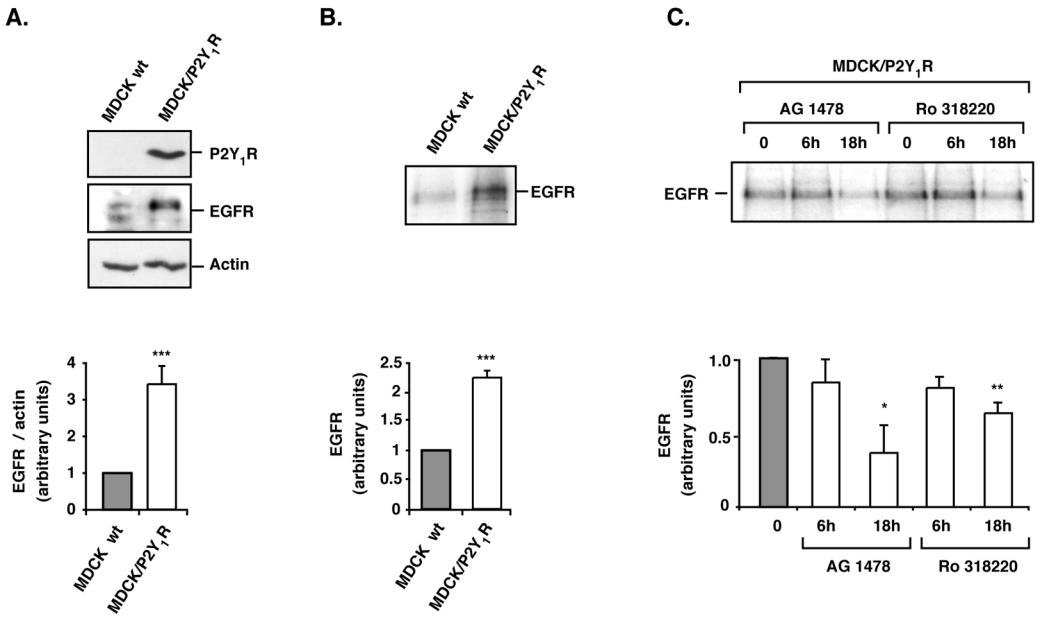

Fig. 10. MDCK $P2_1R$ (MDCK/$P2_1R$) cells display increased expression levels of the endogenous EGFR. (A) Ectopic expression of $P2Y_1R$ increases EGFR levels. Immunoblots of total cell extracts, densitometrically quantified in four independent experiments and normalized with respect to actin show a threefold increased level of EGFR in MDCK P2Y1R cells. (B) Ectopic expression of $P2Y_1R$ increases EGFR biosynthesis. Newly synthesized EGFR was immunoprecipitated from cells metabolically labeled for 2 hours with [^{35}S]Met/Cys (200 μCi/ml). Graph shows twofold increase in synthesis in MDCK P2Y1R cells (n=3). For A and B, values are the means ± s.e.m. ***P<0.001; paired t-test compared with wild-type cells. (C) Increased EGFR expression in MDCK P2Y1R cells depends on EGFR and PKC activity. Cells were treated with either 100 nM AG1478 or 10 nM Ro318220 for 6-18 hours before and during the metabolic labeling. Graph show the densitometric quantification of three independent experiments. Values are the means ± s.e.m. *P<0.05, **P<0.01; Dunnett's test compared with untreated cells.

Zsembery, 2003), their simultaneous stimulation is unnecessary for eliciting a nucleotide-mediated proliferative response, although both can increase the final outcome (Mishra et al., 2006). In FRT cells, the mitogenic effect of UDP, which also requires EGFR kinase activity (not shown), was not inhibited by MRS2179 and, therefore, compromises another P2YR subtype distinct from $P2Y_1R$, very likely $P2Y_6R$. MDCK cells that do not express $P2Y_1R$, responded to ATP and UTP increasing their intracellular calcium concentration, thus suggesting that they do express the $P2Y_2R$. However, apyrase did not decrease their proliferation rate, indicating that $P2Y_2R$ does not contribute to basal mitogenesis in these cells. These results and the longer half-life of extracellular ADP than ATP point to $P2Y_1R$ as a predominant contributor to epithelial cell proliferation under physiological conditions.

