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Rapid constitutive and ligand-activated endocytic trafficking of $P2X_3$ receptor

Fabrizio Vacca,*,¶,[1] Michela Giustizieri,† Maria Teresa Ciotti,‡ Nicola Biagio Mercuri†,§ and Cinzia Volonté*,‡

*From Laboratory of Cellular Neurobiology, Santa Lucia Foundation, Rome, Italy

†Laboratory of Experimental Neurology, Santa Lucia Foundation, Rome, Italy

‡CNR, Institute of Neurobiology & Molecular Medicine, Rome, Italy

§Department of Neuroscience, University of Rome 'Tor Vergata', Rome, Italy

¶Department of Biochemistry, University of Geneva, Geneva, Switzerland

Abstract

P2X receptors mediate a variety of physiological actions, including smooth muscle contraction, neuro-endocrine secretion and synaptic transmission. Among P2X receptors, the $P2X_3$ subtype is expressed in sensory neurons of dorsal root- and trigeminal-ganglia, where it performs a well-recognized role in sensory and pain transmission. Recent evidence indicates that the strength of $P2X_3$-mediated responses is modulated in vivo by altering the number of receptors at the plasma membrane. In the present study, we investigate the trafficking properties of $P2X_3$ receptor in transfected HEK293 cells and in primary cultures of dorsal root ganglion neurons, finding that $P2X_3$ receptor undergoes rapid constitutive and cholesterol-dependent endocytosis. We also show that endocytosis is accompanied by preferential targeting of the receptor to late endosomes/lysosomes, with subsequent degradation. Furthermore, we observe that at steady state the receptor localizes predominantly in lamp1-positive intracellular structures, with a minor fraction present at the plasma membrane. Finally, the level of functional receptor expressed on the cell surface is rapidly up-regulated in response to agonist stimulation, which also augments receptor endocytosis. The findings presented in this work underscore a very dynamic trafficking behavior of $P2X_3$ receptor and disclose a possible mechanism for the rapid modulation of ATP-mediated responses potentially relevant during physiological and pathological conditions.

Keywords: α,β-methyleneATP, biotinylation, endocytosis, internalization, purinergic, ubiquitin.

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The function of plasma membrane (PM) proteins can be regulated by altering their cell surface expression through regulation of exocytosis and/or endocytosis rates, in response to particular biological requirements (Royle and Murrell-Lagnado 2003; Bohn 2007). For instance, in the CNS, short- and long-lasting changes in the number of neurotransmitter receptors at glutamatergic synapses can be regulated in processes such as learning and memory (Groc and Choquet 2006), and the fine regulation of these processes often involves cycling of receptors between PM and intracellular pools (Ehlers 2000; Park *et al.* 2004). Moreover, the dynamics of receptor trafficking and the destiny of internalized receptors (i.e. degradation vs. recycling) are processes tightly regulated by neuronal maturation (Washbourne *et al.* 2004) and activity (Ehlers 2000; Martin and Henley 2004).

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Address correspondence and reprint requests to Fabrizio Vacca, Santa Lucia Foundation. Via del Fosso di Fiorano 65, 00143 Rome, Italy. E-mail: fabrizio.vacca@unige.ch

[1]The present address of Fabrizio Vacca is University of Geneva, Department of Biochemistry, Quai Ernest Ansermet, 30, 1211 Geneva, Switzerland. Email: fabrizio.vacca@unige.ch

Abbreviations used: αβmeATP, α,β-methyleneATP; βMCD, βmethyl-cyclodextrin; biotin-XX, SE, 6-((6-((biotynoyl)amino)hexanoyl)amino)-hexanoic acid, succinimidyl ester; CH, cycloheximide; ConA, concanavalinA; DRG, dorsal root ganglia; GGA, Golgi localizing γ-adaptin ear homology domain ARF-interacting proteins; MESNA, sodium 2-mercaptoethanesulfonic acid; PAO, phenylarsine oxide; PM, plasma membrane; Sulfo-NHS-SS-Biotin, sulfosuccinimidyl-2-(biotinamido-ethyl-1,3-dithiopropionate.

P2 receptors are a family of receptors for extracellular purine and pyrimidine nucleotides (Volonté *et al.* 2006, 2008; Burnstock 2007; Volonté and D'Ambrosi 2009). The family is composed of seven P2X ($P2X_{1–7}$) ion channel receptors and eight P2Y ($P2Y_{1,2,4,6,11–14}$) G protein-coupled receptors. Within the P2X sub-family, endocytic trafficking studies conducted on the $P2X_1$ subtype have shown how this receptor can internalize and recycle back to the PM in response to agonist application (Dutton *et al.* 2000; Ennion and Evans 2001). On the other hand, the $P2X_4$ subtype, constitutively internalizes and recycles at high rate, behavior that has been shown to be dependent on a non-canonical endocytic motif present in its C-terminal tail (Bobanovic *et al.* 2002; Royle *et al.* 2005). Moreover, at least in certain cell types, the $P2X_4$ receptor is mainly localized in lysosomes and its surface expression can be directly regulated by the exocytosis of these organelles (Qureshi *et al.* 2007). Within the P2Y sub-family, ligand-induced internalization and endocytic trafficking in $P2Y_1$ (Tulapurkar *et al.* 2006) and $P2Y_2$ (Tulapurkar *et al.*, 2005) receptors have been studied. In particular, the internalization of $P2Y_1$ receptor has been shown to be dependent on the activity of CaMKII.

The $P2X_3$ subtype is abundantly expressed in sensory neurons of dorsal root (DRG) (Vulchanova *et al.* 1997) and trigeminal ganglia (Llewellyn-Smith and Burnstock 1998), where it performs a well-established role in sensory and pain transmission in physiological and pathological conditions (Inoue 2007; Wirkner *et al.* 2007). It generally assembles as a trimer that can aggregate to form larger complexes (Nicke *et al.* 1998), and it is enriched in specialized sub-membrane compartments such as lipid rafts (Vacca *et al.* 2004). Under basal conditions, the $P2X_3$ subtype is a fast-desensitizating receptor but it can produce larger currents under pathological pain states (Paukert *et al.* 2001; Xu and Huang 2002; Giniatullin *et al.* 2008). Sensitization of $P2X_3$ receptor can depend on post-translational modifications such as phosphorylation occurring on both extracellular (Wirkner *et al.* 2005) and intracellular (Paukert *et al.* 2001; D'Arco *et al.* 2007) sites. Nevertheless, such enhanced responses can also depend on augmented trafficking from intracellular compartments to the PM, for instance in DRG (Xu and Huang 2004) and trigeminal ganglion neurons (Fabbretti *et al.* 2006).

