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Influence of the Calcium Voltage-Gated Channel Auxiliary Subunit (CACNA2D1) Absence on Intraocular Pressure in Mice

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Abstract

The etiology of elevated intraocular pressure (IOP), a major risk factor for glaucoma (optic nerve atrophy), is poorly understood despite continued efforts. Although the gene variant of *CACNA2D1* (encoding α2δ1), a calcium voltage-gated channel auxiliary subunit, has been reported to be associated with primary open-angle glaucoma, and the pharmacological mitigation of α2δ1 activity by pregabalin lowers IOP, the cellular basis for α2δ1 role in the modulation of IOP remains unclear. Our recent findings reveled readily detectable levels of α2δ1 and its ligand thrombospondin in the cytoskeletome fraction of human trabecular meshwork (TM) cells. To understand the direct role of α2δ1 in the modulation of IOP, we evaluated α2δ1 null mice for changes in IOP and found a moderate (~10%) but significant decrease in IOP compared to littermate wild type control mice. Additionally, to gain cellular insights into α2δ1 antagonist (pregabalin) induced IOP changes, we assessed pregabalin's effects on human TM cell actin cytoskeletal organization and cell adhesive interactions in comparison with a Rho kinase inhibitor (Y27632), a known ocular hypotensive agent. Unlike Y27632, pregabalin did not have overt effects on cell morphology, actin cytoskeletal organization, or cell adhesion in human TM cells. These results reveal a modest but significant decrease in IOP in α2δ1 deficient mice, and this response appears to be not associated with the contractile and cell adhesive characteristics of TM cells based on the findings of pregabalin effects on isolated TM cells. Therefore, the mechanism by which pregabalin lowers IOP remains elusive.

Keywords

Ocular hypertension; Trabecular meshwork; Cytoskeleton; CACNA2D1; Pregabalin

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Author contributions

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Glaucoma, one of the leading causes of blindness worldwide, results from optic nerve degeneration and the loss of retinal ganglion cells (Weinreb et al., 2014). Although, the etiology of optic nerve axonal damage and retinal ganglion cell death is poorly understood, elevated intraocular pressure (IOP) due to impaired aqueous humor (AH) drainage through the conventional (trabecular) pathway is a major risk factor for glaucoma (Weinreb et al., 2014). Furthermore, lowering of IOP is a mainstay of treatment available to prevent vision loss in glaucoma patients (Weinreb et al., 2014). Therefore, there is a great deal of interest in understanding different cellular and molecular mechanisms regulating AH outflow and identifying new molecular targets to develop efficacious IOP lowering agents (Acott et al., 2021; Rao et al., 2017).

Recently, in our attempt to identify the major cytoskeletal and cytoskeletal-associated proteins of trabecular meshwork (TM) cells cultured under normal and dexamethasone treated conditions, as some of these proteins are involved in the modulation of AH outflow and IOP (Rao et al., 2017), we readily detected the peptides for the protein product (α2δ1) of the *CACNA2D1* gene, one of the subunits of the voltage-gated calcium channel (L-type calcium channel) and its ligand thrombospondin in the cytoskeletome fraction of human TM cells (Catterall, 2011; Maddala et al., 2023; Risher and Eroglu, 2012). Interestingly, the gene variant and mutation of *CACNA2D1* and thrombospondin are associated with glaucoma, respectively (Chintalapudi et al., 2017; Fu et al., 2022). However, their role in the modulation of IOP is not well understood. For example, pregabalin, which mitigates α2δ1 interaction with different physiological ligands including thrombospondin, has been shown to lower IOP in mice and rabbits (Chintalapudi et al., 2017; Ibrahim et al., 2019). However, the cellular and molecular basis for how pregabalin lowers IOP is not clear, and whether the IOP lowering response of this drug is due to impaired calcium signaling or changes in TM and ciliary muscle contractile and cell adhesive characteristics or due to reduced AH secretion are unknown (Chintalapudi et al., 2017). Moreover, the direct role of α2δ1 in IOP fluctuation has not been fully addressed (Chintalapudi et al., 2017).

