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# Purinergic receptors expressed in human skeletal muscle fibres

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**Abstract** Purinergic receptors are present in most tissues and thought to be involved in various signalling pathways, including neural signalling, cell metabolism and local regulation of the microcirculation in skeletal muscles. The present study aims to determine the distribution and intracellular content of purinergic receptors in skeletal muscle fibres in patients with type 2 diabetes and age-matched controls. Muscle biopsies from vastus lateralis were obtained from six type 2 diabetic patients and seven age-matched controls. Purinergic receptors were analysed using light and confocal microscopy in immunolabelled transverse sections of muscle biopsies. The receptors $P2Y_4$, $P2Y_{11}$ and likely $P2X_1$ were present intracellularly or in the plasma membrane of muscle fibres and were thus selected for further detailed morphological analysis. $P2X_1$ receptors were expressed in intracellular vesicles and sarcolemma. $P2Y_4$ receptors were present in sarcolemma. $P2Y_{11}$ receptors were abundantly and diffusely expressed intracellularly and were more explicitly expressed in type I than in type II fibres, whereas $P2X_1$ and $P2Y_4$ showed no fibre-type specificity. Both diabetic patients and healthy controls showed similar distribution of receptors. The current study demonstrates that purinergic receptors are located intracellularly in human skeletal muscle fibres. The similar cellular localization of receptors in healthy and diabetic subjects suggests that diabetes is not associated with an altered distribution of purinergic receptors in skeletal muscle fibres. We speculate that the intracellular localization of purinergic receptors may reflect a role in regulation of muscle metabolism; further studies are nevertheless needed to determine the function of the purinergic system in skeletal muscle cells.

**Keywords** P2Y receptors · P2X receptors · Type 2 diabetes · Muscle metabolism

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## Introduction

Extracellular nucleotides (adenosine triphosphate, ATP; adenosine diphosphate; uridine triphosphate, UTP; uridine diphosphate) are important signalling molecules in cardiovascular and metabolic regulation [1, 2]. Nucleotides mediate their biological response via two main categories of cell surface receptors, P2X and P2Y, based on their pharmacological properties. P2Y is a family of G-protein-coupled receptors, whereas P2X is a group of ligand-gated ion channel receptors. Currently, eight subtypes of P2Y and seven subtypes of P2X family have been cloned and characterised [1, 3–5].

Purinergic receptors have been demonstrated both luminally and interstitially in skeletal muscles [6–8]. Location varies with the different species and tissues under study. In human tissue, purinergic receptor expressions have primarily been determined by mRNA measurements, thereby not assuring the location of, merely demonstrating

the presence of purinergic receptors [6, 8] Immunohistochemical analysis of human skeletal muscle biopsies have revealed the location of $P2Y_2$ receptors in the endothelium of capillaries and in endothelium and smooth muscle cells of microvessels [8]. Furthermore, $P2X_1$ receptors have been found in the skeletal muscle sarcolemma and potentially in the endothelium of capillaries [8]. These findings were confirmed in a study of type 2 diabetic patients and age-matched controls [7], where a functional difference in vasodilatatory response between the two groups had no relation to localization of vascular $P2Y_2$ and $P2X_1$ receptors nor the mRNA amount of the $P2Y_2$ receptors in skeletal muscle tissue.

In human skeletal tissue, interstitial ATP concentrations have been demonstrated to increase in proportion to exercise intensity [9]. In vitro studies of myotubes, showing ATP to facilitate translocation of glucose transporter type 4 (GLUT4) to the plasma membrane, suggest a metabolic role of extracellular nucleotide [10].

Chronic diseases may result in changes in amount or function of different receptors and agonists, as exemplified in the down-regulation of beta-adrenergic receptors in patients with heart failure and insulin-resistance in patients with type 2 diabetes.

The present study aims to investigate the intracellular content of purinergic receptors in skeletal muscle cells of middle-aged diabetic patients and corresponding healthy controls using confocal microscopy in order to, firstly, determine which receptors are expressed in skeletal muscle cells, and secondly, to clarify whether type 2 diabetes affects the amount and localization of intracellular purinergic receptors.

## Materials and methods

### Subjects

Six diabetic subjects (male/female: 3/3, 48±3 year (±SD), 176±12 cm, 96±23 kg, BMI=31±5 kg/m$^2$) and seven healthy subjects (male/female: 3/4, 51±7 year, 176±7 cm, 76±9 kg, BMI=25±3 kg/m$^2$) participated in this study. The diabetic patients were recruited from outpatient clinics and selected according to World Health Organization criteria [11]. The study was approved by the local ethical committee of City Councils of Frederiksberg, Denmark and conducted in accordance with the Helsinki II Declaration. Prior to giving their written consent to participate, all subjects had been fully informed orally and in writing about risks and discomforts associated with the experiment. On the day of the experiment, the subjects arrived at the laboratory having had only a light breakfast and having refrained from alcohol and caffeine for >12 h. The diabetic subjects maintained their usual antidiabetic treatment during the study.