In the present study, we investigate the trafficking dynamics of the $P2X_3$ receptor expressed exogenously in HEK293 cells and endogenously in DRG sensory neurons. We find that the receptor undergoes rapid constitutive endocytosis and targeting to the late endosomal/lysosomal system, with resultant overall localization in these organelles at steady state and high degradation rate. This very dynamic trafficking behavior can thus represent a possible mechanism for the rapid modulation of ATP-mediated physiopathological responses in sensory neurons.

Materials and methods

Cell cultures, transfections and treatments

The human HEK293 cell line was cultured in Dulbecco's modified Eagle Medium (Sigma, Milano, Italy), supplemented with 2 mM L-Glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated calf serum (Invitrogen, San Giuliano Milanese, Italy). Cells were transfected with full length cDNA coding for rat $P2X_3$ receptor inserted in the pcDNA3 vector ($P2X_3$-pcDNA3) (gift of Prof. A. North); control cells were transfected with the pcDNA3 vector (Invitrogen). Cells were transfected with Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. Briefly, cells at 80–90% confluence in 35 mm-well dishes were transfected with 5 μL lipofectamine 2000, and 4 μg of plasmid DNA. DNA-lipofectamine 2000 containing medium was replaced after 4 h, and experiments were performed approximately 24 h later.

Preparation of DRG primary cultures

Primary cultures of DRG neurons were prepared with minor modifications to a reported method (Hu and Li 1997). Briefly, lumbar ganglia were excised from 15-days old Wistar rats and placed into phosphate-buffered saline (PBS) without calcium or magnesium. After removing the roots, DRGs were minced and incubated at 37°C for 35 min with trypsin (0.5 mg/mL) and DNAse (0.1 mg/mL). After enzymatic digestion, the cells were then placed in D-MEM plus 10% fetal bovine serum and dissociated with a 10 mL syringe, centrifuged at 1000 rpm, at 25°C. Cells were then re-suspended with D-MEM (plus 10% fetal bovine serum, penicillin, streptomycin, and 0.25 mg/mL nerve growth factor), and plated on poly-l-lysine-coated Petri dishes and cultured at 5% CO_2. Cells were used 2 days after plating. Animals were used in accordance with the European Directive 86/609/EEC requirements, and all efforts were made to minimize the number of animals used and their suffering.

Immunofluorescence

HEK293 cells were fixed for 30 min at 4°C in 3% paraformaldehyde and permeabilized for 5 min with 0.1% Triton X-100 in PBS at 25°C. Nonspecific binding sites were blocked with 10% normal donkey serum (Jackson Immunoresearch, West Baltimore Pike, PA, USA) and 1% bovine serum albumin (BSA) in PBS, for 30 min at 25°C. Plates were then incubated sequentially for 1 h at 25°C with rabbit-polyclonal anti-$P2X_3$ (1 : 1000) and monoclonal antibodies either anti-EEA-1 (1 : 500) (BD biosciences, San diego, CA, USA) or anti Lamp-1 (1 : 200) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa city, IA, USA). After two washes with 1% BSA in PBS, the plates were incubated for 1 h at 25°C with a mixture of Cy2-conjugated donkey anti-mouse IgG (1 : 100), or Cy3-conjugated donkey anti-rabbit IgG (1 : 100) (Jackson Immunoresearch). All antibodies were diluted in 1% BSA in PBS. After three washes with PBS, plates were mounted with glass coverslips an anti-fading gel mount (Biomeda, Foster City, CA, USA). Double immunofluorescence was analyzed with a laser scanning confocal microscope (LSM 510; Zeiss, Jena, Germany).

Surface biotinylation and isolation of biotinylated proteins

HEK293 cells (24 h after transfection) and DRG neurons (2 DIV) were washed twice with Dulbecco's modified PBS (D-PBS) containing 0.6 mM $MgCl_2$ and 1,8 $CaCl_2$. Surface proteins were biotinylated by incubating the cells with 0,5 mg/mL 6-((6-((biotynoyl)amino)hexanoyl)amino) -hexanoic acid, succinimidyl ester (biotin-XX, SE) (Invitrogen) dissolved in D-PBS containing 0.6 mM $MgCl_2$ and 1,8 $CaCl_2$ for 30 min at 4°C. Cells were then washed for three times with D-PBS, 100 mM glycine and either directly lysed with lysis buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin) or returned to 37°C in D-MEM, to allow protein internalization and degradation before lysis. Reversible biotinylation was obtained with 0.5 mg/mL sulfosuccinimidyl-2-(biotinamido)ethyl-1,3 dithiopropionate (Sulfo-NHS-SS-Biotin) (Pierce, Milano, Italy) in D-PBS with 0.6 mM $MgCl_2$ and 1.8 $CaCl_2$ for 30 min at 4°C. After three washes with ice-cold D-PBS plus 100 mM glycine, the cells were returned to 37°C in D-MEM, to allow protein internalization. Biotin linked to proteins on the cell surface was then stripped using ice-cold 100 mM sodium 2-mercaptoethanesulfonic acid (MESNA) in D-PBS (plus 0.6 mM $MgCl_2$, 1.8 $CaCl_2$) (2 × 25 min). Residual MESNA was quenched by 10-min incubation with 120 mM iodoacetamide (3 min). The cells were then washed for three times in ice-cold D-PBS and lysed. Cell lysates were centrifuged at 15 000 × *g* for 10 min at 4°C. Cell lysates were quantified for protein content with the Bradford method (Bio-Rad Laboratories, Segrate, Italy) and normalized lysate volumes were used in subsequent steps. Biotinylated proteins were precipitated by incubating lysates for 2 h at 4°C with streptavidin-coupled agarose beads (Invitrogen). Beads were washed for three times with lysis buffer and then boiled for 5 min after the addition of 2X Laemmli sample buffer (15 μL/sample). In order to verify the potential biotinylation of intracellular proteins as a consequence of cellular permeabilization by the different treatments, all the blots were probed with anti β-actin antibody.

