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Agonist-stimulated internalisation of the ligand-gated ion channel $P2X_1$ in rat vas deferens

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Abstract Using cell surface biotinylation and Western blotting, we investigated the extent to which native $P2X_1$ receptors in rat vas deferens are internalised after exposure to agonist. Exposure to 100 μM α,β-meATP 30 min prior and during a 10 min biotinylation period resulted in a ∼50% reduction in the amount of biotinylated $P2X_1$ receptor indicating that activation of the receptor by agonist induces receptor internalisation. Furthermore, biotinylation under saturating conditions suggests that once internalised, a rapid recycling of $P2X_1$ receptor back to the cell surface occurs. The physiological implications of these mechanisms in terms of receptor function are discussed.

Key words: P2X; Receptor internalisation; ATP; Vas deferens

1. Introduction

P2X receptors are membrane cation channels gated by the binding of extracellular ATP. Seven distinct mammalian isoforms of the receptor ($P2X_{1-7}$) have been cloned each with different biophysical and pharmacological properties [1]. Differences in native P2X receptor responses result not only from the differential expression of different P2X isoforms but also from the formation of heteromultimers between different isoforms [2] and possibly through the existence of alternatively spliced isoforms [3].

The characteristic features of P2X-mediated responses in smooth muscle cells are a high sensitivity to α,β methylene ATP (α,β-meATP, $EC_{50} \sim 1$ μM) and a rapid desensitisation of the response during the continued presence of agonist. This corresponds closely to the phenotype of recombinant homomeric $P2X_1$ receptors [4,5] and studies on $P2X_1$ knockout mice have shown that the $P2X_1$ receptor is essential for P2X-mediated responses in vas deferens [6]. The underlying mechanism of desensitisation of the $P2X_1$ receptor is unclear and could possibly involve receptor internalisation. Agonist-stimulated internalisation is a common mechanism for G protein-coupled receptors [7]. However, the extent to which internalisation plays a role in the regulation of ligand-gated ion channels is unclear. Examples of ligand-gated ion channels that undergo receptor internalisation include the $GABA_A$ and AMPA receptors [8,9]. Recent studies using over-expressed $P2X_1$ receptor–green fluorescent protein (GFP) chimeras have shown that 80–90% of the fluorescence can be internalised following agonist stimulation [10,11]. Therefore, by inference a component of receptor recovery from desensitisation is dependent on the recycling of the receptors back to the surface. However, whether $P2X_1$ receptor internalisation occurs in native tissue with physiological levels of receptor and if this plays a role in the tachyphalaxis of $P2X_1$ receptor responses remains to be determined. In order to address these questions we have used cell surface biotinylation to measure the membrane localisation of $P2X_1$ receptors [4] to estimate the extent of agonist-induced receptor internalisation in the vas deferens and to compare this to the recovery of P2X receptor-mediated contractile responses following agonist stimulation.

2. Materials and methods

2.1. Surface protein biotinylation

Male Wistar rats (250–300 g) were killed by cervical dislocation. Vasa deferentia were cut into four, washed in phosphate-buffered saline (PBS) and placed in 940 μl of PBS in 24 well culture dishes (two vas deferens segments per well). Biotinylating agent (EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce, USA)) was added in a 50 μl volume to give the appropriate final concentration (see figure legends). After biotinylation tissues were washed extensively in PBS and placed in 550 μl of homogenisation buffer [4] on ice. For agonist studies α,β-meATP (Sigma, UK) was applied 30 min before addition of biotinylating agent in a 10 μl volume to give a final concentration of 100 μM. Incubations and washes were conducted at room temperature. Tissues were homogenised with a Polytron homogeniser and centrifuged at $16\,000 \times g$ (4°C) for 2 min. A 5 μl aliquot of the supernatant was assayed for total protein content using Bradford reagent and the remaining supernatant diluted to 5 mg/ml in buffer H. For quantification of total $P2X_1$ receptor (biotinylated and non-biotinylated), a 30 μl aliquot was mixed with an equal volume of gel loading buffer. For isolation of biotinylated proteins, 30 μl of streptavidin agarose beads (Sigma) were added to 400 μl of supernatant and mixed on a rolling shaker at 4°C for 3 h. Beads were washed twice in homogenisation buffer, three times in PBS and 30 μl of gel sample buffer added.