Therefore, to gain more insights into the role of α2δ1 in the modulation of IOP, and TM actin cytoskeletal organization, cell adhesion and contractile activity, in this study, we addressed the direct role of α2δ1 in modulation of IOP by evaluating the changes in IOP in *CACNA2D1* null mice, and the effects of pregabalin, an antagonist of α2δ1, on human TM cell morphology, actin cytoskeletal organization, focal adhesion, and myosin light chain phosphorylation in comparison with a Rho kinase inhibitor. Rho kinase inhibitors have been demonstrated to lower IOP and are used to treat ocular hypertension in glaucoma patients with a known mechanism of action (Inoue and Tanihara, 2013; Kopczynski and Heah, 2018; Rao et al., 2017). The IOP lowering response of Rho kinase inhibitors has been shown to associate with TM relaxation, decreased actin stress fibers, focal adhesions, and ECM production (Rao et al., 2017).

We have detected several peptides of α2δ1 protein in the cytoskeletome fraction of human TM cells based on mass spec analysis (Maddala et al., 2023). α2 and δ1, which are encoded by the *CACNA2D1* gene are extracellular membrane bound glycoproteins and linked to each other through a disulfide band (Ablinger et al., 2020; Catterall, 2011). While α2 is located

extracellularly, the δ1 subunit is membrane-attached through a glycosylphosphatidylinositol (GPI) anchor (Ablinger et al., 2020). α2δ1 can be a subunit of the voltage-gated calcium channel and can participate in the regulation of L-type calcium channel activity (Catterall, 2011). Interestingly, an increasing number of studies also reveal calcium channel complex-independent functions for the α2δ1 subunit and its role in various neurological disorders (Ablinger et al., 2020; Eroglu et al., 2009). We confirmed the findings of mass spectrometry-based detection of α2δ1 in the human TM cell cytoskeletome fraction by immunoblot analysis using the cytoskeletome protein fraction derived from three independent strains (20, 27, and 48-year-old donors, passage 3) of TM cells (Fig. 1A) (Maddala et al., 2023). Interestingly, the levels of α2δ1 were significantly increased under dexamethasone treatment (0.5 μM for 7 days with alternative days changing the media as described previously (Maddala et al., 2023)), compared to control cells (treated with ethanol, 1μl/ml cell culture medium) in the cytoskeletome fraction based on immunoblot analysis (Figs. 1A & 1B). Dexamethasone is a well-characterized ocular hypertensive agent (Dibas and Yorio, 2016). However, in this study, we did not test the effects of other known ocular hypertensive agents, such as TGF-β2, on the levels of α2δ1 in TM cells. Our focus here centered on understanding the role of this protein in the modulation of IOP, rather than its regulation. As expected, α2δ1 was detected primarily in the membrane-enriched fraction (100,000xg pellet) of human TM cells (based on samples derived from the duplicates of two TM cell strains of 41 and 38-year-old donors grown under normal culture conditions with 10% fetal bovine serum) as shown in Fig. 1C based on immunoblot analysis. Intriguingly, in contrast to α2δ1, in the cytoskeletome fraction of human TM cells (based on mass spectrometry analysis), we did not detect the peptides of any other subunits of L-Type calcium channel complex (Maddala et al., 2023). Further, α2δ1 was also readily detectable in the TM of a human donor eye (90 year-old) based on immunofluorescence analysis performed using paraffin embedded tissue sections and α2δ1 monoclonal antibody obtained from Novus Biologicals (Fig. 1D) as we described previously (Choi et al., 2022). Additionally, in the cytoskeletome fraction of human TM cells (Mass spectrometry data), we detected several peptides for various isoforms of thrombospondin with thrombospondin-1 being more prominent based on a peptide count (Maddala et al., 2023). Thrombospondins are some of the well-characterized ligands of α2δ1 and together are demonstrated to regulate synaptogenesis (Eroglu et al., 2009; Risher et al., 2018). All cell culture experiments involving human TM cells in this study were conducted in accordance with the protocols established by Keller et al. (Keller et al., 2018).