### Practical procedures

The muscle biopsies were obtained from the middle portion of the vastus lateralis muscle using the percutaneous needle technique with suction [12]. The muscle biopsies were immediately separated into two parts. One part used for transverse section analysis was mounted in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands), frozen in isopentane, cooled to its freezing point in liquid nitrogen and stored at −80°C until analysed. The other part used for single fibre analysis was placed in Krebs–Ringer buffer containing 0.1% procaine hydrochloride for 2–3 min, pinned down at resting length in petri dishes coated with Sylgard 184 (Dow Corning, Germany), fixed with 2% depolymerised paraformaldehyde and 0.15% picric acid in 0.1 M phosphate buffer at room temperature for half an hour, left for an additional 4.5 h in the fixative at 4°C, transferred to 50% glycerol in PBS and stored at −20°C until analysed.

### Fluorescence immunohistochemistry on transverse cryosections

Ten micrometer transverse sections from muscle biopsies mounted in Tissue-Tek were cut at −22°C using a Leica CM3050 S cryostat (Leica Microsystems, Denmark), collected on SuperFrost® Plus glass slides (Thermo Scientific, Denmark), within seconds placed in 4% depolymerised paraformaldehyde and 0.15% picric acid in 0.1 M phosphate buffer at 4°C for 1 h, rinsed several times in PBS and processed for immunohistochemistry.

Sections were blocked for 10 min with 50 mM glycine in PBS, incubated for 10 min with immunobuffer (PBS with 50 mM glycine, 0.25% bovine serum albumin, 0.03% saponin, and 0.05% sodium azide) and labelled for 90 min with primary antibodies diluted in immunobuffer. The following primary antibodies were used, either alone or two together (rabbit+mouse): Rabbit polyclonal anti-$P2X_1$ (APR-001, Alomone Labs, Israel; 1:500; previously used in the following studies [6, 8, 13–19]); mouse polyclonal anti-$P2X_4$ (H00005025-B01P; Abnova, Taiwan; 1:500); rabbit polyclonal anti-$P2Y_1$ (APR-009, Alomone Labs; 1:500; previously used in the following studies [18, 20–22]); polyclonal anti-$P2Y_2$ (APR-010, Alomone Labs; 1:500; previously used in the following studies [6, 8, 18, 22–24]); rabbit polyclonal anti-$P2Y_4$ (P6497, Sigma-Aldrich, Denmark; 1:250); rabbit polyclonal anti-$P2Y_{11}$ (APR-015, Alomone Labs; 1:500; previously used in the following studies [18, 25–30]); rabbit polyclonal anti-$P2Y_{12}$ (HPA013796, Sigma-Aldrich; 1:30; previously used in

the following study [31]). The $P2X_1$ antibody was highly specific and directed against the epitope corresponding to amino acid residues 382–399 of rat $P2X_1$ (human 15/18 residues identical); the $P2Y_{11}$ antibody was corresponding to amino acid residues 357–373 of human $P2Y_{11}$; western blots for anti-$P2X_1$ and anti-$P2Y_{11}$ are shown on the Alomone homepage. The $P2Y_4$ antibody corresponded to the second extracellular loop of human $P2Y_4$. A mouse monoclonal anti-skeletal slow myosin heavy chain 1 (MHC1) antibody (M8421, Sigma-Aldrich; 1:500) was used to identify type I muscle fibres. Mouse monoclonal anti-alpha sarcoglycan (IVD3(1)A9, developed by Kevin P. Campbell, University of Iowa, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by University of Iowa, Department of Biology, Iowa City, IA 52242; 1:50). It was used to mark the plasma membrane on single muscle fibres and was also used as a marker of sarcolemma. Mouse monoclonal anti-CD31 (M0823, Dako, Denmark; 1:500) was used as an endothelial marker and microtubules were stained using mouse monoclonal anti-α-tubulin (clone DM1A, T-9026, Sigma-Aldrich, Denmark; 1:500).