2.2. Gel electrophoresis and quantification

Protein samples (20 μg/well) were heated to 80°C for 5 min before separation on a 10% SDS–PAGE gel. The gel was transferred to Hybond-P membrane (Amersham, UK) and processed for antibody staining (anti-$P2X_1$ (Alamone, Israel) 1:1000 or extracellular signal-regulated kinase (ERK) (Santa Cruz, CA, USA) 1:500 dilution) as described elsewhere [4]. Protein bands were visualised using an ECL (Plus) kit and with Hyperfilm MP (Amersham). Images were captured using a Bio-Rad GS-670 densitometer and protein bands quantified using the NIH Image computer.

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2.3. Organ bath analysis of contractile responses in rat vas deferens

Vas deferens contractions were measured as described previously [6]. Agonist (α,β-meATP, 10 μM final concentration) was added to the organ bath at 30 or 10 min intervals and removed by three washes.

3. Results

Sulfo-NHS-LC-Biotin can be used to label primary amines of surface proteins [12] and has been used to characterise surface expression of recombinant P2X receptors [4,13]. In this study, we have used this technique in combination with streptavidin agarose bead precipitation and Western blot analysis with an anti-$P2X_1$ antibody to study surface $P2X_1$ receptor levels in the rat vas deferens. A single protein band of ~60 kDa corresponding to the glycosylated form of the $P2X_1$ receptor [14,15] was detected by the $P2X_1$ antibody (Figs. 1, 3 and 4). Biotinylation of membrane-localised $P2X_1$ receptors was time and concentration dependent and saturated at 500 μg/ml Sulfo-NHS-LC-Biotin following 10 min biotinylation (Figs. 1 and 3). In order to rule out the possibility that cellular damage from dissection could allow biotinylation of intracellular proteins, we assayed the exclusively cytoplasmic protein ERK. Two ERK isoforms were detected from total protein samples, however, biotinylated forms were not detected (Fig. 2). This confirms that the technique is selective for extracellular proteins and we can therefore use the level of biotinylation as a measure of surface localisation to determine whether activation of the receptor results in subsequent internalisation of native $P2X_1$ receptors.

In order to investigate the extent of receptor internalisation we quantified the levels of biotinylated $P2X_1$ receptors under control conditions and following agonist stimulation (30 min prestimulation and during biotinylation). In initial studies at a saturating concentration of Sulfo-NHS-LC-Biotin (500 μg/ml) there was no difference between the levels of biotinylated $P2X_1$ receptors following 30 min biotinylation (agonist treatment 97.1 ± 2.5% of non-treated controls, $n = 4$ experiments). The biotinylation technique does not give a snapshot measure of surface localisation at any one time but an estimate of the number of receptors that have been at the surface during the