Having found readily detectable levels of α2δ1 in human TM cells and tissue, we asked whether IOP is altered under deficiency of α2δ1 since the antagonist of α2δ1 (pregabalin) has been reported to lower IOP in mice and rabbits (Chintalapudi et al., 2017; Ibrahim et al., 2019). To investigate this question, we utilized a well-established α2δ1 null mouse model (Eroglu et al., 2009; Risher et al., 2018), which was maintained on a C57BL/6 genetic background (n=12). We measured intraocular pressure (IOP) changes in adult mice (3-4 months old, both males and females) by recording diurnal variations specifically between 1 and 2 PM. This investigation involved comparing these changes in the aforementioned α2δ1 null mice to their respective littermate controls (wild type) (n=7; determined through genotyping using tail DNA). The IOP measurements were conducted using a rebound

tonometer under isoflurane sedation conditions. The mouse experiments described in this study were carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Association for Research in Vision and Ophthalmology. The animal protocol (A213-19-10) was approved by the Institutional Animal Care and Use Committee of the Duke University School of Medicine. The α2δ1 null mice grew normally, and their body weights were very similar to the littermate controls (Fig. 1E & F). There were no noticeable phenotype differences between the α2δ1 null and control mouse eyes (Fig. 1E). We also validated the absence of α2δ1 expression in α2δ1 null mice through immunofluorescence analysis. Cryosections from eye tissues (n=3), including the iridocorneal angle and cornea, were compared with corresponding sections from the littermate wild type control mice. This comparison, depicted in Fig. 1G, confirmed the lack of α2δ1 expression in the null mice. IOP recordings were repeated on the same mice with a one-week interval. Fig. 1H shows a moderate but significant and consistent decrease (by ~5.4 & 11.2% in repeat measurements with a one-week interval between the two separate sets of readings on the same mice) and the two sets combined (by ~10%) in IOP in α2δ1null mice compared to littermate controls based on nonparametric Wilcoxon rank sum test of differences between the medians. IOP values (based on the average of 4 to 5 individual recordings derived from the rebound tonometer) of the individual eyes were plotted. The IOP values (mean ± SD, mmHg) recorded for the littermate control and α2δ1 KO ($^{-/-}$) mice are as follows: 17.75 ± 2.40 and 15.79 ± 2.43 (first week, Left panel, Fig. 1H); 18.83 ± 2.38 and 16.90 ± 2.95 (second week, middle panel, Fig. 1H); and 18.33 ± 2.41 and 16.34 ± 2.72 (first and second week combined, right panel, Fig. 1H), respectively. There were no differences in the histology of the AH outflow pathway, including the iridocorneal angle between the α2δ1 null and littermate control mice based on light microscope examination (Fig. 1I).

The absence of α2δ1 revealed a modest but statistically reduced IOP in *CACNA2D1* null mice similar to the reduced IOP of BXD mice harboring the *D* genotype of α2δ1 (Chintalapudi et al., 2017). In the Chintalapudi study, mice that were wild type for α2δ1 (harbored the *B* genotype) were responsive to pregabalin and IOP was reduced in a dose dependent manner (Chintalapudi et al., 2017). In this study, we sought to determine if the mechanism of action of pregabalin was via the effects on the actin cytoskeletal reorganization. To do so, we evaluated the effects of pregabalin on human TM cell actin cytoskeletal organization and cell adhesive interactions in comparison with a Rho kinase inhibitor. Rho kinase inhibitors are used to lower IOP in glaucoma patients, and the ocular hypotensive response of these inhibitors correlates well with altered cell shape, decreased actin stress fibers, cell adhesive interactions, contractile activity, and extracellular matrix (ECM) accumulation in cultured TM cells (Inoue and Tanihara, 2013; Kopczynski and Heah, 2018; Rao et al., 2017). Interestingly, human TM cells (maintained in culture medium containing 2% fetal bovine serum, passage 4-6, strains from 20 and 71 year-old donors as we described earlier, (Maddala et al., 2023)) treated with 10 and 100 μM pregabalin (ALP Pharm, Beijing Co. Ltd (Beijing, China) for 24 hrs did not exhibit noticeable effects on cell morphology, actin stress fibers (Rhodamine-phalloidin fluorescence) or focal adhesions (vinculin immunofluorescence) (Fig. 2A). Whereas the same strain of human TM cells maintained under similar culture conditions and treated with Rho kinase inhibitor (10 μM

for 24 hrs) as has been reported earlier (Rao et al., 2001), exhibited dramatic changes in cell shape in association with decreased actin stress fibers and focal adhesions (Fig. 2A). The reported half-maximal inhibitory concentration (IC50) values for pregabalin and Y27632 ranged between 0.1 and 0.3 μM and 0.3 and 1 μM, respectively (Fink et al., 2002; Uehata et al., 1997).