Electrophoresis and immunoblotting

For SDS-PAGE, cellular lysates were diluted in 4X Laemmli sample buffer, containing a final 2% (v/v) β-mercaptoethanol as reducing agent, and proteins were separated on 7.5% or 12.5% polyacrylamide gels. Blotting to nitrocellulose membranes (Amersham Biosciences, Milano, Italy) was performed using TRIS-glycine transfer buffer with 20% (v/v) CH_3OH. Non-specific binding was prevented by incubating the blotted membranes in 5% (w/v) non-fat dry milk in TBS-T (Tris–buffered saline, 0,2% (v/v) tween-20). Primary antibodies were incubated for 2 h at room temperature or overnight at 4°C ($P2X_3$ from Alomone, Jerusalem, Israel; ubiquitin from Santa Cruz Biotech, Santa Cruz, CA, USA; transferrin receptor from Invitrogen). Secondary peroxidase-linked antibodies were used for 50 min at 25°C. The antibodies were diluted in TBS-T with 3% (w/v) non-fat dry milk. Immunostained bands were visualized using the ECL detection system (Amersham Biosciences) with Kodak 440CF Image Station; band intensities were measured on the raw images (which were never saturated during acquisition) with 1D image analysis software (Eastman Kodak Company, Rochester, NY, USA).

Electrophysiological recordings

For electrophysiological experiments, HEK293 cells were plated on glass coverslips and co-transfected with $P2X_3$-pcDNA3 and a green-fluorescent protein expressing plasmid (Invitrogen) (8 : 1 molar ratio). In mock-transfection control experiments, the $P2X_3$-pcDNA3 plasmid was substituted with the pcDNA3 empty vector. Coverslips were placed in a recording chamber, on the stage of an upright microscope (Axioscope FS, Zeiss, Germany) equipped for infrared video microscopy (Hamamatsu, Hamamatsu City, Japan) and submerged in a continuously flowing (2.5 mL/min) solution at 37°C. This solution contained 126 mM NaCl, 2.5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 2.4 mM $CaCl_2$, 10 mM glucose, and 18 mM $NaHCO_3$, gassed with 95% O_2-5% CO_2. Whole cell patch clamp recordings were obtained from green-fluorescent protein-positive cells visualized by epifluorescence at 480 ± 40 nm excitation wavelength. Borosilicate glass electrodes (WPI 1.5 mm) were pulled with a PP 83 Narishige puller. The resistance of the pipette was ∼4 MΩ when filled with a standard solution containing (in mM) 145 KC1-gluconate, 0.1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 0.75 EGTA, 2 Mg-ATP, and 0.3 Na_3GTP, pH 7.3. The membrane voltage and currents were acquired using pClamp and Axoscope software (Axon Instruments, Sunnyvale, CA, USA). Agonist (100 μM αβmeATP) was applied via a patch pipette that was positioned in close vicinity of the cell body and was connected to a pressure application system (Picospritzer, 20–30 psi, 0.1–10 s). Numerical data in the graph are expressed as means ± SD. Unpaired Student's *t*-test was used to compare data and $p < 0.05$ was considered significant.

Results

Rapid constitutive endocytosis of $P2X_3$ receptor

In HEK293 cells, the transiently transfected $P2X_3$ receptor imaged by confocal microscopy shows intense immunofluorescence within intracellular structures with vesicular appearance; actually, low levels of the protein are detected at the PM (Fig. 1a). To further confirm the PM versus intracellular distribution of $P2X_3$ receptor, we labeled PM-exposed receptor with the cell-impermeant biotinylation reagent Biotin-XX-SE. The amount of biotinylated receptor was then compared to scalar dilutions of the total cell lysate. In this way, it is possible at least to estimate the percentage of receptor present on the cell surface, which was found to be around 12% (Fig 1b). Even if this methodology cannot exactly quantify the PM fraction of the receptor, it can provide a realistic approximation. The $P2X_3$ protein is visible as two distinct bands around 60 KDa, probably due to heterogeneous post-translational modifications. We never find differences in ability of these two forms to reach the PM. Furthermore, we find PM fractions for transfected $P2X_1$ and $P2X_7$ receptors of 50% and 60% respectively (not shown), thus ascertaining the suitability of this methodology for detecting higher expression levels at the PM.

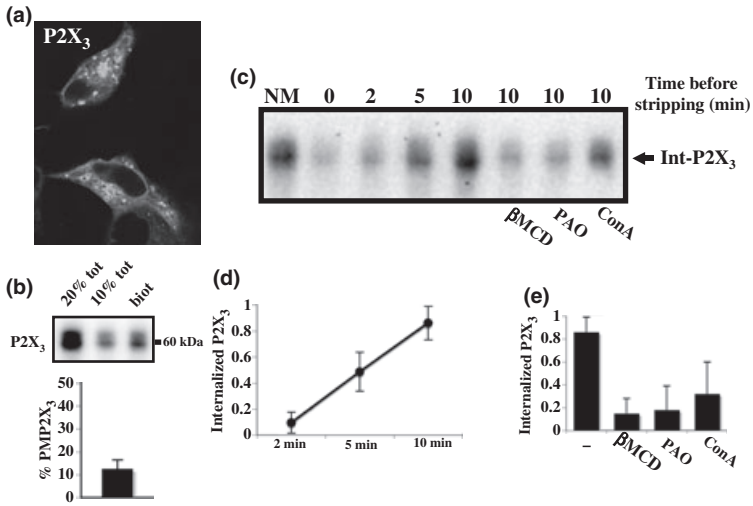

Fig. 1 Intracellular localization and rapid constitutive endocytosis of $P2X_3$ receptor in HEK293 cells. (a) Transiently transfected HEK293 cells were fixed and stained with anti-$P2X_3$ receptor antiserum. Immunofluorescence was detected with laser scanning confocal microscope. (b) PM receptor was labeled with biotin-XX-SE at 4°C and precipitated with agarose–streptavidin beads. The streptavidine-precipitated $P2X_3$ (60 KDa) was compared with different amounts of total cell lysate (given as a percentage of the quantity used for precipitation). Bands were quantified and the estimated percent of PM $P2X_3$ is shown. The graph represents results from three independent experiments. (c) After PM receptor labeling with NHS-SS-biotin at 4°C, HEK293 cells were returned to 37°C for the indicated times and extracellularly exposed biotin was then stripped with MesNa (100 mM). After lysis, internalized proteins (protected from stripping) were precipitated with streptavidin beads and probed for $P2X_3$ protein after western blot (Internalized-$P2X_3$). NM= no MesNa treatment. PAO (5 μM), ConA (300 μg/mL), βMCD (10 mM) were added during the incubation at 37°C. (d) Quantification of internalization kinetics, values are expressed relative to NM, taking time 0 as background and give averages plus SD from four independent experiments. (e) Quantification of the inhibitory effect of PAO, ConA and βMCD on the internalization process at the time point of 10 min (averages and SD from 3 independent experiments).