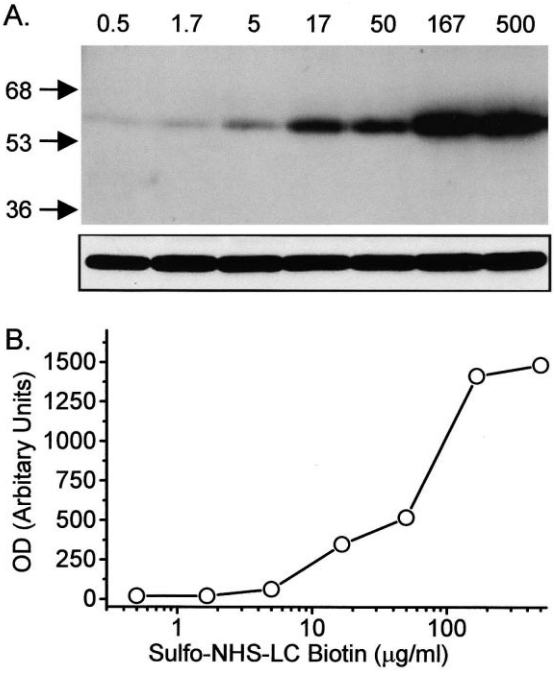

Fig. 1. Biotinylation of rat vas deferens $P2X_1$ receptors with Sulfo-NHS-LC-Biotin is concentration dependent. A: Purified biotinylated $P2X_1$ (inset shows total $P2X_1$). B: Optical density of the biotinylated $P2X_1$ protein bands in A plotted against concentration of biotinylating agent (10 min incubation). Lane markers correspond to the concentration of Sulfo-NHS-LC-Biotin in μg/ml. Size markers are in kDa.

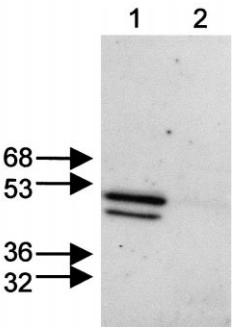

Fig. 2. The cytoplasmic protein ERK is not biotinylated. Rat vasa deferentia were exposed to Sulfo-NHS-LC-Biotin (500 μg/ml) for 10 min. Figure shows a representative ($n = 4$) Western blot probed with anti-ERK antibody which detects the cytoplasmic proteins ERK1 (44 kDa) and ERK2 (42 kDa). Lane 1, total protein sample. Lane 2, purified biotinylated proteins. Molecular weight markers in kDa.

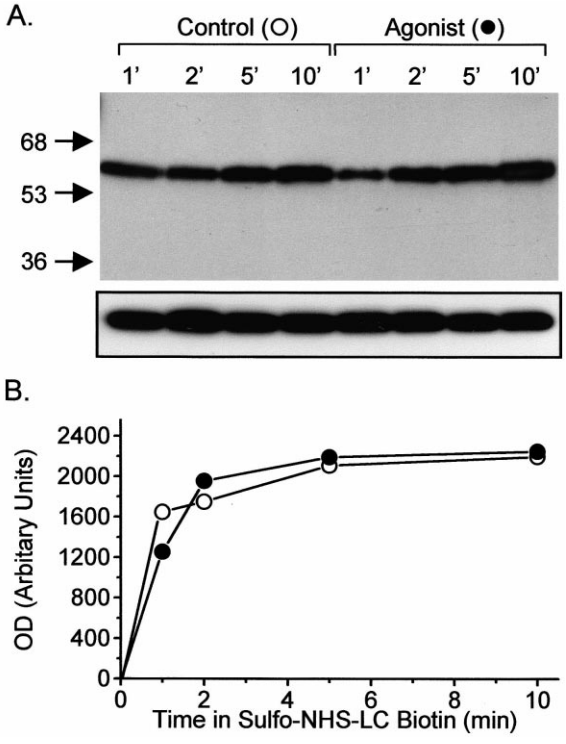

Fig. 3. Effect of agonist stimulation on surface localisation of $P2X_1$ receptors. Vasa deferentia were exposed to agonist (100 μM α,β-meATP) or no agonist for 30 min followed by incubation in 500 μg/ml Sulfo-NHS-LC-Biotin for the time indicated (minutes) in the continued presence of agonist A. Purified biotinylated $P2X_1$ (inset shows total $P2X_1$). B: Optical density of the biotinylated $P2X_1$ protein bands in A plotted against time in biotinylating agent (open circles: no agonist, closed circles: agonist-stimulated). Size markers are in kDa.