After incubation with primary antibody, the sections were washed 3×5 min with PBS and labelled with secondary antibodies for 1 h. The secondary antibody solution contained fluorescent Alexa Flour® 488 goat anti-rabbit IgG (A-11034, Invitrogen, Denmark; 1:750), Alexa Flour® 568 goat anti-mouse IgG (A-11031, Invitrogen, Denmark; 1:750) and occasionally TO-PRO®-3 iodide (T3605; Invitrogen, Denmark; 1:1,000) was added as a nuclei stain. After incubation with secondary antibody solution nuclei were always stained with Hoechst 33342 (0.5 μg/ml) in blocking buffer (irrespectively of prior staining with TO-PRO-3 or not) for 3 min, washed 3× 5 min with PBS and mounted in Vectashield (Vector Laboratories, Burlingame, CA) and the cover slip sealed using nail polish. Slides were stored at −20°C until analysed. Staining without primary antibody or without primary and secondary antibodies served as negative controls.

### Fluorescence immunohistochemistry on single muscle fibres

This was performed essentially as previously described [32]. In short, around 20 bundles of one to three fibres were teased from each fixed muscle biopsy (stored at −20°C in 50% glycerol in PBS) with fine forceps, placed in 50 mM glycine in PBS and incubated with immunobuffer (composition as described for cryosections) for at least 30 min. Fibres were then incubated overnight with primary antibodies diluted in immunobuffer. The primary antibodies, either alone or two together (rabbit+mouse), were the same as those used for cryosections. After three washes of 30 min each with immunobuffer, fibres were incubated for 2 h with appropriate secondary antibodies conjugated to either Alexa-488 or Alexa-568 (Invitrogen) diluted in immunobuffer. Nuclei were stained for 5 min with Hoechst 33342 (0.5 μg/ml) diluted in immunobuffer, then washed three times for 30 min each with immunobuffer, rinsed in PBS, and finally mounted in Vectashield; the cover slip was sealed using nail polish. Slides were stored at −20°C until analysed. Staining without primary antibody or without primary and secondary antibodies served as negative controls.

### Confocal image acquisition and analysis

Slides were inspected with conventional epifluorescence microscopy and specimens localized and evaluated by their Hoechst staining. Images were collected with a confocal laser-scanning microscope (TCS SP2, Leica), using either a Plan-Apo 40×/1.00 or a Plan-Apo 63×/1.32 oil objective. For comparison reasons, all images of a given antibody combination were recorded with identical settings, no threshold (off set) for the collection photomultipliers was applied and care was taken that only a small fraction of pixels showed saturation intensity values. For each cryosection six to eight confocal images were collected as a z-stack, and consecutive optical sections were spaced either 0.35 μm (63× objective) or 0.50 μm (40× objective) apart from each other in the *Z*-plane. As to whole mounts ("single fibres"), similar recordings were done from the surface of muscle fibres as well as from one-third inside the fibres. Maximum projections of z-stacks were constructed solely for visualization purposes.

The use of fluorescent conjugated secondary antibodies, in contrast to enzymatically (e.g., HRP or AP) conjugated secondary antibodies, in immunohistochemistry has definitive advantages with regard to quantification and multiplexing. However, earlier research results has shown that the use of immunofluorescence microscopy in studying human muscle fibres, at least in adults, also contains some treacherous pitfalls. This is due to the presence of small autofluorescence structures, lipofuscin granules, which are very "eye-catching", and which may easily be mistaken for true positive specific staining. Lipofuscin inclusions are formed from damaged cell components and accumulated in postmitotic cells in relation to oxidative stress and ageing [33–36]. In contrast hereto, lipofuscin granules are absent in healthy muscle in laboratory animals like rats and mice. When studying human skeletal muscle through immunofluorescence microscopy, corrective measures therefore must be employed. This can practically be done by taking advantage of dissimilarity in fluorescence emission pat-

terns. Although lipofuscin and purinergic receptors are excited with the same laser, it is possible to collect images without receptor fluorescence but not images without lipofuscin fluorescence. After collecting the two sets of pictures, it is possible to subtract the lipofuscin staining from the receptor staining.

### Western blot analysis

The specificities of the three key antibodies in this study, $P2X_1$; $P2Y_4$, and $P2Y_{11}$ were examined using Western blotting (Fig. 1).

Biopsies were freeze-dried and dissected fat free prior to homogenisation in magnesium buffer (10% glycerol, 20 mM sodium pyrophosphate, 150 mM NaCl, 50 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), 1% nonyl phenoxylpoly-ethoxylethanol (NP-40), 20 mM β-glycerophosphate, 2 mM sodium orthovanadate ($Na_3VO_4$), 10 mM natrium fluoride (NaF), 2 mM phenylmethylsulphonyl flouride, 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 3 mM benzamidine). Samples were rotated end over end for 60 min at 4°C and then centrifuged for 30 min at 17,000×*g* at 4°C. The lysate was collected and protein concentration was determined by a BSA protein assay (Pierce Biotechnology, Inc., Rockford, IL, USA). Lysate proteins were separated on 16.5% Tris-Tricine gels (BioRad) and transferred semi-dry to PVDF-membranes (Millipore AMC, US). The membranes were incubated with primary $P2X_1$, $P2Y_4$, or $P2Y_{11}$ antibody all diluted 1:200. Secondary antibody horseradish-peroxidase (HRP)-conjugated goat anti-rabbit (P-0448, Dako, Glostrup, Denmark) 1:5,000 was used for detection of $P2X_1$, $P2Y_4$ or $P2Y_{11}$ protein. Proteins were then analysed (Kodak Image Station, 2000MM) and detected (Kodak Molecular Imaging software).