This distribution can arise from incomplete PM delivery from the secretory pathway or, alternatively, from a high rate of receptor endocytosis resulting in low $P2X_3$ protein on the PM at steady state. To test this last hypothesis, we directly measured the rate of receptor endocytosis under basal conditions. PM exposed receptor was first labeled for 30 min at 4°C with the cell-impermeant biotinylation reagent NHS-SS-biotin. The cells were then returned to 37°C for various lengths of time and the extracellular biotin, labeling the PM exposed receptor was thus stripped with the reducing reagent MesNa. Under these conditions, we find that after 10 min at 37°C, all the labeled receptor is present in the intracellular compartment, completely protected from stripping, therefore indicating that complete receptor internalization already occurs in this time lapse (Fig. 1c–e). The general inhibitors of endocytosis, phenylarsine oxide (PAO) (Beaumont *et al.*1998; Zwaagstra *et al.* 2001) or concanavalinA (ConA) (Beaumont *et al.*1998; Jayanthi *et al.* 2004) within different extent block the $P2X_3$ receptor internalization, which is also inhibited when cholesterol is extracted from the PM by βmethyl-cyclodextrin (βMCD). Furthermore, when we prevent receptor endocytosis by maintaining the cells in the presence of PAO, ConA or βMCD at 37°C for different times (5–15 min) before biotinylation, we observe even a gradual increase in the level of $P2X_3$ receptor at the PM, indicating that accumulation of the receptor indeed occurs at the PM when endocytosis is inhibited (Fig. 2a and d). The effect of βMCD is in accord with the involvement of cholesterol-rich domains in receptor endocytosis, as well as in $P2X_3$ receptor functioning (Vacca *et al.* 2004). In order to evaluate also the potential contribution of classical clathrin-dependent endocytosis, we treated the cells with high-sucrose medium, a treatment that is well known to block the assembly of clathrin coats (Heuser and Anderson 1989). Under this condition, we do not observe a PM accumulation of $P2X_3$ receptor, even if we can clearly see an augmented PM expression of transferrin receptor (Fig. 2b and d).

Since we observe constitutive internalization, we also investigated the possibility that this phenomenon could be due to autocrine release of ATP from HEK293 cells, continuously stimulating the receptor. Since we do not find PM accumulation of $P2X_3$ following treatment of the cells with apyrase (Fig. 2c and d), this hypothesis is likely to be excluded. Consistently, we do not observe any significant change in the $P2X_3$ receptor levels at the PM, after treatment with the cellular ectonucleotidase inhibitor ARL 67156 (Lévesque *et al.* 2007) (Fig. 2c and d).

We next aimed to establish how functional is the PM accumulation of $P2X_3$ receptor when its endocytosis is inhibited. We measured inward currents elicited by αβmeATP

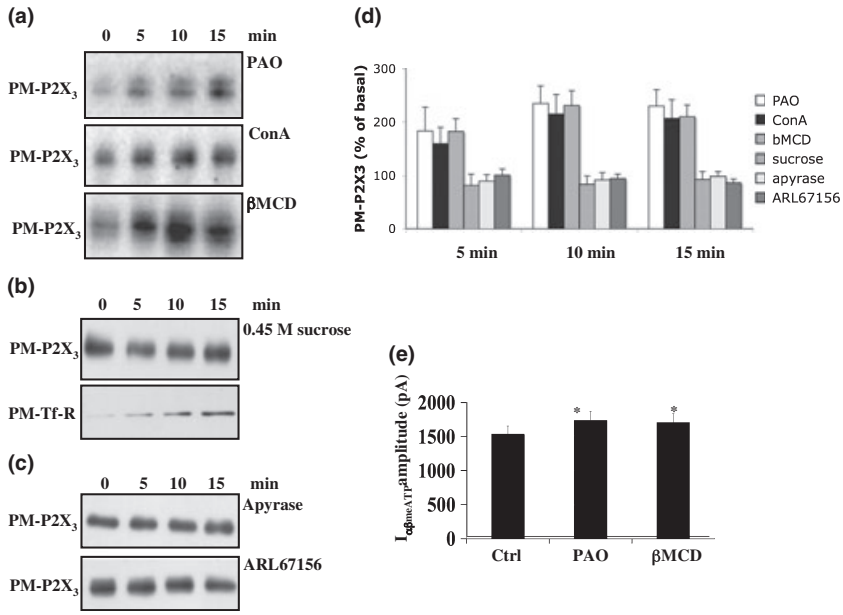

Fig. 2 Increased PM levels of $P2X_3$ receptor after endocytosis inhibition and cholesterol depletion. Cells were treated for the indicated times at 37°C with PAO (5 μM), ConA (300 μg/mL) and βMCD (10 mM) (a), with sucrose 0,45 M (b), with apyrase (10 U/mL) or ARL67156 (50 μM) (c). The PM proteins were labeled with biotin-XX-SE at 4°C, then precipitated with agarose-streptavidin beads, and probed for $P2X_3$ protein (PM-$P2X_3$) or transferrin receptor. (d) Quantitative data from experiments in panels a, b and c. Data represent the averages plus SD from 5 (βMCD), 4 (PAO) or 3 (ConA, sucrose, apyrase and ARL67156) independent experiments. Data are shown as percentage increase on relative controls. (e) Inward currents recorded from $P2X_3$ receptor-transfected HEK293 cells stimulated with 100 μM αβmeATP, pre-treated or not for 10 min with PAO (5 μM) ($n = 8$), βMCD (10 mM)($n = 9$) or vector ($n = 19$). *$p < 0.05$ relative to control in unpaired Student's *t*-test.

after pre-treatment with PAO or βMCD. As shown in Fig. 2e, even if there is a slight increase in ligand-elicited currents, it does not match the highly augmented $P2X_3$ receptor levels observed in biotinylation experiments. This would suggest that the receptor targeted for internalization is, for large part, no longer active, even if artificially constrained at the PM by treatment with PAO or βMCD.

