

Article

Doxycycline and Minocycline act as Positive Allosteric Modulators of the PAC1 Receptor and Induce Plasminogen Activators in RT4 Schwann Cells

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Featured Application: Targeting the PAC1-receptor has the potential to exert neuroprotective effects against a range of neurotoxic, neurodegenerative and inflammatory insults. The novel discovery of doxycycline and minocycline to act as positive allosteric modulators of the PAC1 receptor provides an exciting opportunity to therapeutically target this receptor. This study provides evidence for the ability of these tetracyclines to promote Schwann cells activities associated with axonal regeneration and neuroprotection through the induction of a PAC1-mediated proteolytic activity of tPA and uPA.

Abstract: Regeneration of peripheral nerves depends on the ability of axons to navigate through an altered extracellular environment. It has been suggested that Schwann cells facilitate this process through their secretion of neuropeptides and proteases. Using the RT4-D6P2T Schwann cell line (RT4), we have previously shown that RT4 cultures endogenously express the neuropeptide PACAP, and respond to exogenous stimulation by inducing the expression of tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) through PAC1 receptor activation. In this study, based on recent findings showing that doxycycline and minocycline act as positive allosteric modulators (PAMs) of the PAC1 receptor, we tested if treatment with these tetracyclines could induce the expression and activity of tPA and uPA in RT4 cells. Using ELISA and zymographic analyses, we demonstrate that doxycycline and minocycline reliably induce the secretion and activity of both tPA and uPA, which is paralleled by an increased expression levels, as shown by immunocytochemistry and Western blots. These actions were mediated, at least in part, by the PAC1 receptor, as PACAP6-38 mitigated tetracycline-induced expression and activity of tPA and uPA. We conclude that doxycycline and minocycline can act as PAMs of the PAC1 receptor to promote proteolytic activity in RT4 cells.

Keywords: pituitary adenylate cyclase-activating peptide (PACAP); PAC1 receptor; doxycycline; minocycline; positive allosteric modulator; Schwann cells, tissue plasminogen activator (tPA); urokinase plasminogen activator (uPA)

1. Introduction

Peripheral nerve injury can arise from systemic diseases or localised damage; however, unlike the central nervous system (CNS), the peripheral nervous system (PNS) displays a high regenerative capacity [1]. This is mainly driven by Schwann cells, the myelinating glia of the PNS that display high plasticity following peripheral nerve injury. Upon nerve injury, Schwann cells spontaneously reprogram to switch from a myelinating phenotype to a progenitor-like state that promotes nerve regeneration [2]. Part of this process includes the secretion of proteases by , like plasminogen activators, whose

proteolytic activities clear any residual cell debris and altered extracellular matrix, creating a clear nerve conduit to allow axon regeneration [3, 4]. Two plasminogen activators, the tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), are involved in this process [3].

There is evidence that the neuropeptide pituitary adenylate cyclase-activating peptide (PACAP) is also involved in this regeneration process [5, 6]. PACAP is widely distributed throughout the central and peripheral nervous systems and is associated with a myriad of biological functions including anti-apoptotic, neuroprotective and anti-inflammatory actions [7-10]. The activity of PACAP is mediated by three G protein-coupled receptors (GPCRs) PAC1, VPAC1 and VPAC2. However, PACAP binds with higher affinity (>100-fold) to the PAC1 receptor [11]. As such, the PACAP preferring receptor PAC1 is thought to mediate most of the anti-apoptotic and neuroprotective effects of PACAP. Based on these findings, the PACAP/PAC1 axis has been investigated as a potential target for neurological diseases, including Alzheimer's disease [12], stroke [13], headaches [14] and other neurological disorders (reviewed in [15-18]).

Emerging studies suggest that the PAC1 receptor might assist in the regeneration and functional recovery following peripheral nerve injury, as it has been shown that both PACAP and PAC1 are upregulated in response to peripheral nerve injury [19]. Moreover, it has been demonstrated that axon regeneration is strongly reduced in PACAP knockout animals [20]. *In vitro*, we have previously demonstrated that RT4 Schwann-cell like cultures express PACAP and PAC1 and stimulation of these cells with PACAP promotes tPA and uPA expression and activity [21, 22]. Additionally, we demonstrated that BDNF mimicked the PACAP-induced upregulation of tPA expression and activity in these cell lines [22].

Recently, it has been shown that doxycycline and minocycline act as positive allosteric modulators (PAMs) of the PAC1 receptor [23]. Doxycycline and minocycline are tetracyclines, broad spectrum antibiotics that share similar structures and functions [24]. Both tetracyclines have shown promising potential as therapeutic targets for neurological diseases due to their high lipophilic nature that allows them to readily cross the blood brain barrier and other biological membranes [25]. Both doxycycline and minocycline have been shown to exert neuroprotection and anti-inflammatory activities in a range of neurological pathologies, similarly to PACAP [25-29]. Most importantly, it has been shown that the neuroprotective activity of doxycycline is enhanced by the activation of the PAC1 receptor [30].