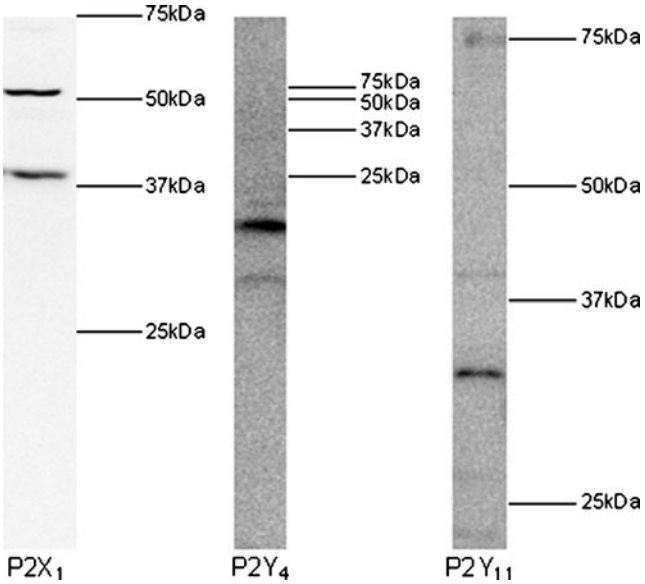


**Fig. 1** Western blotting of $P2X_1$; $P2Y_4$, and $P2Y_{11}$ in human muscle homogenate. Molecular weight markers are given

## Results

### Western blot

The $P2Y_{11}$ antibody gave rise to a single band at 33 kDa on Western blot (Fig. 1). Previous research using the $P2Y_{11}$ Alomone antibody has shown that the molecular weight of the band varies within the interval 34–45 kDa, depending on the tissue or cell under study [25–28].

$P2Y_4$ also gave rise to a single band (~25 kDa). Using a different $P2Y_4$ antibody, Wang et al. [6] have previously found a band of approximately 33 kDa in smooth muscle cells. The reason for the difference in molecular weight between tissues may be due to protein modifications such as glycosylation.

$P2X_1$ gave rise to two bands on the Western blot at approximately 39 and 54 kDa, where the 39 kDa band likely represents non-specific binding to another protein. The 54 kDa band is likely to be specific for $P2X_1$ as previously evidenced by use of a control peptide on smooth muscle cell preparations [6]. Therefore, it cannot be ruled out that staining with this antibody, in part, may be due to binding to another unknown protein.

### Immunohistochemistry

According to initial immunohistochemical screenings of muscle biopsies from healthy subjects, the distribution of purinergic receptors is given in Table 1. Of the seven tested antibodies, there was positive intracellular muscle fibre staining for $P2X_1$ and $P2Y_{11}$ receptors, and positive staining for $P2X_1$ and $P2Y_4$ receptors in the muscle plasma membrane. Staining for $P2Y_1$, $P2Y_2$, $P2Y_{12}$ and $P2X_4$ was absent in skeletal muscle fibres but the receptors were present in the vasculature. $P2Y_2$ was found in EC of vessels; $P2Y_1$ and $P2X_4$ receptors were only found in capillaries. The $P2Y_{12}$ receptor was present in the lumen of blood vessels as evident in longitudinal sections; here the $P2Y_{12}$ receptor was most likely located in thrombocytes. However, since no specific staining for thrombocytes was performed, we cannot fully determine the $P2Y_{12}$ localization in vascular structures.

Muscle fibre associated purinergic receptors, $P2X_1$, $P2Y_4$, and $P2Y_{11}$, were selected for a more thorough morphological investigation. Results of transverse sections

**Table 1** Distribution of purinergic receptors in healthy subjects

| | Intracellularly | Sarcolemma | Endothelial | Capillary |
|---|---|---|---|---|
| $P2X_1$ | ++[a] | +[a] | +[a] | +[a] |
| $P2X_4$ | − | − | − | + |
| $P2Y_1$ | − | − | − | + |
| $P2Y_2$ | − | − | + | nd |
| $P2Y_4$ | − | + | + | + |
| $P2Y_{11}$ | ++ | −/+ | + | + |
| $P2Y_{12}$ | − | − | − | + (thrombocytes) |

++/+/− denotes high expression/medium expression and no expression. *nd* not determined

[a]It cannot be ruled out that staining with this antibody, in part, may be due to binding to another unknown protein

were supported by analyses of teased fibres. $P2X_1$ receptors were located in intracellular vesicles, in sarcolemma, and in the endothelium of capillaries (Fig. 2). $P2Y_4$ receptors were visible in moderate amounts in the sarcolemma and in the endothelium of the capillaries (Fig. 3). $P2Y_{11}$ receptors were diffusely expressed intracellularly in type I fibres whereas the expression in type II fibres was considerably less (Fig. 4). This receptor was only sparsely present in sarcolemma but clearly evident in the endothelium of surrounding capillaries.