Internalized $P2X_3$ receptor is targeted to a degradative pathway

Having established a high rate of $P2X_3$ receptor constitutive endocytosis, we next investigated the fate of internalized receptor and the potential mechanisms responsible for maintaining its steady-state distribution. In order to analyze the degradation rate of the internalized protein, we labeled the PM $P2X_3$ protein with biotin at 4°C and then returned the cells to 37°C for various lengths of time, permitting to the biotin-labeled receptor to eventually follow the endocytic pathway until degradation. After 40 min at 37°C, we already observe a significant overall decrease of $P2X_3$ biotinylated protein, supporting a fast degradation rate (Fig. 3a and b) that is moreover consistently delayed (but not prevented) by the inhibitor of phosphatidylinositol-3-kinase wortmannin, and by the inhibitor of microtubule polymerization, nocodazole. These compounds are indeed well known to respectively inhibit the insertion of internalized receptors into lumenal vesicles of multi-vesicular bodies (Petiot *et al.* 2003), and the microtubule-dependent transport of these organelles to late endosomes and lysosomes (Le Blanc *et al.* 2005). As a control, receptor degradation is completely unaffected by treatment with the compound MG132, therefore excluding proteasomal degradation of biotinylated $P2X_3$ receptor (Fig. 3a and b).

Late endosomal/lysosomal targeting is further confirmed by the observation that at steady state the intracellular $P2X_3$ receptor strongly localizes in perinuclear vesicular structures labeled by the late endosome/lysosome marker lamp-1. On the contrary, there is little or no colocalization with the early endosomal marker EEA-1, probably indicating a rapid transit through the early endosomal system (Fig. 4). The high turnover of the receptor was also confirmed by treatment with the protein synthesis inhibitor cycloheximide (CH) (Fig. 3c and d). Indeed, after 60 min in the presence of CH we observe a drastic fall in both PM and total $P2X_3$ receptor content, thus indicating that new protein synthesis is necessary to maintain the steady-state level of $P2X_3$ protein also at the PM. This does not occur, for instance, for the highly related $P2X_1$ receptor subtype (Fig. 3c and d).

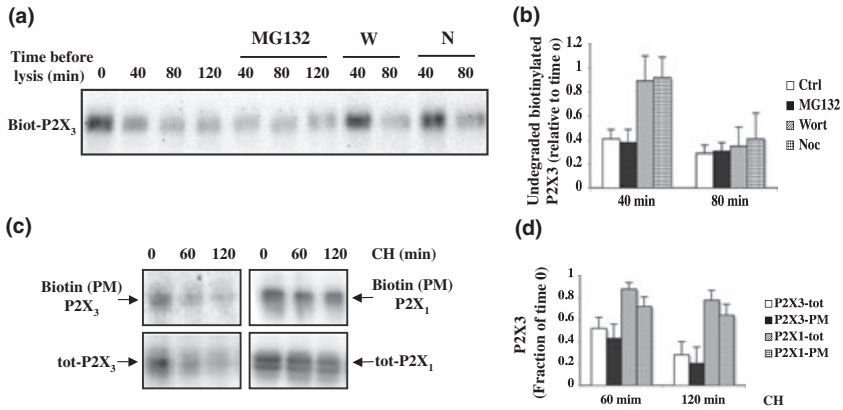

Fig. 3 (a) Internalized $P2X_3$ receptor is targeted to a degradative pathway. After PM receptor labeling with biotin-XX-SE, the cells were returned to 37°C for the indicated times, in the presence or absence of various compounds (MG132, 5 μM; wortmannin (W, 100 nM); nocodazole (N, 10 μM)). After lysis, biotinylated proteins were precipitated with streptavidin beads, and biotinylated $P2X_3$ receptor (biotin-$P2X_3$) was detected by western blot. (b) Quantitative results from panel a, expressed as average levels of biotinylated receptor relative to time 0 with SD from three independent experiments. (c) Cells were treated with cycloheximide (CH) for the indicated times. The PM proteins were then labeled with biotin-XX-SE, then streptavidin-precipitated (PM-$P2X_{1,3}$) and total cell extracts (tot-$P2X_{1,3}$) were probed for $P2X_1$ and $P2X_3$ proteins after western blot. (d) Quantitative results from panel c representing average protein levels relative to control and SD from three independent experiments.

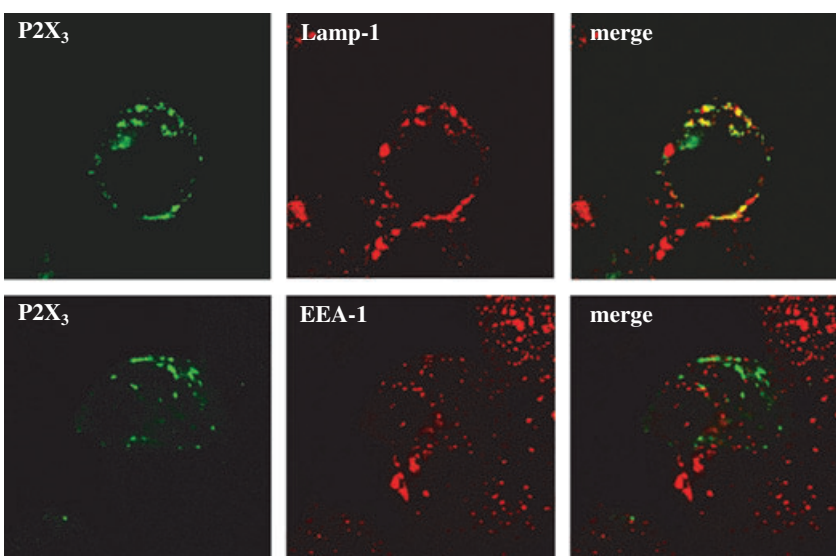

Fig. 4 Localization of $P2X_3$ in late endosomes/lysosomes. Transiently transfected HEK293 cells were fixed and stained with an anti-$P2X_3$ antiserum (Cy2, green) together with either anti-EEA-1 or anti-lamp-1 (Cy3, red) antibodies. Immunofluorescence was detected from two channels, with laser scanning confocal microscope.

