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A new role for $P2X_4$ receptors as modulators of lung surfactant secretion

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In recent years, P2X receptors have attracted increasing attention as regulators of exocytosis and cellular secretion. In various cell types, P2X receptors have been found to stimulate vesicle exocytosis directly via Ca^{2+} influx and elevation of the intracellular Ca^{2+} concentration. Recently, a new role for $P2X_4$ receptors as regulators of secretion emerged. Exocytosis of lamellar bodies (LBs), large storage organelles for lung surfactant, results in a local, fusion-activated Ca^{2+} entry (FACE) in alveolar type II epithelial cells. FACE is mediated via $P2X_4$ receptors that are located on the limiting membrane of LBs and inserted into the plasma membrane upon exocytosis of LBs. The localized Ca^{2+} influx at the site of vesicle fusion promotes fusion pore expansion and facilitates surfactant release. In addition, this inward-rectifying cation current across $P2X_4$ receptors mediates fluid resorption from lung alveoli. It is hypothesized that the concomitant reduction in the alveolar lining fluid facilitates insertion of surfactant into the air–liquid interphase thereby "activating" it. These findings constitute a novel role for $P2X_4$ receptors in regulating vesicle content secretion as modulators of the secretory output during the exocytic post-fusion phase.

Keywords: $P2X_4$ receptor, lamellar body, alveolar epithelial cell, exocytosis, calcium, cellular secretion, pulmonary surfactant

INTRODUCTION

In recent years, P2X receptors have attracted increasing attention as regulators of exocytosis and cellular secretion in a wide variety of organs including the lungs (Burnstock et al., 2012). P2X receptors are membrane cation channels that are activated by extracellular adenosine triphosphate (ATP), the molecular and functional properties of which have been reviewed in detail elsewhere (Surprenant, 1996; North, 2002; Khakh and North, 2006; Burnstock and Kennedy, 2011; Kaczmarek-Hajek et al., 2012). ATP has been known to stimulate cellular secretion for several decades (Rodriguez Candela and Garcia-Fernandez, 1963; Diamant and Kruger, 1967). One of the earliest indications for involvement of P2X receptors in stimulating secretion came from the studies of Cockcroft and Gomperts (1979a,b, 1980). They found that ATP triggers degranulation and histamine release in mast cells via activation of P_{2Z} (Cockcroft and Gomperts, 1980), which later turned out to be $P2X_7$ (Surprenant et al., 1996). Since the first cloning of P2X receptor subunits in 1994 (Brake et al., 1994; Valera et al., 1994), P2X receptors have been found to stimulate and modulate various cellular secretion pathways, including fluid secretion in exocrine glands and epithelia (Novak, 2011), secretion of cytokines via release of plasma-derived microvesicles (Solini et al., 1999; MacKenzie et al., 2001) or exosomes (Qu et al., 2007; Qu and Dubyak, 2009).

Moreover, several members of the P2X family have been implicated in regulating exocytosis of secretory organelles in a variety of cell types (Gu and MacDermott, 1997; Ulmann et al., 2008; Jacques-Silva et al., 2010; Gutierrez-Martin et al., 2011; Huang et al., 2011). Substantial evidence suggests that P2X receptor activation stimulates exocytosis directly via influx of Ca^{2+} from the extracellular space and elevation of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$; Kim et al., 2004; Shigetomi and Kato, 2004; Jacques-Silva et al., 2010; Hayoz et al., 2012). It is well established that a series of Ca^{2+}-dependent steps during the exocytic pre-fusion stage is essential for fusion of exocytic vesicles with the plasma membrane (Burgoyne and Morgan, 1998; Sudhof, 2004; Neher and Sakaba, 2008). Ca^{2+} can either enter through P2X receptor pores themselves or through voltage-gated Ca^{2+} channels, which are activated as a consequence of the P2X receptor-mediated membrane depolarization (Khakh and North, 2006). In line with these findings, several studies proposed a role for $P2X_4$ receptors in exocytosis that is mediated via an increase in the intracellular Ca^{2+} concentration. $P2X_4$ receptors have a relatively slow desensitization (5–10 s) and a high Ca^{2+} permeability, Ca^{2+} contributes 8% of the whole current in human $P2X_4$ (Wang et al., 1996; Garcia-Guzman et al., 1997; North, 2002; Egan and Khakh, 2004). Hence, activation of $P2X_4$ receptors can generate sufficient increases in $[Ca^{2+}]_c$ to stimulate regulated exocytosis. Indeed, insulin secretion from pancreatic islets (Ohtani et al., 2011) and exocytic response in parotid acinar cells (Bhattacharya et al., 2012) following stimulation with ATP were augmented in the presence of ivermectin, a selective potentiator of $P2X_4$ receptor currents (Khakh et al., 1999). $P2X_4$ activation was also found to modulate glutamate and gamma-aminobutyric acid (GABA) release in hypothalamic neurons (Vavra et al., 2011) and brain-derived neurotrophic factor (BDNF) in microglial cells (Trang et al., 2009).