There were no differences in expression levels nor distribution between controls and diabetic subjects for any of the three receptors using this semi-quantitative approach.

## Discussion

The present study demonstrates that purinergic receptors, $P2Y_4$, $P2Y_{11}$, and likely $P2X_1$, are expressed in human skeletal muscle fibres. The amounts of the three purinergic receptors and their localization indicate either a function of transport, metabolism or signalling in the skeletal muscle cell.

We found no differences in the amount of intracellular purinergic receptors between diabetic subjects and controls, although, similar amounts do not exclude differences in sensitivity or function in different patient groups.

Based on our experiments on specificity, we conclude that the $P2Y_4$ and $P2Y_{11}$ antibodies specifically bind to the respective receptors whereas, due to binding of the antibody to an additional protein of lower molecular weight, the results of the staining with $P2X_1$ antibody must be interpreted with caution.

During exercise, glucose uptake is enhanced in skeletal muscle cells, even in the absence of insulin [37]. Since ATP levels increase in the interstitium during muscle contractions [8, 38], we speculate whether ATP and the intracellular P2 receptors may be involved in glucose uptake, potentially by translocation of glucose transporters, and this independently of insulin activity. In vitro stimulation of $C_2C_{12}$ myoblasts with ATP resulted in a dose-dependent transporter-mediated glucose uptake [10]. On stimulation with ATP, a translocation of GLUT1 and GLUT4 was shown suggesting that ATP is a facilitator of the translocation of glucose transporters from cytosol to the plasma membrane [10]. When stimulated with ATP and insulin, the increase in glucose uptake was additive, suggesting that ATP and insulin may act both in common and by different mechanisms [10].

Arterial infusion of the NO donor, sodium nitroprusside, increases basal leg glucose uptake in healthy humans [39]. Studies of NO-inhibition during exercise have shown a reduction in glucose uptake [40] and this reduction was even larger in patients with type 2 diabetes [41], thereby indicating that glucose uptake in patients with diabetes could be more dependent on NO than in healthy individuals.

In vitro stimulation with the P2X-receptor agonist, 2-MeSATP, and the P2Y agonist, UTP, resulted in nNOS translocation of neuronal nitric oxide synthase (nNOS) from cytosol to the cell membrane, indicating that receptors sensitive to both ATP and UTP may be involved in NOS translocation in neurons [42]. The mu isoform of nNOS (nNOSμ) is the primary form expressed in skeletal muscle and its activity, and the resulting NO-production, increases by 1.5–2-fold with contraction [43]. Interestingly, a reduced content of nNOSμ has been found in humans with impaired glucose homeostasis [40]. It would thus be of interest to examine whether also nNOS in skeletal muscle cells is susceptible to stimulation with purinergic agonists.

The observed fibre-type differences in $P2Y_{11}$ receptor localization could indicate a relation to muscle cell metabolism. nNOS is expressed in the sarcolemma of all skeletal muscle fibre types, yet stronger immunoreactivity of nNOS is found in the sarcolemma and cytoplasm of type I muscle fibres [44]. Type I fibres (red, slow twitch) have a high capacity for lipid oxidation; moreover, they and are the predominant targets of insulin action.

Another potential function of the purinergic receptors in human skeletal muscle is contractility. In vitro studies of mouse cardiomyocytes demonstrated a positive inotropic effect of ATP stimulation via the $P2Y_{11}$ receptor [45]. In the