$P2X_3$ receptor is ubiquitinated in vivo

Several membrane receptors are internalized and targeted to lysosomes by multi-ubiquitination occurring in their cytoplasmic moiety (Haglund *et al.* 2003). If we analyze the sequence of the $P2X_3$ receptor (Fig. 5a), we notice in the short cytoplasmic C-terminal tail several conserved lysine residues as potential targets for ubiquitination, together with a consensus sequence (DSGψXS) known to be essential for regulated ubiquitination for instance of β-catenin and Ikb-α (Aberle *et al.* 1997). In addition, after immunoprecipitation of the $P2X_3$ receptor from HEK293 cells, we distinguish by SDS-PAGE several anti-ubiquitin-reactive protein bands with molecular masses matching the expected molecular weight of multi-ubiquitinated $P2X_3$ receptor (Fig. 5b). Internalized ubiquitinated receptors are known to be deubiquitinated concurrently with the insertion into lumenal vesicles of multi-vesicular bodies (Agromayor and Martin-Serrano 2006). If we inhibit the insertion of proteins into internal vesicles by the use of wortmannin (Petiot *et al.* 2003), or if we block the proteins in the sorting endosome by incubation of the cells at 19°C for 45 min (Parton *et al.* 1989), we find that the same ubiquitinated protein bands immunoprecipitated by the anti-$P2X_3$ receptor antiserum strongly increase. When the $P2X_3$ immunoprecipitate is blotted with the same

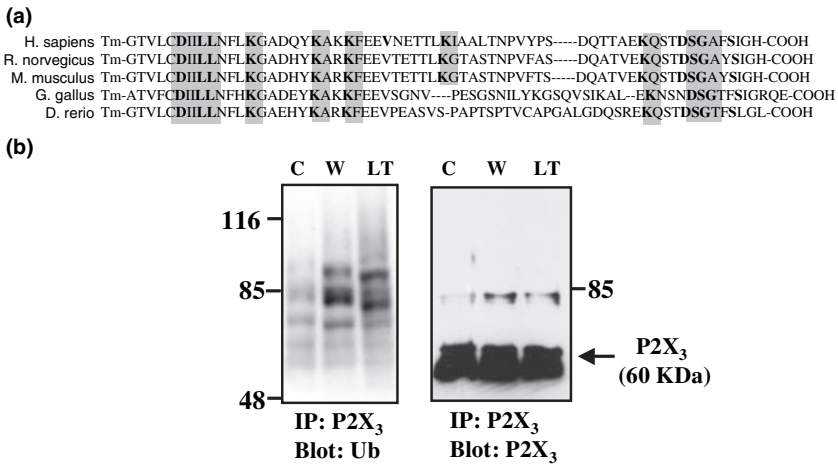

Fig. 5 $P2X_3$ receptor is ubiquitinated in vivo in HEK293 cells. (a) Comparison of C-terminal $P2X_3$ protein sequence from different species. Conserved lysine residues and consensus sequences for ubiquitination (DSGΨXS) and GGA domain binding (DXXLL) are indicated. Protein sequences obtained from National Center Biotechnology Information Protein Database (http://www.ncbi.nlm.nih.gov). Accession numbers: NP_002550 (H. sapiens); CAA62594 (R. norvegicus); NP_663501 (M. musculus); XP_426413 (G. gallus); NP_571698 (D. rerio). B) $P2X_3$ receptor was immuno-precipitated from HEK293 transfected cells, subjected to SDS-PAGE and western blot and probed with anti-ubiquitin antibody or $P2X_3$ antibody as indicated. The bands recognized by the anti-Ub Ab (in the 70–90 KDa range) are consistent with the predicted molecular mass of the unmodified receptor (60–65 KDa) containing respectively one to four units of covalently-linked ubiquitin (7 KDa). One band, around 80 KDa is recognized by the $P2X_3$ Ab. In order to preserve ubiquitination of $P2X_3$ protein, the cells were treated with wortmannin (100 nM) or maintained at 19°C (LT) for 45 min, in order to prevent sorting and concomitant de-ubiquitination of the receptor in multi-vesicular bodies. The bands corresponding to Ig heavy chains were stained with poinceau red and cut off from the filter before $P2X_3$ hybridization, in order to avoid cross-reaction with secondary antibodies.

$P2X_3$ antiserum, we recognize one additional band of 80 KDa, that also increases after 19°C and wortmannin treatments. We fail to observe the same three bands pattern observed with the anti-Ub antibody. It is possible the additional Ub reactive bands belong to a protein different $P2X_3$. An alternative explanation could be a weak affinity of the P2X3 antibody for an ubiquitinated epitope, giving that the P2X3 antibody is raised against a synthetic peptide in the same C-terminal region of the receptor.

Effect of ligand on $P2X_3$ receptor trafficking

It is well known that several PM receptors are rapidly internalized following ligand binding. Therefore, we directly tested if the internalization rate of the $P2X_3$ receptor could be modulated by the selective agonist αβmeATP. Even though $P2X_3$ receptor *per se* undergoes rapid constitutive endocytosis (Figs 1 and 2), the treatment with the ligand clearly accelerates the extent of internalization (Fig. 6a and b). Quite surprisingly, when we analyze the level of $P2X_3$ receptor at the PM after ligand binding, we also observe a transient, but consistent increase that is dependent on receptor activation, being completely prevented by the $P2X_3$ receptor antagonist NF023 (Soto *et al.* 1999) (Fig. 6c and d). Interestingly, we also observe a similar transient increase in the $P2X_3$ receptor at the PM after treatment with the calcium ionophore ionomycin (Fig. 6c and d), thus suggesting that receptor mobilization could be mediated by receptor-evoked calcium entry, which has been already observed to be $P2X_3$-dependent in transfected HEK293 cells (Fischer *et al.* 2003). Noteworthy, the externalization of the receptor by ligand binding (Fig 6b) is accompanied by a consistent increase in the inward current response to a second sequential ligand stimulus, indicating that the ligand-increased receptor expressed at the PM is indeed functional (Fig. 6e). No inward current was elicited by αβmeATP in mock-transfected cells, proving that the observed signals do not depend on other purinergic receptors endogenously expressed in HEK293 cells (not shown).