There is evidence suggesting that GPCRs can act as oncoproteins (Li et al., 2005; Marin and Chen, 2004; Marinissen and Gutkind, 2001; Parnot et al., 2002). GPCRs with activating mutations have been detected in several human cancerous tumors (Dhanasekaran et al., 1998; Marinissen and Gutkind, 2001). Interestingly, the ectopic expression or overexpression of wild-type GPCRs, such as the serotonin 1C (Julius et al., 1989), muscarinic acetylcholine (Gutkind et al., 1991), thrombin (Whitehead et al., 1995) and glutamate receptor 1 (Pollock et al., 2003) receptors, can transform cells in the presence of their respective ligands. Several neuropeptides acting through their cognate GPCRs promote cell proliferation and transformation (Cardona et al., 1991; Cuttitta et al., 1985; Gutkind, 1998; Heasley, 2001), indicating that GPCRs can also mediate growth-stimulatory autocrine loops. We could not find any evidence of $P2Y_1R$ expression in MDCK cells, neither by immunodetection nor by functional assays, such as nucleotide-induced proliferation or rise in intracellular Ca^{2+}. Therefore, we used these cells to study the effects of ectopic expression of $P2Y_1R$. Permanently transfected epithelial MDCK cells ectopically expressing $P2Y_1R$ (MDCK $P2Y_1R$), even at lower levels than the endogenous receptor in FRT cells, acquire a highly proliferative phenotype, manifested under basal conditions and crucially dependent on EGFR tyrosine-kinase activity.

Unexpectedly, MDCK $P2Y_1R$ cells display an increased expression level of EGFR. To our knowledge, a similar phenomenon has not been previously described for any other GPCR. The ectopically expressed $P2Y_1R$ probably establishes an autocrine stimulatory loop fed by endogenously released nucleotides, leading to increased signaling and transmodulation of the EGFR. The endogenous expression of the EGFR is known to increase because of activation of the PKC and the EGFR itself (Bjorge and Kudlow, 1987; Bjorge et al., 1989; Earp et al., 1988). Both PKC (Ralevic and Burnstock, 1998) and the EGFR (this work) participate in the $P2Y_1R$-signaling pathway. We demonstrated here that blockade of any of these proteins reduced the expression of EGFR in MDCK/$P2Y_1R$ cells. Thus, besides the role of $P2Y_1R$ in mediating the acute nucleotide transactivation of the EGFR, $P2Y_1R$ seems also to participate in the regulation of EGFR expression, through a mechanism involving PKC and EGFR activity. Indeed, these two $P2Y_1R$ roles have important implications for EGFR function in development and carcinogenesis.

Most human cancers derive from epithelia (Peto, 2001), and the proliferation and differentiation of epithelial cells are crucially dependent on the EGFR (Arteaga, 2002; Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995; Yarden, 2001). The EGFR plays fundamental roles in carcinogenesis. EGFR overexpression or activating mutations as well as over activity of other elements of the EGFR signaling pathway are common abnormalities in tumoral processes (Yarden, 2001). Our results disclose a novel mechanism by which abnormalities in a GPCR function could potentially contribute to cancer by promoting a proliferative phenotype that is highly dependent upon EGFR activity and increased EGFR expression. P2Y$_1$R could potentially mediate altered mitogenic responses to environmental nucleotides. Exaggerated release of nucleotides is expected to occur with injuries such as chronic inflammation – a condition associated with cancer (Coussens and Werb, 2002). Anomalies in P2Y$_1$R leading to its ectopic expression, overexpression or constitutive activation could promote highly proliferative phenotypes as suggested by our observations in MDCK P2Y$_1$R cells. This may also extend to other GPCRs.

Because EGFR function is crucial in processes of cell differentiation, migration and apoptosis, and the EGFR is also an effective target for novel antitumor drugs (Gschwind et al., 2004), we envision a broad range of regulatory roles for extracellular nucleotides and P2Y$_1$R that remain to be explored.

Materials and Methods

Reagents

DMEM containing high glucose, protein-A-Sepharose, butyric acid, 2-MeSADP, ADP, ATP, UDP, UTP, Apyrase from potato and MRS2179 were purchased from Sigma. Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT). All other cell-culture reagents were from Invitrogen (Carlsbad, CA). [^{3}H]thymidine and the enhanced chemiluminescence (ECL) system were from Amersham (Piscataway, NJ). Ro318220, PP2 and AG1478 were purchased from Calbiochem (La Jolla, CA). Ilomastat was from Chemicon (Temecula, CA). Human recombinant EGF produced by Chiron (Emeryville, CA) was kindly provided by Pablo Valenzuela and Carlos George-Nascimento (Austral Biologicals, San Ramon, CA).