The biochemical analysis for the levels of phospho-paxillin, phospho-MYPT1 (substrate of Rho kinase), and phospho-myosin light chain (MLC) which regulate the contractile and cell adhesive interactions did not change under pregabalin treatment compared to controls (Fig. 2B and C). In contrast, all these biochemical attributes were significantly decreased under Rho kinase treatment (Fig. 2B and C). One exception is that with 100 μM pregabalin, approximately twice the concentration of pregabalin measured in the aqueous humor after 21 days of dosing (Ibrahim et al., 2019), there was a significant decrease in the levels of p-MLC compared to the controls. There were no changes in total MLC in both pregabalin and Rho kinase inhibitor-treated TM cells (Fig.2B and C). Lower than 10 μM pregabalin did not reveal any effects on the above-described TM cell characteristics (data not shown).

Collectively, these results reveal that similar to BXD mice carrying the *D* genotype of α2δ1 with IOP modulation that was unresponsive to pregabalin, α2δ1 null mice show a modest but statistically significant decrease in IOP. Under the conditions of gene targeting, particularly with a global absence of the targeted gene expression, one can expect some adaptive homeostatic mechanisms to kick in, which can be different from the pharmacological targeting of a given protein as a possible explanation for the discrepancy observed between the pharmacological effects verses gene deletion regarding IOP changes recorded in α2δ1 null mice verses pregabalin treated mice (Chintalapudi et al., 2017). Alternatively, it is also plausible that pregabalin-induced ocular hypotensive response might not be solely due to the mitigation of α2δ1 activity, and there may be other unknown targets for pregabalin in the trabecular and unconventional (uveoscleral) outflow pathways (Park and Lee, 2009; Sills, 2006).

This study also uncovers that the reported IOP lowering response of pregabalin might not be related to the changes in TM cell morphology, contractile characteristics, and cell adhesive interactions because unlike the Rho kinase inhibitor, Y27632, pregabalin treatment did not lead to changes in TM cell shape, actin stress fibers, and focal adhesions. Interestingly, in the cytoskeletome-enriched fraction of human TM cells, while we reported the detection of peptides of different channel proteins, we did not detect the peptides for α, β and γ subunits of L-type calcium channels. In contrast, there were more than 30 peptides detected for the α2δ subunit (Maddala et al., 2023). These findings indicate that within TM cells, the α2δ1 subunit of L-Type channels may exert its effects independently of other subunits, potentially suggesting a calcium-independent modulation of intraocular pressure (IOP). However, it is crucial to emphasize that the α2δ1 subunit has demonstrated regulatory roles in Rac1 GTPase activity (Risher et al., 2018), lipid rafts (Robinson et al., 2011), as well as interactions with thrombospondin (Risher and Eroglu, 2012) and perlecan (Reyes Fernande et al., 2022). Additionally, it plays a role in cell migration and adhesion (Garcia et al. 2008). Despite these known functions, it remains unclear whether any of these aspects contribute to the observed decrease in IOP documented in α2δ1 knockout (KO) mice. Consequently,

further mechanistic studies are warranted to elucidate the factors underlying the reduced IOP observed in α2δ1 KO mice. Moreover, the ocular hypotensive activity of pregabalin, as reported in various species (Ibrahim et. al. 2019), adds another layer of complexity to the understanding of these mechanisms. Therefore, there is a need for additional investigations to comprehensively unravel the intricate relationship between the α2δ1 subunit, decreased IOP in α2δ1 KO mice, and the ocular hypotensive effects of pregabalin across different species.