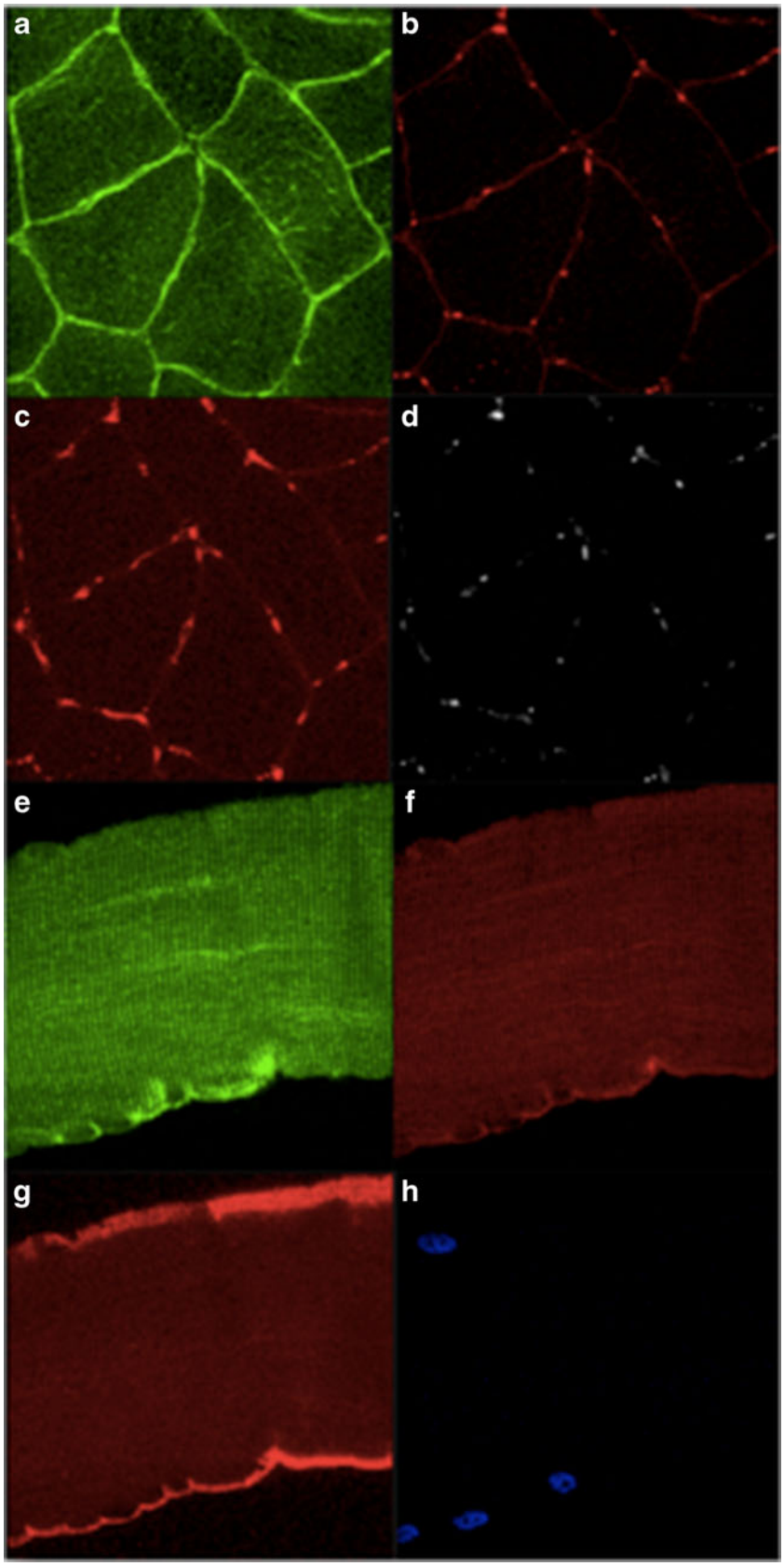


**Fig. 2** Staining of $P2X_1$. Panel **a**–**d** transverse sections. Panel **a** $P2X_1$; panel **b** $P2X_1$ background; panel **c** CD31; panel **d** CD31 background. Panels **e**–**h** single fibre. Panel **e** $P2X_1$; panel **f** $P2X_1$ background; panel **g** $\alpha$-Sarcoglycan; panel **h** nuclei staining