Plasmids and antibodies

The human P2Y$_1$R cDNA (GenBank Acc. no. NM_002563) tagged at the N-terminus with a HA epitope (HA-P2Y$_1$R) (Wolff et al., 2005), was extracted from the plasmid pLXSN and subcloned between the *Eco*RI and *Xho*I restriction sites of the pDCNA3 expression vector (Invitrogen). Antibodies EGFR984 and EGFR1176 were raised in rabbits by injecting the described immunogenic peptides $_{984}$DDVVDADEYLIPQ$_{996}$ and $_{1176}$VAPQSSEFIGA$_{1186}$ of the EGFR cytosolic tail (Kris et al., 1985; Margolis et al., 1990), coupled to mollusc *C. concholepas* hemocyanin (Blue Carrier, from Biosonda Biothechnology, Santiago, Chile). The EGFR984 antibody has been previously characterized (Salazar and Gonzalez, 2002). Polyclonal antibodies against human P2Y$_1$R were similarly obtained with the C-terminal immunogenic peptide CPEFKQNGDTSL (Moore et al., 2000). Hybridomas producing monoclonal anti-EGFR antibodies HB8506 reacting with the extracellular domain of the EGFR were from American Type Culture Collection (Manassas, VA). Anti-phosphotyrosine 4G10 monoclonal antibody was a gift from María Rosa Bono (Universidad de Chile, Santiago, Chile). IgG fraction was purified using the Econo-Pac Serum IgG purification kit (Bio-Rad). Anti-phospho ERK and anti-total ERK were from Sigma, anti-actin was from abCam (Cambridge, UK) and anti-cyclin D from BD Pharmingen (Franklin Lakes, NJ).

Cell culture and transfections

Cells were cultured in DMEM (HeLa and MDCK) or F12 (FRT) media supplemented with 7.5% FBS and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone), maintained at 37°C in a humidified atmosphere (95% air, 5% CO_2) and passaged every 3-4 days. Before the assays, cells were maintained in serum-free medium for 24 hours. MDCK cells were transfected with HA-P2Y$_1$R using the Lipofectamine 2000 method and subjected to selection in 0.8 mg/ml geneticin sulfate (G418) to obtain stable transfectants, then maintained in 0.4 mg/ml G418.

Immunoblotting and immunoprecipitation

For immunoblot assays, 60 μg protein from total cell extracts prepared as described (Salazar and Gonzalez, 2002) were resolved on 8-10% polyacrylamide SDS gels and transferred onto nitrocellulose (Schleicher and Schuell, Germany). Proteins recognized by the primary antibodies anti-P2Y$_1$R (10 μg/ml), EGFR984 (1:500), 4G10 (1:1000), anti-actin (1:5000), anti-cyclinD (1 μg/ml), anti-phospho ERK (1:1000) and anti-total ERK (1:1000) were detected using the ECL system and horseradish peroxidase conjugated anti-rabbit (1:5000) or anti-mouse (1:5000) IgGs as secondary antibodies. The EGFR was immunoprecipitated with the monoclonal antibody HB8506 in HeLa cells and with EGFR1176 antibodies in FRT cells, resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine monoclonal antibody 4G10, as described (Salazar and Gonzalez, 2002). For total EGFR detection, membranes were stripped and incubated with the polyclonal antibody EGFR984 (Salazar and Gonzalez, 2002).

To assess the biosynthetic levels of EGFR in MDCK cells, cells were first incubated in Met/Cys-free DMEM (Gibco) and 1% FBS for 30 minutes at 37°C and then metabolically labeled with 200 μCi/ml [^{35}S] Met/Cys (Perkin Elmer) for 2 hours at 37°C. EGFR was immunoprecipitated with EGFR1176 antibody, resolved by SDS-PAGE and visualized by fluorography as described (Bravo-Zehnder et al., 2000; Salazar and Gonzalez, 2002) using Amplify (Amersham). To assess the dependency of EGFR expression on EGFR and PKC activity, cells were treated with either 100 nM AG1478 or 10 nM Ro 318220 for 6 or 18 hours before and during metabolic labelling.

The relevant bands on immunoblots and gels were digitized, and their relative intensities estimated with NIH or Image J software. In the immunoblots, we used actin, unphosphorylated EGFR or unphosphorylated ERK as loading reference to standardize the densitometric intensity. In immunoprecipitation assays of metabolically labeled EGFR, an aliquot of total cell extracts was precipitated with 20% trichloroacetic acid and counted by liquid scintillation, to discard alterations in total [^{35}S]Met/Cys incorporation.