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Data Availability:

Data will be made available on request

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Highlights

- CACNA2D1 null mice exhibit a moderate reduction in IOP.
- Pregabalin, a CACNA2D1 antagonist, demonstrates minimal impact on the morphology of TM cells.
- Pregabalin induces minimal changes in the contractile and adhesive traits of TM cells.
- The mechanism underlying the ocular hypotensive effect of pregabalin remains unclear.

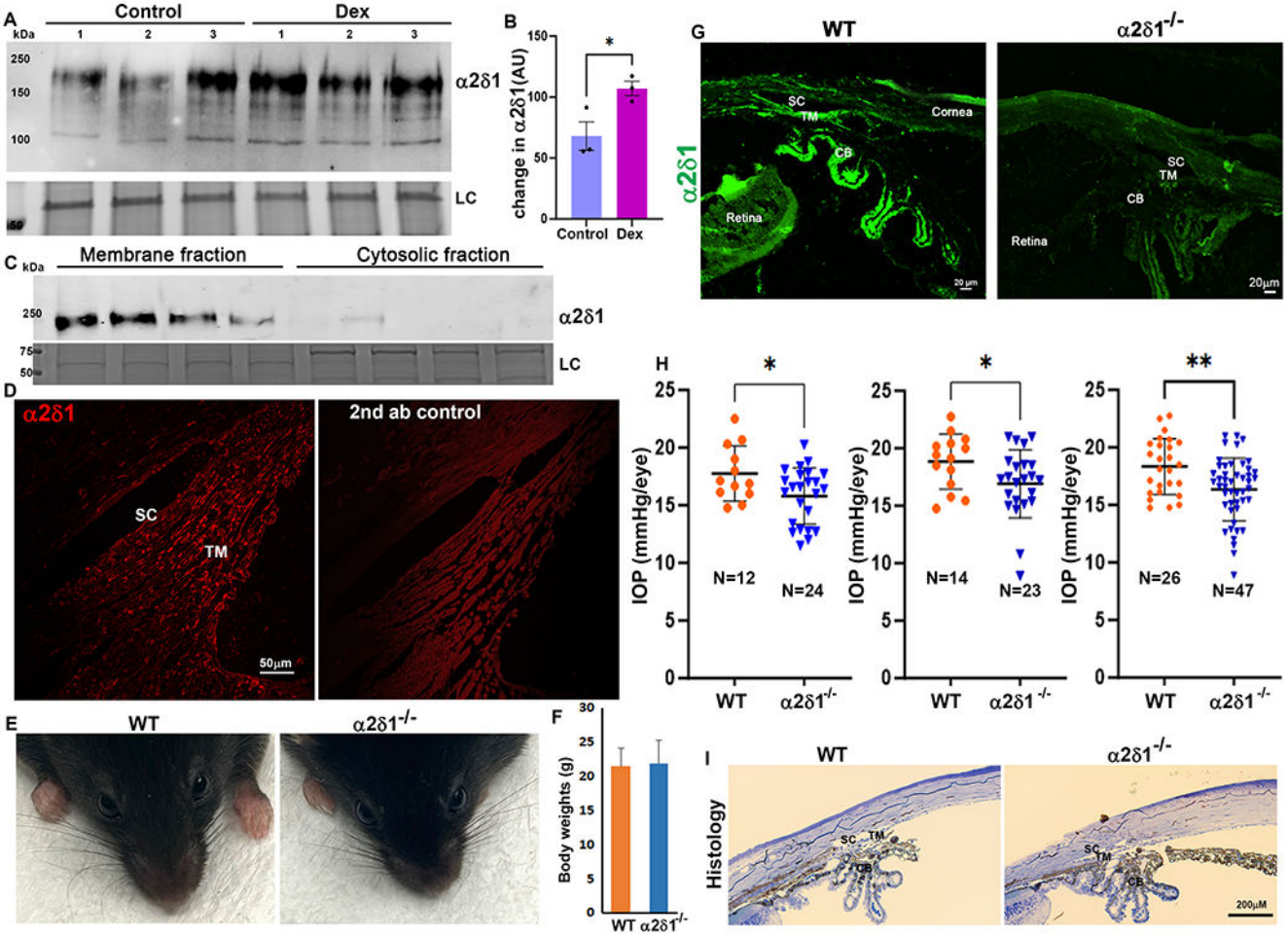

Figure. 1. Distribution of α2δ1 in human TM cells and tissue, and IOP changes in α2δ1 null mice.