In the present study we investigated the novel role of doxycycline and minocycline as potential PAMs of PAC1 receptor. To achieve this goal, we tested if exogenous administration of either tetracycline would induce tPA and/or uPA expression and activity of tPA and uPA in a similar way as exogenous PACAP. In addition, we assessed if PAC1 receptor blockade prevented these effects in RT4 Schwann cell-like cultures.

2. Materials and Methods

2.1 RT4 Schwann Cell Line

This study used the rat Schwann cell line RT4-D6P2T (ATCC number CRL-2768) obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich, Castle Hill, NSW, Australia). Cells were incubated at 37°C in a humidified environment with 5% CO₂. Cells were grown to 80-85% confluence and treated as indicated in the corresponding experiments.

2.2 ELISA tPA and uPA activity

The concentrations of uPA and tPA were determined by Enzyme Linked Immune Sorbent Assay (ELISA) commercial kits (Abcam, Cambridge, MA, USA; tPA [ab198510] and uPA [ab108917]) according to the manufacturer's instructions. RT4 cells were either left untreated (controls) or treated with increasing concentrations of either doxycycline or minocycline (10, 50, 100, 500 and 1000 ng/mL) and tPA and uPA levels assessed 24 hours later. Each condition was tested using five different batches of cells (n=5/group).

2.2 Cell viability assay

To assess cell viability, we used the cell proliferation kit I (Sigma-Aldrich, NSW, Australia). Cells were treated with increasing doses of either doxycycline or minocycline for 24 h as above. 10 μ L of MTT labelling reagent was added to each well and incubated for 4 h. 100 μ L of solubilisation solution was added to each well and incubated overnight at 37°C. Absorbance was measured at 565 nm in the TECAN infinite M1000-PRO ELISA reader (ThermoFisher Scientific, Victoria, Australia).

2.3 Zymography of tPA and uPA activity

The activity of tPA and uPA was determined by zymographic analysis using a procedure previously published [22, 31]. Briefly, following indicated treatment, cells were collected with 1% Triton X-100 in PBS buffer. Equal amounts of protein were loaded onto a 12% polyacrylamide gel containing 2mg/ml casein in the presence of 5 μ g/ml plasminogen. After electrophoresis, enzyme reaction was initiated by incubating the gel in 0.1 M glycine-NaOH (pH 8.3) at 37°C for 18 h, and lytic areas were identified after staining the gel with a solution of 30% methanol, 10% glacial acetic acid and 0.5% Coomassie blue G250. The gels were then destained and scanned with Bio-Rad Imaging system. Images were assessed semi quantitatively using ImageJ software. To exclude interference by matrix metalloproteinase, EDTA (2 mM) was included in the glycine-NaOH buffer during incubation. Gels that did not containing plasminogen were also run and failed to produce lytic areas corresponding to plasminogen activators (data not shown). Bands were identified based on their relative molecular weight.

2.4 Immunocytochemistry

To determine the expression of tPA in RT4 cells, immunocytochemistry was performed. Briefly, 1×10^4 cells were seeded in poly-L-lysine (Sigma-Aldrich, Castle Hill, NSW, Australia) pre-coated tissue culture coverslips (22mm \varnothing , Sarstedt, SA, Australia). Cells were fixed with 4% filtered paraformaldehyde (PFA: 4% in PBS pH 7.4) (Sigma-Aldrich, Castle Hill, NSW, Australia). Coverslips were then washed three times with ice cold PBS. Cell were then permeabilised for 10 minutes in PBS containing 0.25% Triton X-100 (Sigma-Aldrich, Castle Hill, NSW, Australia), followed 3 \times washes in PBS for 5 minutes. Thereafter, cells were soaked in 1.5% H₂O₂ / PBS solution for 15 min to quench endogenous peroxidases. Non-specific binding of antibodies was then prevented by incubating coverslips with 1% BSA in PBST for 30 minutes. Once completed, the cells were incubated in diluted rabbit anti-tPA primary antibody (Abcam, Cambridge, MA, USA [ab157469]; diluted 1:500 in PBST and 1% BSA) in a humidified chamber overnight at 4°C. The next day, the primary antibody was removed with 3 \times washes in PBS for 5 minutes. Cells were then incubated with secondary antibodies (goat anti-rabbit IgG H&L (HRP) [ab97051] at 1/5000 dilution) in 1% BSA in PBST for 2 h at room temperature with gentle oscillation. Secondary antibody solution was then removed, and cells were washed again three times with PBS for 5 minutes. A drop streptavidin peroxidase was then added to each well (containing the coverslip) and allowed to incubate for 30 min at room temperature, followed by 3 \times 5 min washes in PBST on an orbital shaker. 3,3'-Diaminobenzidine (DAB) substrate (Sigma-Aldrich, Castle Hill, NSW, Australia) was added to each cell coverslip under a fume hood. Once the cells started turning brown, the reaction was stopped with 2 \times washes

in PBS for 5 minutes each on a shaker. Counterstaining of nuclei was performed by dipping each coverslip into a staining dish of hematoxylin for 10 sec, followed by an acid bath (200 mL UltraPure water and 1–3 drops of acetic acid). Stained coverslips were then imaged using a Nikon Eclipse TS2 inverted microscope (magnification 20×). Scale bar is 60µm.