[^{3}H]thymidine incorporation assays

FRT or HeLa cells (2.5×10^3 cell/well) seeded in 48-well plates and maintained for 24 hours in medium plus 7.5% FBS, were serum-starved for 24 hours to induce quiescence. This lowered by 60% the basal [^{3}H]thymidine incorporation in a reversible manner. After incubation with different stimuli (nucleotides, EGF or FBS) in the indicated conditions of each experiment, the medium was removed and the cells were then incubated with 2 μCi /ml [^{3}H]thymidine for 16 hours at 37°C, washed three times with 1 ml ice-cold PBS and incubated for 20 minutes with 5% trichloroacetic acid at room temperature. Then, cells were washed twice with ice-cold ethanol, extracted with 0.5 M NaOH for 30 minutes at 37°C, neutralized with 0.5 M HCl and [^{3}H]thymidine incorporation was assessed by liquid scintillation. Incubations with antagonists were made 30 minutes before and during agonist treatment. To assess the effect of AG1478, apyrase or MRS2179 upon basal proliferation rate, the drugs were maintained during the 16 hour [^{3}H]thymidine incorporation period.

Viability assays and doubling time calculation

Population doubling time of the cell lines was determined by seeding 5000 cells/well in 24-well plates. Cells were trypsinized every 24 hours and counted using a hemocytometer. Viability was assessed based on Trypan Blue exclusion. Doubling time was calculated from the graph of viable cell number versus time (linear zone), using the formula: doubling time=$(\ln 2 \times t)/(\ln X/Xo)$, where X corresponds to the cell number at time t and Xo corresponds to the initial cell number.

Extracellular nucleotide measurement

FRT and HeLa cells seeded in 24-well tissue-culture plates (40,000 cells/well) and grown for 72 hours in media supplemented with 7.5% FBS, were placed in 200 μl of Tyrode buffer 4 hours before the nucleotide assay. ATP, ADP, AMP and adenosine concentrations in the media were assessed at steady state (basal conditions) or after cell injury provoked by a cross scratch made with a 1 mm tip. The media were collected and extracellular nucleotides were derivatized for sensitive quantification of adenyl purines as fluorescent 1, *N*6-etheno species, by adding 100 μl buffer phosphate-citrate (pH 4) and 10 μl chloro-acetaldehyde and then heating for 40 minutes at 80°C, as described (Lazarowski et al., 2004; Todorov et al., 1996). The reaction was stopped by incubation on ice for 5 minutes. After 24 hours at 4°C, an automated Merck/Hitachi HPLC apparatus equipped with a fluorescence detector was used for the identification and quantification of ethenylated species, at excitation and emission wavelengths of 260 nM and 410 nM, respectively. Each sample (100 μl) was injected into a Lichrocart 125-4 column equilibrated with the mobile phase (100 mM KH_2PO_4, 5 mM tetrabutylammonium, 4% acetonitrile, pH 3) at 1 ml/minute. Data from test samples were compared against known concentrations of derivatized adenosine, AMP, ADP and ATP analyzed in parallel. Typical elution times of these etheno standards were 1.7 minutes for ϵ-adenosine, 1.9 minutes for ϵ-AMP, 3.3 minutes for ϵ-ADP and 8.2 minutes for ϵ-ATP.

Calcium measurements

The rise in intracellular Ca^{2+} induced by nucleotide agonists was measured in wild-type MDCK cells, as described (Yerxa et al., 2002). In brief, cells seeded at a density

of 30,000 cells/well in 96-well plates, were grown for 24 hours and incubated with 2.5 μM Fluo-3-AM (Molecular Probes) for 1 hour at 37°C. Intracellular Ca^{2+} levels were monitored as changes in fluorescence intensity using a fluorescent light imaging plate reader (FLIPR, Molecular Devices). The peak fluorescence achieved by each nucleotide concentration was used to plot the concentration-response curve using GraphPad Prism version 3.00 for Windows (Graph Pad Software, San Diego, CA). Nucleotides (0.1 nM to 100 μM) were applied for 3 minutes, fluorescence was continually recorded a minute before and during nucleotide addition. The maximal fluorescence attained with the largest ligand concentration was used to normalize the curves. Each protocol was performed in duplicate and repeated in three to four batches of cells.

Statistical analysis

Values were expressed as the mean ± s.e.m. Dunnett's tables were used to compare several treatments against the same control and analysis of variance, or paired *t*-test was applied when necessary. Curves were plotted using GraphPad Prism version 3.00 for Windows (Graph Pad Software, San Diego, CA). The number of experiments is indicated by *n* in each figure.

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