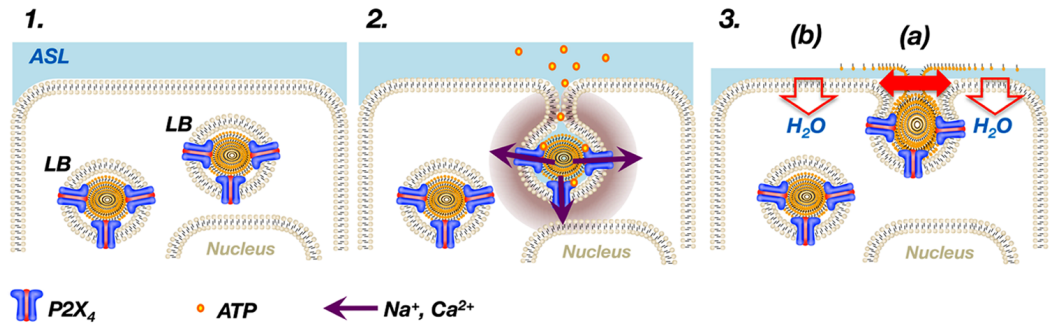

FIGURE 1 | $P2X_4$ receptors on LBs modulate surfactant secretion. $P2X_4$ receptors are expressed on LBs, large storage organelles for pulmonary surfactant in ATII cells **(1)**. Upon exocytosis of LBs, $P2X_4$ receptors readily become part of the apical membrane and activation of $P2X_4$ by extracellular ATP results in a transient, non-selective, inward-rectifying, cation current at the site of the fused vesicle causing a local increase in Ca^{2+} around the fused vesicle **(2)**. The local increase in Ca^{2+} promotes fusion pore expansion **(3a)**. In addition, the inward-rectifying cation current on the apical side results in vectorial ion transport across ATII cells, which in turn promotes fluid resorption and thereby facilitates adsorption of newly released surfactant into the air–liquid interface **(3b)**. ASL = alveolar surface liquid.

In all of these systems, activation of P2X receptors adjusts the secretory output predominantly by modulating the number of vesicles that fuse with the plasma membrane. Depending on the cell type and the shape of the Ca^{2+} signal, the rise in $[Ca^{2+}]_c$ triggers fusion of secretory vesicles with the plasma membrane, but also affects maturation and trafficking of secretory vesicles to the plasma membrane (Neher and Sakaba, 2008; Dolensek et al., 2011; Gutierrez-Martin et al., 2011).

VESICULAR $P2X_4$ RECEPTORS PROMOTE SURFACTANT SECRETION VIA FACE – "FUSION-ACTIVATED Ca^{2+}-ENTRY"

Apart from regulating secretion via adjusting the number of fusing organelles the amount and composition of the secretory output is – at least for exocytosis of large secretory granules and secretion of bulky vesicle contents – modulated following fusion of the vesicle with the plasma membrane during the so-called exocytic "post-fusion" phase. Recent evidence also suggests a role for $P2X_4$ receptors therein. It has been demonstrated that activation of $P2X_4$ receptors following vesicle–plasma membrane fusion modulates the secretion and activation of pulmonary surfactant (Miklavc et al., 2011; Dietl et al., 2012; Thompson et al., 2013).

Pulmonary surfactant is secreted via exocytosis of lamellar bodies (LBs), large lysosome-related storage organelles in alveolar type II (ATII) epithelial cells. Surfactant is stored in LBs as densely packed membranous structures that do not readily diffuse out of fused LBs following opening of the exocytic fusion pore. Rather, surfactant is so insoluble, that it may remain entrapped within the fused vesicle for many minutes and the slowly expanding fusion pore acts as a mechanical barrier for the release (Dietl et al., 2001; Haller et al., 2001; Singer et al., 2003; Dietl and Haller, 2005; Miklavc et al., 2012).

Miklavc et al. (2010) initially discovered that exocytosis of LBs results in localized Ca^{2+} influx at the site of vesicle fusion which they termed "FACE" for "fusion-activated Ca^{2+}-entry". Subsequently, they found that FACE is mediated via activation of $P2X_4$ receptors expressed on the limiting membranes of LBs (Miklavc et al., 2011). Upon exocytosis of LBs, the $P2X_4$ receptor is readily part of the apical membrane as soon as membrane fusion is completed (Miklavc et al., 2009). Activation of $P2X_4$ in the presence of extracellular ATP then results in a transient, non-selective, inward-rectifying, cation current at the site of the fused vesicle (Miklavc et al., 2011; Thompson et al., 2013) (**Figure 1**). The relatively high Ca^{2+} permeability of $P2X_4$ receptors (North, 2002) causes a local, transient rise of $[Ca^{2+}]_c$ around the fused vesicle which promotes fusion pore expansion (Miklavc et al., 2011). In ATII cells, vesicle content (i.e., surfactant) release is tightly regulated via Ca^{2+}-dependent fusion pore expansion (Haller et al., 2001) and it has been demonstrated that FACE via $P2X_4$ receptors on LBs directly facilitates surfactant release in the alveolus (Miklavc et al., 2011).