Trafficking of $P2X_3$ receptor in dorsal root ganglion neurons

The $P2X_3$ receptor is endogenously expressed *in vivo* in sensory DRG neurons. We thus aimed at confirming and extending to primary cultures the data obtained with transfected cells. Interestingly, the receptor encounters a high rate of internalization also in DRG neurons and the internalization is also partially inhibited by PAO and βMCD (Fig. 7a), thus proving that in transfected cells this intrinsic property of the $P2X_3$ receptor is not due to protein over-expression. Furthermore, the internalized receptor is apparently sorted to a degradative pathway in a wortmannin- and nocodazole-sensitive manner, as in HEK293 cells (Fig. 7b). Finally, the receptor seems to have a relatively high turnover rate. Indeed it almost completely disappears from

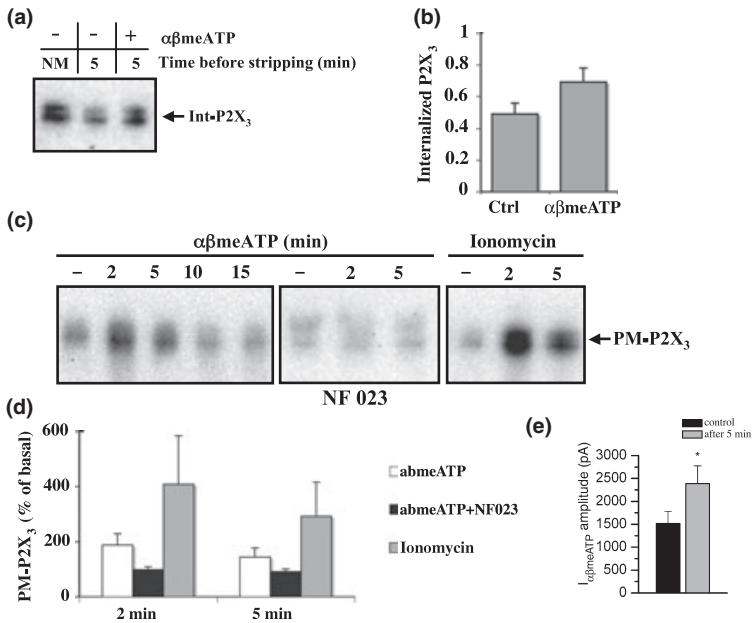

Fig. 6 Effect of ligand on $P2X_3$ receptor trafficking. (a) After PM receptor labeling with NHS-SS-biotin, HEK293 cells were returned to 37°C for the indicated times, in the presence or absence of αβmeATP (50 μM), and extracellularly exposed biotin was stripped with MesNa. After lysis, internalized proteins (protected from stripping) were precipitated with streptavidin beads and probed for $P2X_3$ protein after western blot (Internalized-$P2X_3$). NM= no MesNa treatment. (b) Quantification of results from panel c representing averages and SD from four independent experiments. (c) Cells were treated for the indicated times with αβmeATP (50 μM) or ionomycin (5 μM). Where indicated, cells were pre-treated (5 min) with the receptor antagonist NF023 (25 μM). The PM proteins were then labeled with biotin-XX-SE, precipitated with agarose–streptavidin beads and probed for $P2X_3$ protein after western blot (PM-$P2X_3$). (d) Quantification of results from panel c representing averages and SD from three independent experiments. (e) Inward currents recorded from $P2X_3$ receptor-transfected HEK293 cells by two subsequent stimuli with 100 μM αβmeATP, separated by a 5 min interval. Data represent recordings from 15 different cells obtained from three independent transfections. *$p < 0.05$ in unpaired Student's *t*-test.

the PM after 120 min of CH treatment and also the total protein level is strongly reduced (Fig. 7c). Nevertheless, we fail to observe in DRG neurons an increase of receptor at the PM after ligand binding (Fig. 7d). A different balance between internalization and secretion in response to ligand activation might thus account for the apparent discrepancy between neurons and HEK293 cells. In this context, it is also interesting to note that the percentage of receptor at the PM is consistently higher in DRG neurons, reaching values around 25–30% (not shown).

Discussion

Trafficking to and from PM often regulates the signaling strength of receptors. While in canonical models the internalization is regulated by selected stimuli, typically agonist binding, recent experimental evidence indicates that several receptors can also undergo constitutive internalization and recycling. These processes have been observed for instance for ion-channel such as α-amino-3-hydroxy-5-methylisoxazole-4-propionate (Park *et al.* 2004) and purinergic $P2X_4$ (Bobanovic *et al.* 2002; Royle *et al.* 2005), as well as for G-protein coupled receptors such as the CB_1 cannabinoid (Leterrier *et al.* 2004), $GABA_B$ (Grampp *et al.* 2007) and the mGluR7 glutamate receptors (Pelkey *et al.* 2005).

With our work, we now show that also the purinergic ionotropic $P2X_3$ receptor subtype, from both transfected HEK293 cells and primary DRG neurons, undergoes rapid constitutive endocytosis, whose inhibition leads to the accumulation of the receptor at the PM. Moreover, at steady-state we find that the rate of $P2X_3$ receptor endocytosis likely overcomes the secretion to the PM, thus limiting the overall number of receptors at the cell surface as also confirmed by the major localization of the $P2X_3$ protein in late endosomes/lysosomes. The internalization is improbably due to the highly unstable extracellular ATP potentially secreted from HEK293 cells, since apyrase treatment has no effect on the level of $P2X_3$ protein at the PM. Since $P2X_3$ receptor endocytosis is strongly inhibited by cholesterol extraction by βMCD, and $P2X_3$ is a lipid raft-resident protein (Vacca *et al.* 2004), we can hypothesize that the receptor might be internalized via a rafts/caveolae-dependent mechanism. It is known that acute cholesterol depletion from PM can also inhibit clathrin-dependent endocytosis (Subtil *et al.* 1999), but the involvement of clathrin-independent pathways

is reinforced by experiments in hypertonic medium, which show no change in PM levels of $P2X_3$ receptor.

After endocytosis, membrane receptors can be either recycled to the PM or targeted to the lysosomes for degradation. When considering the trafficking and localization properties of the $P2X_3$ protein, we conclude that this receptor follows predominantly the lysosome degradation route: first, the turnover of the biotinylated receptor is relatively rapid (40–80 min). At the same time, new protein synthesis is necessary to maintain the steady-state level of $P2X_3$ protein even at the PM. Second, reduction of endo-lysosomal trafficking with wortmannin and nocodazole significantly delays biotinylated receptor degradation. Finally, at steady state the main fraction of the $P2X_3$ receptor localizes in lamp1-positive intracellular organelles with a minor, barely detectable, fraction at the PM.

Several membrane receptors are internalized and targeted to lysosomes by multi-ubiquitination occurring in their cytoplasmic mojety. Consistently, in the short cytoplasmic C-terminal tail of the $P2X_3$ receptor several conserved lysine residues are present as potential targets for ubiquitination. In addition, there is a consensus sequence (DSGψXS) known to be essential for regulated ubiquitination for instance of β-catenin and Ikb-α proteins (Aberle *et al.* 1997). Finally, in the $P2X_3$ receptor immunoprecipitated from HEK293 cells we notice several ubiquitin-containing protein bands, corresponding to the predicted molecular masses of poly-ubiquitinated $P2X_3$ receptor. One of these bands is also recognized by the $P2X_3$ antiserum and increased in conditions supposed to preserve ubiquitination in the endocytic pathway, thus reinforcing the hypothesis that $P2X_3$ receptor could be indeed ubiquitinated *in vivo*. Although further proofs will be required to establish the direct involvement of ubiquitination in the trafficking of the receptor, this feature could provide a possible molecular mean for rapid receptor endocytosis, and a possible mechanism for regulation.