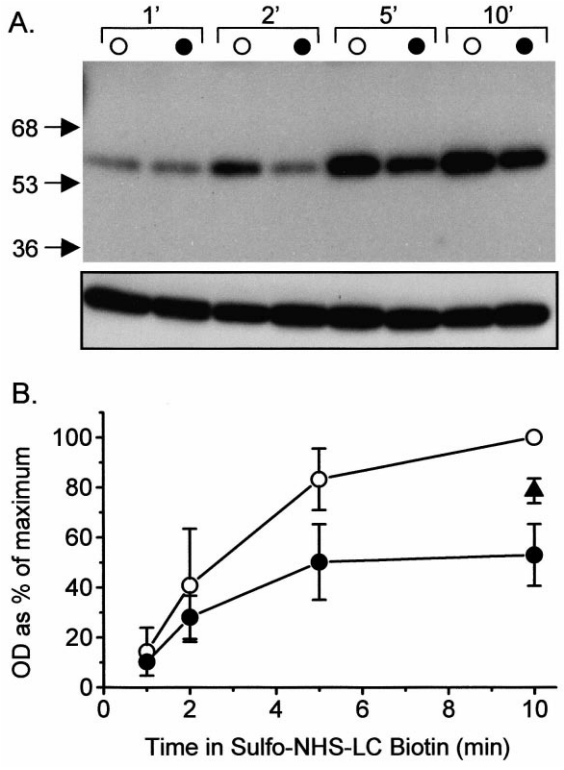

Fig. 4. Agonist stimulation decreases surface localisation of the $P2X_1$ receptor. Vasa deferentia were exposed to agonist (100 μM α,β-meATP) (●) or no agonist (○), for 30 min followed by incubation in 50 μg/ml Sulfo-NHS-LC-Biotin for the time indicated (minutes). A: Purified biotinylated $P2X_1$ (inset shows total $P2X_1$). B: Optical density of the biotinylated $P2X_1$ protein bands in A (and from three other duplicate experiments not shown) plotted against time in biotinylating agent (● agonist-stimulated; ○ no agonist). The closed triangle represents a single point (gels not shown) for samples which had the agonist washed away immediately prior to biotinylation. Points are plotted as % maximum value (optical density for the band corresponding to 10 min biotinylation, no agonist for that particular experiment) ± S.E.M. ($n = 4$ experiments, two rats per experiment). Size markers are in kDa.

incubation period, i.e. it incorporates a component of receptors that may have been recycled to the surface. Therefore we looked at the time-course of biotinylation to determine if any changes in the level of surface $P2X_1$ receptor following agonist stimulation could be resolved. Biotinylation was very rapid; ~80% of surface $P2X_1$ receptors were labelled within the first 2 min (Fig. 3B, open symbols). There was some indication that the level of biotinylated $P2X_1$ receptors was reduced compared to control tissues following a 1 min incubation in Sulfo-NHS-LC-Biotin. However, on longer incubation with biotinylating agent the levels of surface expression were indistinguishable between agonist-stimulated and control tissues, as was the case in the initial studies. Thus, if vas deferens $P2X_1$ receptors are internalised during the presence of agonist, there must be a continual shuttling of receptor back and forth from the membrane surface in order to give equivalent levels of biotinylation with non-agonist-stimulated cells. In order to overcome such a masking effect we reduced the biotinylating agent to submaximal concentrations (~30%) in order to slow the rate of the reaction and therefore reduced the probability of labelling transient surface $P2X_1$ receptors.

When the concentration of Sulfo-NHS-LC-Biotin was reduced to 50 μg/ml (~30% maximum labelling), a clear decrease in $P2X_1$ receptor biotinylation was observed in the presence of agonist indicating internalisation of $P2X_1$ receptors (Fig. 4). This difference was apparent after 5 min biotinylation and by 10 min there was a 47.0 ± 12.3% decrease in the amount of receptor biotinylation in agonist-stimulated compared to control tissues ($n = 4$ experiments). When α,β-meATP was washed off prior to biotinylation the level of $P2X_1$ receptor expression recovered to 78.6 ± 4.9% of the control response (see closed triangle point in Fig. 4B) and showed that the decrease in biotinylation was not due to the α,β-meATP interfering with the ability of the Sulfo-NHS-LC-Biotin to react with the cell surface receptors.