Ca^{2+} channels localized in the membranes of the secretory vesicles that respond to changes in the membrane potential or extracellular agonists upon fusion are ideally suited for generating a localized rise in Ca^{2+} and selectively affect the individual fused vesicle. Yet, so far such mechanisms have only been known in invertebrates (Smith et al., 2000; Yao et al., 2009; Miklavc and Frick, 2011) and $P2X_4$ receptors on LBs resemble the first analog mechanism in mammals. It will be interesting to see whether a similar role for P2X receptors is present in other secretory cells. Similar to LBs in ATII cells, many different cell types harbor secretory lysosomes or lysosome-related organelles to store for secretory products that are released via exocytosis of these organelles (Dell'Angelica et al., 2000; Blott and Griffiths, 2002; Luzio et al., 2007). Many of these contain rather bulky, macromolecular vesicle contents and release is modulated via the exocytic post-fusion phase (Thorn, 2009). In addition, it is well established that P2X receptors, in particular $P2X_4$, are predominantly located within lysosomal compartments and inserted into the cell surface upon exocytosis (Qureshi et al., 2007; Toyomitsu et al., 2012).

VESICULAR $P2X_4$ RECEPTORS FACILITATE "ACTIVATION" OF SURFACTANT

Following release into the alveolar hypophase surfactant maintains its compact organization, constituting lamellar body-like particles (LBPs; Haller et al., 2004). To gain its vital function of reducing the surface tension within alveoli, it needs to be inserted

into the air–liquid interface. Freshly released LBPs disintegrate when they contact an air–liquid interface, leading to instantaneous spreading and insertion of surfactant material at this interface (Dietl and Haller, 2005). Thompson et al. (2013) demonstrated that, in addition to facilitating fusion pore dilation, FACE via $P2X_4$ also drives fluid resorption from the alveolar lumen. The $P2X_4$ mediated inward-rectifying cation current on the apical side results in vectorial ion transport across ATII cells, which in turn promotes apical to basolateral fluid transport (Thompson et al., 2013) (**Figure 1**). FACE-dependent transepithelial fluid resorption is a rather transient process which requires the presence of luminal ATP or other $P2X_4$ agonists and hence it is unlikely that it is a major contributor to regulation of alveolar liquid homeostasis under physiological conditions (Folkesson and Matthay, 2006). However, it has been suggested that this localized alveolar fluid resorption results in temporary thinning of the alveolar hypophase which in turn promotes contact between LBPs and the interphase and facilitates adsorption of newly released surfactant into the air–liquid interface (Thompson et al., 2013). Hence, activation of $P2X_4$ and FACE (which in order to embrace the true nature of FACE should now be referred to as "fusion-activated cation entry") facilitates surfactant release via fusion pore opening and contributes to "activation" or "functionalising" of surfactant. Such a temporal and local coordination of surfactant secretion and reduction of alveolar lining fluid could constitute a powerful mechanism for fine-tuning surfactant replenishment – the integrators being vesicular $P2X_4$ receptors and extracellular ATP.

ATP AS INTEGRATOR FOR SURFACTANT SECRETION AND "ACTIVATION"

It is intriguing that extracellular ATP plays multiple functions for surfactant secretion in the alveolus. Apart from $P2X_4$ receptors, ATII cells also express $P2Y_2$ receptors (Garcia-Verdugo et al., 2008; Burnstock et al., 2012) and activation thereof is one of the most potent stimuli for LB exocytosis and surfactant secretion (Rice and Singleton, 1987; Frick et al., 2001; Andreeva et al., 2007; Dietl et al., 2010). Hence, ATP is integrating the entire secretion process from stimulating LB exocytosis to facilitating surfactant release and "activating" surfactant during the post-fusion phase.

Despite this importance of ATP for lung function, the origins of ATP in the alveoli are still elusive. It has been reported that ATP is present in the pulmonary hypophase (Patel et al., 2005), however, the estimated concentration under resting conditions is in the low nM range (Bove et al., 2010), well below the EC_{50} values for $P2X_4$ activation (North, 2002) or $P2Y_2$ activation (Lazarowski et al., 1995; Brunschweiger and Muller, 2006).