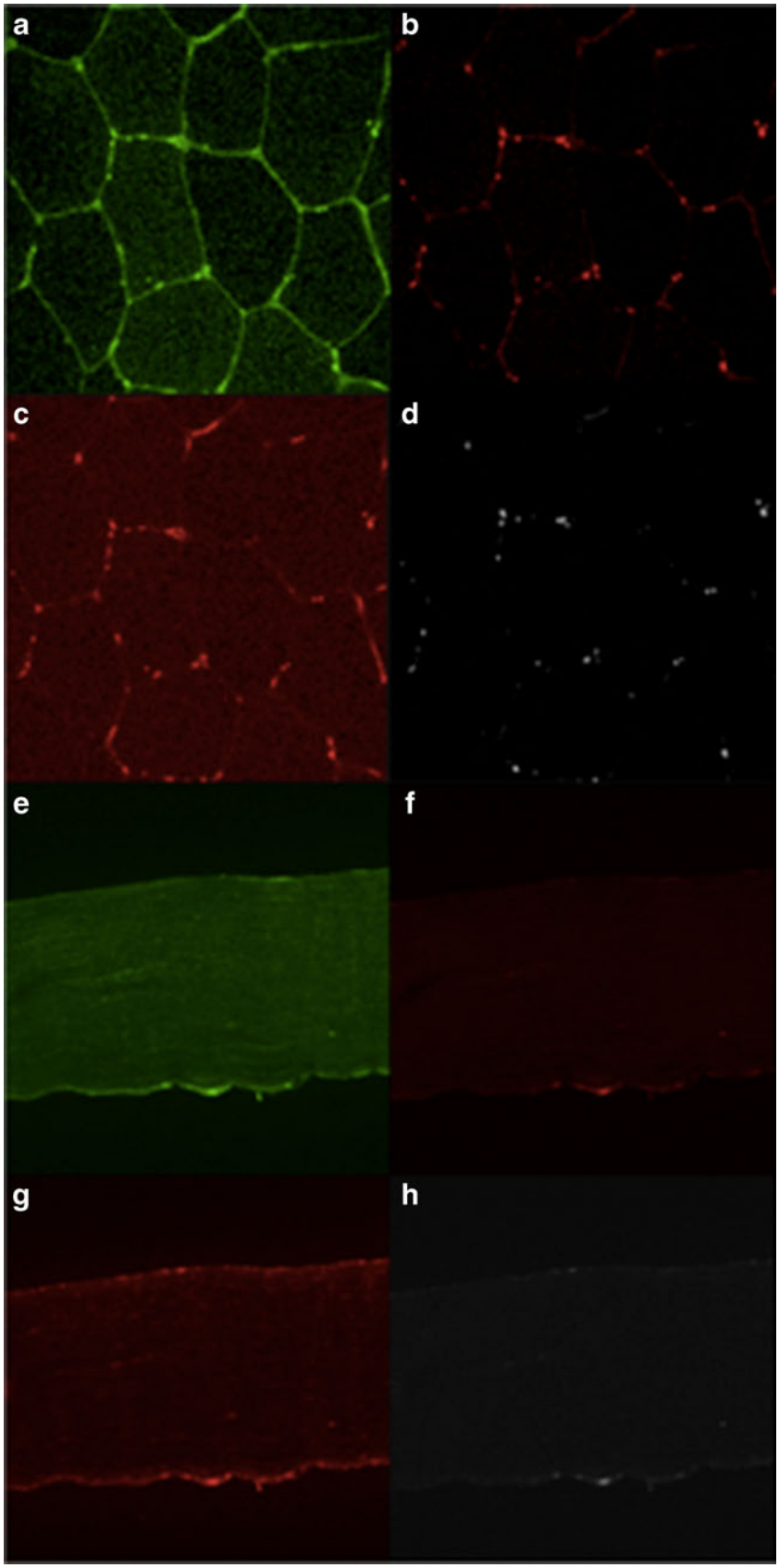


**Fig. 3** Staining of $P2Y_4$. Panel **a**–**d** transverse sections. Panel **a** $P2Y_4$; panel **b** $P2Y_4$ background; panel **c** CD31; panel **d** CD31 background. Panel **e**–**h** single fibre. Panel **e** $P2Y_4$; panel **f** $P2Y_4$ background; panel **g** $\alpha$-tubulin; panel **h** $\alpha$-tubulin background