Contraction studies were used to compare the changes in surface $P2X_1$ receptor expression with recovery of functional P2X receptors. The metabolically stable ATP analogue, α,β-meATP (10 μM), evoked transient contractile responses of the vas deferens that decayed during the continued presence of agonist (Fig. 5A). Following 10 min washout of α,β-meATP the response to a subsequent application of agonist evoked a contraction that was ~40% of the control response (in contrast to ~80% recovery of surface expression). Full recovery of contractile responses following agonist stimulation required a 30 min post wash period (Fig. 5). There was no significant difference in percentage recovery between a 2 min application

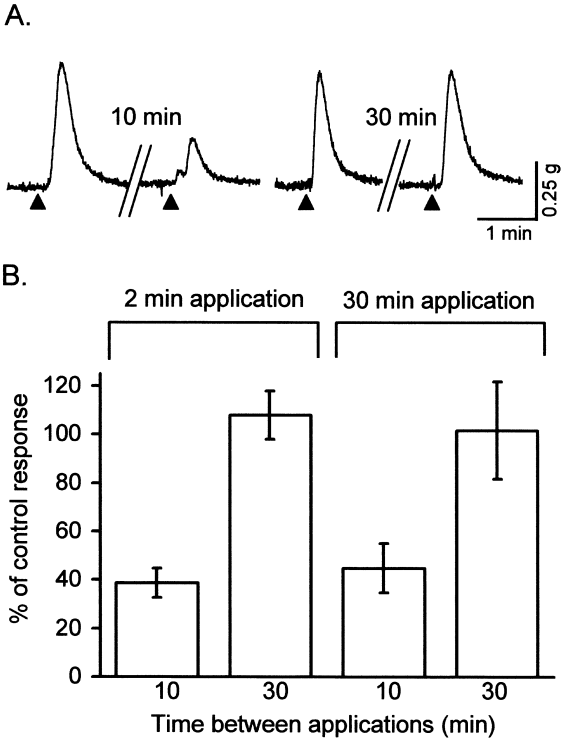

Fig. 5. Time dependent recovery of $P2X_1$ receptor-mediated contractions. A: Representative traces of isometric tensions induced by a 2 min application of 10 μM α,β-meATP (p) recorded from rat vas deferens in organ baths. Recovery times are given above the broken lines. B: Mean peak values (± S.E.M.) as a percentage of initial response (2 min application, 30 min recovery) for 2 min and 30 min applications of agonist with either a 10 min or 30 min recovery time. Note the recovery of the response after 10 min is independent of the application time indicating that desensitisation of the receptor (either internalisation of physical conformational changes) occurs within the first 2 min of agonist exposure.

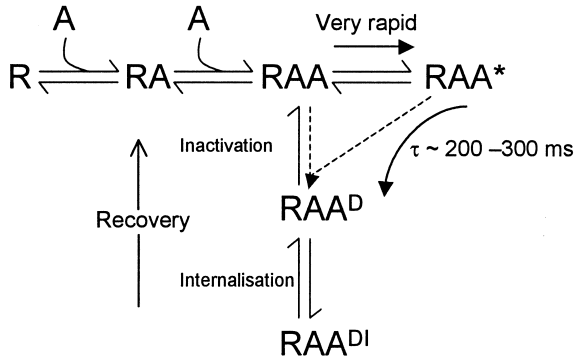

Fig. 6. Model of state changes of the $P2X_1$ receptor following agonist stimulation. R = receptor, A = agonist, RA = agonist bound receptor, RAA = receptor bound with two agonists, RAA* = agonist bound open/activated receptor, RAA^D = agonist bound desensitised receptor, RAA^I = agonist bound desensitised/internalised receptor. The Hill coefficient of ATP binding to P2X receptors indicates that at least two molecules of ATP are required for receptor activation. It is unclear at present whether the transition to RAA^D is directly from RAA or from RAA* (this has been indicated by the dashed line). Based on the work of Dutton et al. [10] suramin does not lead to receptor internalisation; coupled with recent work showing that amino acids associated with ATP and suramin binding are different [4] it is considered unlikely that antagonist binding proceeds to a state equivalent to RAA and that antagonist binding will not lead to receptor internalisation.