Even if the high rate of receptor endocytosis that we reported here fits well with the overall late endosomal/lysosomal sub-cellular localization of the $P2X_3$ receptor in HEK293 cells, it is impossible to exclude that at least a fraction of the receptor could reach the late endosomes bypassing the PM, given the emerging number of routes that link secretory and endocytic pathways (Hinners and Tooze 2003). Interestingly, in the C-teminal tail of the $P2X_3$ receptor there is also a conserved di-leucine sorting motif (DXXLL), which is a consensus-binding site for proteins

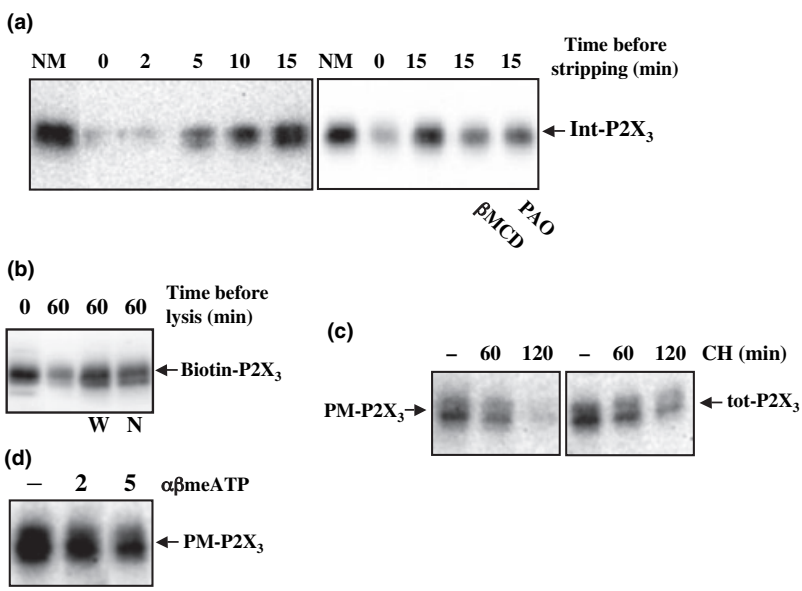

Fig. 7 $P2X_3$ receptor trafficking in DRG sensory neurons. (a) After PM receptor labeling with NHS-SS-biotin, DRG sensory neurons were returned to 37°C for the indicated times and extracellularly exposed biotin was stripped with MesNa. After lysis, internalized proteins (protected from stripping) were precipitated with streptavidin beads and probed for $P2X_3$ after western blot (Internalized-$P2X_3$). NM = no MesNa treatment. When indicated, PAO (5 μM) or βMCD (10 mM) were added during the incubation at 37°C. (b) After PM receptor labeling with biotin-XX-SE, cells were returned to 37°C for 60 min, in the presence or absence of 100 nm wortmannin (W), or 10 μM nocodazole (N). After lysis, biotinylated proteins were precipitated with streptavidin beads and non-degraded $P2X_3$ protein (biotin-$P2X_3$) was detected by western blot and immunoreaction. (c) Cells were treated with cycloheximide (CH) for the indicated times. The PM proteins were then labeled with biotin-XX-SE, then streptavidin-precipitated (PM-$P2X_3$) and total cell extracts (tot-$P2X_3$) were probed for $P2X_3$ proteins after western blot. (d) DRG neurons were treated for the indicated times with αβmeATP. The PM proteins were then labeled with biotin-XX-SE, precipitated with agarose–streptavidin beads and probed for $P2X_3$ after western blot (PM-$P2X_3$).

trafficking between *trans*-Golgi network and endosomes (Bonifacino and Traub 2003).

A key issue in receptor dynamics is the cellular challenge with specific agonists, which generally increases the internalization rate of several signaling receptors (Ennion and Evans 2001; Tsao *et al.* 2001). Quite surprisingly, after agonist stimulation of HEK293 cells we observe a transient increase in the $P2X_3$ receptor at the PM, as confirmed by both biotinylation experiments and electrophysiological recordings. Since also endocytosis is concurrently increased after αβmeATP challenge, the most obvious explanation is that agonist binding induces further secretion of the $P2X_3$ receptor from an intracellular pool to the PM. Since only a minor fraction of $P2X_3$ receptor is present at the PM at steady state, the secretion of only a limited amount of the intracellular pool should be sufficient to considerably increase the relative level of the receptor at the PM, as we observe. Moreover, since a similar transient increase of $P2X_3$ protein at the PM also occurs after treatment with the calcium ionophore ionomycin, a positive feedback loop between ligand-evoked Ca^{2+} influx and recruitment to the cell surface might even exist. This hypothesis is moreover consistent with results reported in DRG by Xu and Huang (Xu and Huang 2004), showing that electrical stimulation induces trafficking of $P2X_3$ receptors to the PM, in a Ca^{2+}/calmodulin protein kinase II-dependent manner. Given that in several cell types the calcium entry can stimulate lysosome secretion as a general membrane repair mechanism (Jaiswal *et al.* 2002) or specialized secretory route (Stinchcombe *et al.* 2004; Zhang *et al.* 2007; Li *et al.* 2008), and given the high steady-state level of the $P2X_3$ receptor in the endo-lysosomal system, we do not exclude that ligand- and ionomycin-mediated receptor externalization could be dependent on lysosomal secretion, as similarly proposed by Qureshi and coworkers for the purinergic $P2X_4$ receptor (Qureshi *et al.* 2007).

In conclusion, the present study highlights the trafficking properties of the $P2X_3$ receptor subtype showing its rapid constitutive endocytosis and targeting to the late endosomal/lysosomal system, which results into overall localization of the receptor in these organelles at steady-state. Although the implications for receptor functional regulation in sensory neurons *in vivo* are still premature, the present findings, describing a highly dynamic constitutive trafficking of the $P2X_3$ receptor, can certainly disclose a possible mechanism for the rapid modulation of ATP-mediated responses potentially relevant during physiological as well as pathological conditions.

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