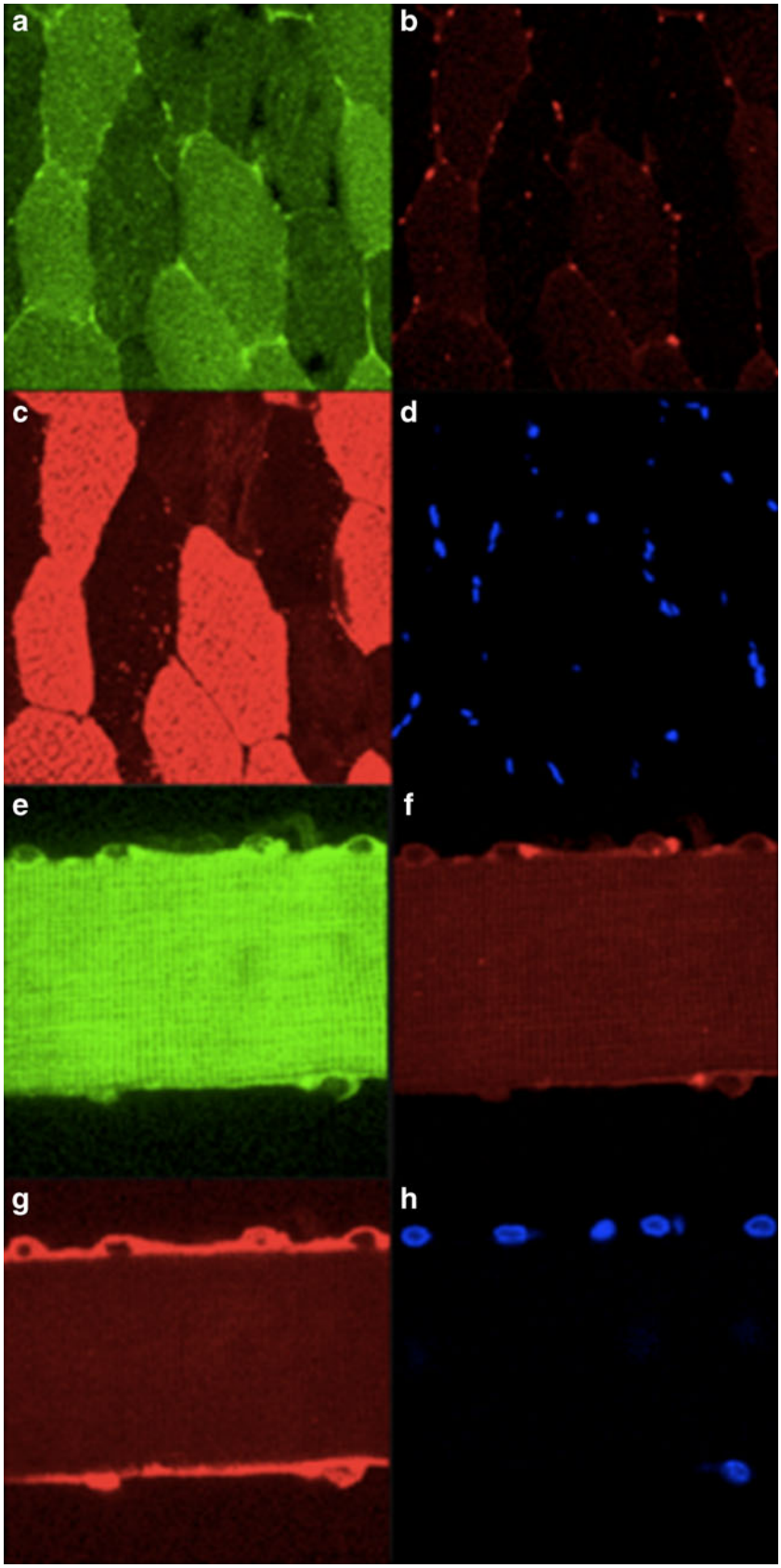


**Fig. 4** Staining of $P2Y_{11}$. Panel **a**–**d** transverse sections. Panel **a** $P2Y_{11}$; panel **b** $P2Y_{11}$ background; panel **c** MHC1 (type I muscle fibers); panel **d** nuclei staining. Panel **e**–**h** single fibre. Panel **e** $P2Y_{11}$; panel **f** $P2Y_{11}$ background; panel **g** α-Sarcoglycan; panel **h** nuclei staining

cardiomyocyte of rats, mRNA for the $P2Y_{11}$ receptor was detected in both atria and ventricle [46]. Thus, $P2Y_{11}$ might also be involved in contractility receptors in human skeletal muscle cells.

## Limitations

To determine the exact location in sarcolemma, that is, whether receptors are placed upon, in, or just below the plasma membrane, it requires the use of electron microscopy. In order to investigate a more precise location in sarcolemma, we tested the approachability of different markers of sarcolemma; however, none of these markers provided us with an improved knowledge of the exact location in sarcolemma, but gave sufficient evidence to conclude that the receptors are closely associated with sarcolemma.

Biopsies represent resting skeletal muscle cells and it may well be that the location of purinergic receptors changes when exposed to their agonists as during exercise. Receptors may be translocated to the outer surface (or internalised) when intra- or extracellular concentration of agonists changes, thus communicating changes between different compartments [47].

## Conclusion

Purinergic receptors are present in human skeletal muscles, $P2Y_4$ and likely $P2X_1$ in the plasma membrane, $P2Y_{11}$ and likely $P2X_1$ intracellular. Positive staining for $P2Y_1$, $P2Y_2$, $P2Y_{12}$, and $P2X_4$ were absent in the skeletal muscle fibres, but the receptors were present in the vasculature consistent with previous findings [1, 2, 6, 48, 49]. Type 2 diabetes does not appear to affect distribution of intracellular purinergic receptors; however, there may be functional differences of the receptors. Further functional studies of purinergic receptors in skeletal muscle cells may clarify the exact actions of these receptors.

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