Cell stretch during deep inflation is considered the most potent if not only physiologically relevant stimulus for surfactant secretion (Wirtz and Dobbs, 2000; Dietl et al., 2004, 2010; Frick et al., 2004) and stretch-induced ATP release from alveolar epithelial cells (Patel et al., 2005; Mishra et al., 2011) could represent a key regulatory element (Dietl et al., 2010). Several possible pathways for ATP release have been described in the respiratory epithelia. ATP can either be released into the hypophase via regulated exocytosis from secretory cells (Kreda et al., 2010; Okada et al., 2011), or in a conductive way via pannexin hemichannels (Ransford et al., 2009; Seminario-Vidal et al., 2011) or $P2X_7$ receptors (Mishra et al., 2011). In particular, local ATP release within individual alveoli may provide an ideal mechanism to gradually adapt local surfactant secretion to local demands. The alveolar epithelium consists of only two cell types; besides surfactant secreting ATII cells, flat alveolar type I (ATI) cells cover most of the alveolar surface. In contrast to primary ATII cells that only express $P2X_4$ receptors (Miklavc et al., 2011) ATI cells express $P2X_4$ and $P2X_7$ receptors (Weinhold et al., 2010; Burnstock et al., 2012). $P2X_7$ knock-out mice fail to increase surfactant secretion in response to hyperventilation and substantial evidence suggests that ATP release via $P2X_7$ receptors on ATI cells maintains alveolar surfactant homeostasis in response to increased alveolar distension by stimulating $P2Y_2$ receptors on ATII cells (Mishra et al., 2011) and, in light of our recent findings, possible activation of $P2X_4$ (Miklavc et al., 2011; Thompson et al., 2013). In addition to responding to mechanical distension of alveoli, alveolar epithelial cells also respond to increased tension forces at the air–liquid interphase with exocytic release of ATP (e.g., upon local depletion of surfactant or when coming in close proximity to the air–liquid interphase following a decrease in alveolar hypophase height; Ramsingh et al., 2011).

Whether ATII cells also release ATP, to act in an autocrine feedback loop, is still unknown. Many secretory vesicles, including lysosome-related organelles, have been found to contain significant amounts of ATP (Bodin and Burnstock, 2001; Praetorius and Leipziger, 2009; Lazarowski et al., 2011) and it has been reported that ATP is released from ATII-like A549 cells, likely via exocytosis (Tatur et al., 2008; Ramsingh et al., 2011). It is tempting to speculate that LBs contain ATP and hence provide the ligand for the $P2X_4$ receptors themselves. In such a scenario, the high degree of pH sensitivity of this receptor (Clarke et al., 2000; Zsembery et al., 2003; Coddou et al., 2011) could prevent intravesicular activation of the receptor in the presence of vesicular ATP (pH of LB is <6.1; Chander et al., 1986).

Also, under pathophysiological conditions resulting from many chronic lung diseases, release of purine nucleotides from respiratory epithelia is significantly increased (Adriaensen and Timmermans, 2004; Lommatzsch et al., 2010). It has been demonstrated that trauma-induced damage of the alveolus leads to substantial ATP release and that extracellular ATP is a key player to rescue alveolar function following damage, including regulation of surfactant secretion (Riteau et al., 2010; Belete et al., 2011). In addition, several studies have demonstrated up-regulation of P2X receptors in various cell types during pathological conditions including inflammation, tumor growth, and injury (Burnstock and Kennedy, 2011) and it has been hypothesized that chronic extracellular ATP may be responsible (Geisler et al., 2013). Such a mechanism could be particularly relevant in the lung, and P2X receptors may play an even greater role in many pathological conditions with chronically increased extracellular ATP levels. Initial evidence came from studies indicating that smoke-induced lung inflammation leads to increased levels of ATP in broncho-alveolar fluid and up-regulation of $P2X_7$ expression (Lommatzsch et al., 2010; Lucattelli et al., 2011). A similar role for $P2X_4$ receptors under pathophysiological conditions is still to be confirmed. However,

it is becoming increasingly evident that purinergic signaling is taking center stage in regulating secretion of pulmonary surfactant and adapting it to local demands under physiological and diseased conditions. $P2X_4$ receptors on LBs provide an ideal mechanism for fine-tuning surfactant secretion via ATP levels in the alveolar hypophase.

Despite recent advances in our understanding how purinergic signaling in the alveolus, in particular activation of vesicular $P2X_4$ receptors, modulates LB exocytosis, surfactant secretion and activation of released surfactant, several important questions still remain to be answered: What is the physiological relevance of such a complex regulatory mechanism? What are the sources of ATP under physiological and more importantly pathophysiological conditions? And – extending the scope from the lung – is purinergic signaling a general mechanism for secretion of large, macromolecular vesicle contents or is it unique to LB exocytosis and surfactant secretion? The answers to these questions warrant further research and certainly promise an increased understanding of the role of P2X receptors in regulating exocytosis and cellular secretion.

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