A & B. The levels of α2δ1 significantly increased in the cytoskeletome fraction of human TM cells (three different strains) following treatment with dexamethasone (Dex, 0.5μM; 7 days) compared to control cells treated with ethanol (vehicle) ((Maddala et al., 2023). This increase was statistically significant ($P<0.05$, N=3). Loading control (LC) was used for normalization, employing equal protein amounts from cytoskeletal fractions separated via sodium dodecyl-sulfate polyacrylamide gel electrophoresis and stained with Coomassie blue. **C.** Immunoblot analysis confirmed the presence of α2δ1 primarily in the membrane-enriched fraction of human TM cells (duplicates of two independent strains) using equal protein amounts derived from the 100,000xg supernatant (soluble) and pellet (membrane-enriched) fractions. LC was employed as described above. **D.** Immunofluorescence analysis revealed the distribution of α2δ1 in human TM and Schlemm's canal (SC) compared to a control specimen (no primary antibody). Red fluorescence shows α2δ1 localization. Image magnification is indicated by the bar. **E & F**. α2δ1 knockout ($^{-/-}$, n=12) mice exhibited a normal ocular phenotype and body weight in comparison to respective littermate wild type control mice (n=7). Values are presented as mean ± SEM. **G.** Validation of α2δ1$^{-/-}$ mice not expressing α2δ1 in comparison with littermate wild type mice based on

immunofluorescence analysis. **H.** $\alpha 2\delta 1^{-/-}$ mice displayed a significant but modest decrease in intraocular pressure (IOP) compared to littermate wild type control (WT) mice. Repeated recordings of IOP changes over one-week intervals (Left and middle panels) in the same mice demonstrated significance ($P<0.04$ and $P<0.03$, respectively). The right panel depicts the combined IOP changes over the first and second week in $\alpha 2\delta 1^{-/-}$ mice compared to their respective control mice. * $P<0.05$, ** $P<0.005$), N= sample number, mean ± SD). **I**. Representative histological images from two independent eyes and quadrants illustrate the iridocorneal angle, encompassing TM & SC in WT and $\alpha 2\delta 1^{-/-}$ mice, based on light microscope imaging. The bars indicate image magnification. WT denotes littermate wild type control; −/− represents knockout. CB; ciliary body, TM; trabecular meshwork, SC: Schlemm's canal.