(39 ± 6%, $n = 13$) and 30 min applications (44 ± 10%, $n = 12$) (Fig. 5). The inability of the vas deferens to elicit a full response after 10 min recovery is due to desensitisation of the P2X receptors rather than an inability of the contractile apparatus to respond since applications of the α-adrenoceptor agonist arterenol (10 μM) given directly after the second applications of α,β-meATP elicit a full contractile response (data not shown).

4. Discussion

In this study we have shown that agonist stimulation leads to $P2X_1$ receptor internalisation and receptor recycling. Using the biotinylation technique it was estimated that >50% of $P2X_1$ receptors were internalised during the continued presence of α,β-meATP (Fig. 4). This is the first time that internalisation of native $P2X_1$ receptors has been reported. Under physiological conditions it is likely that ATP released from sympathetic nerves will similarly result in $P2X_1$ receptor internalisation. The decrease in surface receptor labelling probably underestimates the true level of receptor internalisation in response to agonist as it will also incorporate labelling of recycled receptors. Our results are consistent with recent studies using over-expressed recombinant $P2X_1$–GFP tagged receptors [10,11]. For $P2X_1$–GFP chimeric receptors there was a >80% decrease in steady-state fluorescence following continued agonist stimulation associated with internalisation of the chimera and quenching of the GFP signal by the acidic environment in the endosomes. The use of the fluorescent chimeric receptors gave a measure of the steady-state levels of surface vs. internalised $P2X_1$ receptors. Our results with a saturating concentration of biotinylating agent (that gives a measure of the number of receptors that have been expressed at the surface during the incubation period) indicate that native smooth muscle $P2X_1$ receptors once internalised are subsequently quickly shuttled to and from the membrane. Work on recycling of the transferin receptor has demonstrated the existence of two compartments for internalised receptors: a peripheral compartment from which rapid recycling is possible and a perinuclear compartment from which recycling is slow [16,17]. Due to the apparent quick recycling rate of internalised $P2X_1$ receptors indicated in this study, it is likely that they are internalised to the rapid recycling peripheral compartment as has been shown for the $GABA_A$ receptor [18].

$P2X_1$ receptor internalisation and shuttling can be added to an existing model [19,20] of receptor desensitisation, to describe the activation, inactivation, internalisation and recovery of $P2X_1$ receptors (Fig. 6). Following agonist binding the channel rapidly opens (in patch clamp studies the rate of rise of P2X currents is as fast as the solution exchange [21] and it is likely that the channel opens almost instantaneously following ligand binding as has been shown for other ligand-gated ion channels). The rapid mono-exponential decay of native and recombinant $P2X_1$ receptor currents ($\tau \sim$ 200–300 ms [22]) corresponds to a single step to a ligand bound inactivated state [23]. From this state the receptor can subsequently be internalised (>80% internalisation in 2–3 min) [10]. The recovery of $P2X_1$ receptors from desensitisation therefore proceeds in two steps, (i) recycling of $P2X_1$ receptors to the surface of the cell followed by (ii) recovery from the inactivated state. Internalisation of the $P2X_1$ receptor is unlikely to be the major determinant of the recovery of responses following agonist stimulation since 10 min after agonist stimulation ~80% (Fig. 4, closed triangle) of the receptors had re-appeared on the cell surface, however, only ~40% of the contractile response was recovered (Fig. 5). Therefore transitions into and out of the inactivated state are most likely to be the rate-limiting step in desensitisation and recovery. We have recently suggested that an intracellular messenger and/or intact cytoskeleton contribute to $P2X_1$ receptor recovery in vascular smooth muscle [23]. Whether it is necessary for receptors to be internalised in order to recover inactivation is unclear. A decrease in recycling of the $P2X_1$ receptor to the surface would provide a mechanism for the longer term down regulation of P2X receptor-mediated responses. Thus smooth muscle cells could regulate responsiveness to repeated ATP stimulation both in the short (receptor inactivation) and long term (decreased surface expression).

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