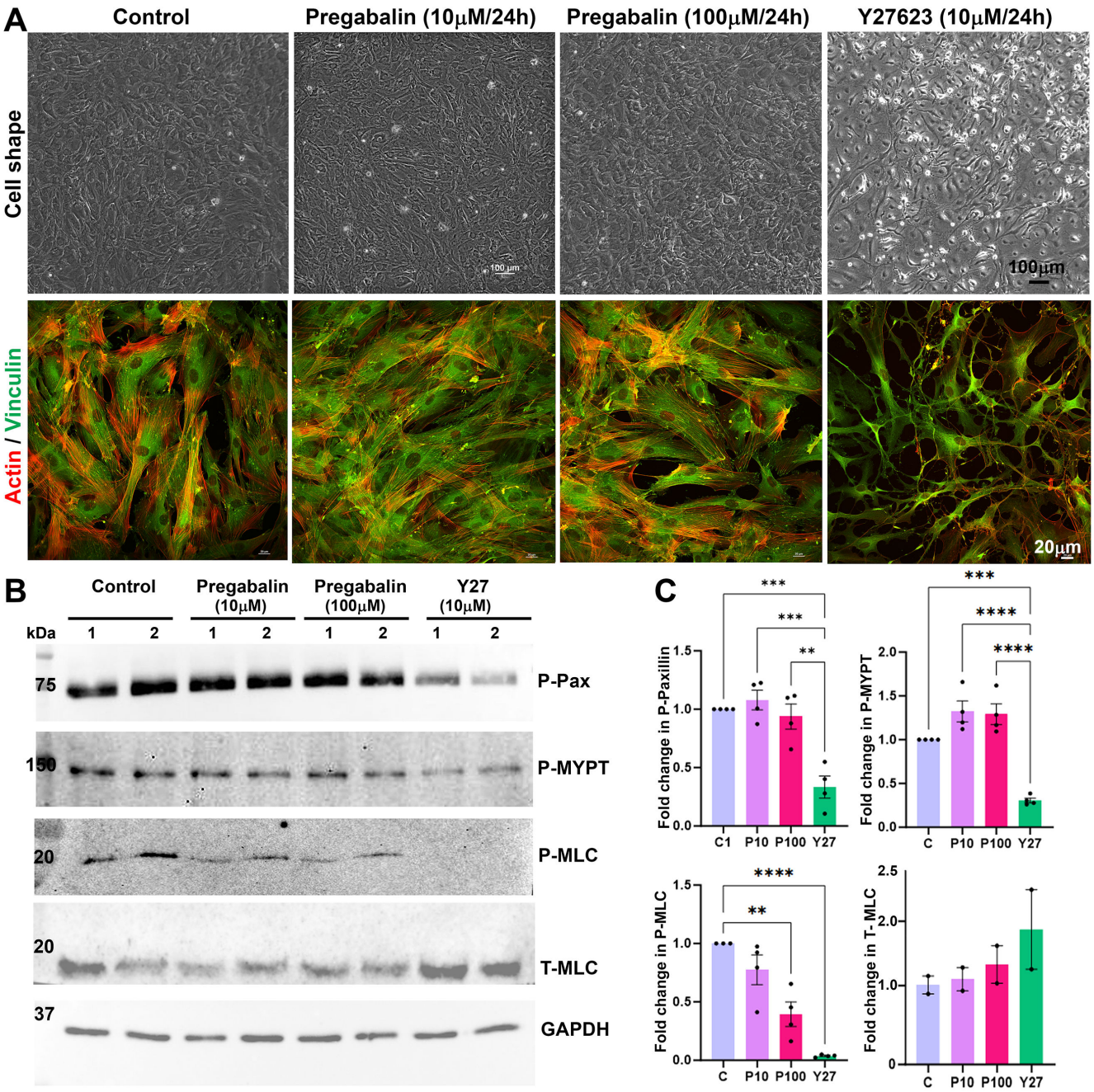

Figure 2. Effects of Pregabalin and Rho Kinase Inhibitor on Human TM Cell Morphology, Actin Stress Fibers, and Focal Adhesions

A. Human TM cells were cultured on gelatin-coated glass coverslips in 2% fetal bovine serum and treated with either pregabalin (10 & 100 μM) for 24 hrs or the Rho kinase inhibitor Y27632 (10 μM) for the same duration. Pregabalin-treated cells exhibited normal cell morphology, akin to control cells. In contrast, cells treated with the Rho kinase inhibitor displayed pronounced changes in cell shape, appearing rounded and filamentous based on phase contrast imaging, markedly distinct from both pregabalin-treated and control cells. Confocal microscopy using a Nikon Eclipse 90i revealed that Rho kinase inhibitor-

treated cells exhibited a significant reduction in actin stress fibers (Rhodamine phalloidin fluorescence) and focal adhesions (Vinculin immunofluorescence), whereas pregabalin-treated cells showed no discernible differences compared to control cells. Representative images comparing the effects of drug-treated samples against their respective controls are depicted. Scale bars indicate image magnification. **B** and **C**. Analysis under the aforementioned treatment conditions demonstrated a significant decrease in phosphorylated-paxillin, p-MYPT1, and p-MLC levels in Y27632-treated TM cells compared to both pregabalin-treated and control cells. Conversely, no significant differences were observed between pregabalin-treated and control cells, except for a notable reduction in p-MLC levels in cells treated with 100 μM pregabalin compared to control cells. Total MLC levels remained consistent with control cells in both pregabalin- and Y27632-treated TM cells. Statistical analyses were conducted using a Šídák's multiple comparisons test in GraphPad prism (version 10.1.2) software, presenting values as mean ± SEM. ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was immunoblotted as a loading control.