2.5 Western blot

Protein lysates were homogenised in RIPA buffer (Sigma-Aldrich, St. Louis, MO), which was supplemented with cOmplete™ULTRA protease inhibitor cocktail (Roche Life Science, North Ryde, NSW). Lysates were then sonicated twice at 50% power for 10 sec using an ultrasonic probe, followed by centrifugation prior to protein quantification. Protein concentrations were determined using the Bicinchoninic Acid (BCA) Assay Kit (ThermoFisher Scientific, Waltham, MA).

Lysates (20µg) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) using 4-20% Mini-PROTEAN® TGX Stain-Free™ protein gels (15 wells) (Bio-Rad, Hercules, CA). Samples were prepared by 3.75µl of 4× Laemmli buffer (Bio-Rad, Hercules, CA) and β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) mix (Laemmli buffer 20:1 with β-mercaptoethanol) to a final volume of 15µl. Samples were then denatured at 70°C for 10min using the T100™ thermal cycler.

Samples were resolved on gels and transferred to a PVDF membrane using the semi-dry approach (Trans-Blot Turbo™ Transfer Pack; Bio-Rad, Hercules, CA). Once proteins were transferred, the membrane was removed from the apparatus and briefly washed in tris-buffered saline containing Tween®20 (TBST) 1× three times before blocking with 5% blocking buffer containing 5% skim milk in TBST 1× for 1h at room temperature at 55-60rpm. The membranes were probed with anti-rabbit primary antibodies recognizing either tPA (1:1000; GeneTex, Irvine, CA, USA; cat. No. GTX103453), uPA (1:750; LifeSpan Biosciences Seattle, WA, USA; cat. No. LS-C193095) or GAPDH (1:1000; BioRad, Hercules, CA; cat. No. VPA00187) (used as loading control) overnight at 4°C under gentle oscillation (55rpm). Following incubation, membranes were washed thoroughly. Finally, membranes were incubated with a goat anti-rabbit secondary antibody (1:10000; BioRad, Hercules, CA; cat. No. STAR208P) for 1h at room temperature and washed again before imaging. To visualize immunoreactive bands, we used Clarity™ Western ECL Blotting Substrate (Bio-Rad, Hercules, CA). Images were acquired on the ChemiDoc™ MP System (Bio-Rad, Hercules, CA). Band intensities were quantified using ImageJ software and values were normalized to GAPDH.

2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7.04 for Windows, GraphPad Software, San Diego California, USA, www.graphpad.com. All experimental data are reported as mean ± S.E.M. To assess statistical differences between three or more groups we used one-way analysis of variance (ANOVA) followed by Tukey *post-hoc* test, unless otherwise stated. P-values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Dose-response effects of doxycycline and minocycline on tPA and uPA secretion in RT4 cells

To investigate if doxycycline and minocycline were able to stimulate the secretion of tPA and uPA by RT4 Schwann-cell like cultures, we measured the amount of plasminogen activators released in culture media by enzyme-like immunosorbent assay (ELISA) after treatment with either antibiotics for 24 h. We utilized increasing concentrations of doxycycline and minocycline (0, 10, 50, 100, 500 and 1000ng/ml) to establish the optimal concentration to use in subsequent testing (**Fig. 1**). PACAP was used as a positive control in all experiments, which significantly increased tPA (**p<0.001 Vs Control) and uPA (*p<0.05

and $**p<0.01$) levels in culture media. We have already reported a dose- and time-dependent increase in plasminogen activators expression and activity by PACAP in RT4 cells in earlier studies [22].

Experimental data demonstrated that both doxycycline and minocycline dose-dependently increased the levels of secreted tPA and uPA in the supernatant of RT4 cells, although uPA induction was less remarkable than tPA (Fig. 1a-b and a'-b', respectively). Doxycycline reliably increased tPA secretion at 50ng/ml ($*p<0.05$ Vs Control, Fig. 1a), however, 100ng/ml doxycycline was required to significantly increase uPA levels as well ($**p<0.01$ Vs Control, Fig. 1a').

Minocycline treatment significantly increased tPA ($**p<0.01$ Vs Control, Fig. 1b) and uPA levels ($*p<0.05$ Vs Control, Fig. 1b') in the culture media at 50ng/ml; however, similarly to doxycycline, minocycline also peaked its activity at 100ng/ml ($**p<0.01$ Vs Control for both tPA and uPA). Accordingly, we identified 100ng/ml as the most effective concentration of both doxycycline and minocycline to use in subsequent experiments.

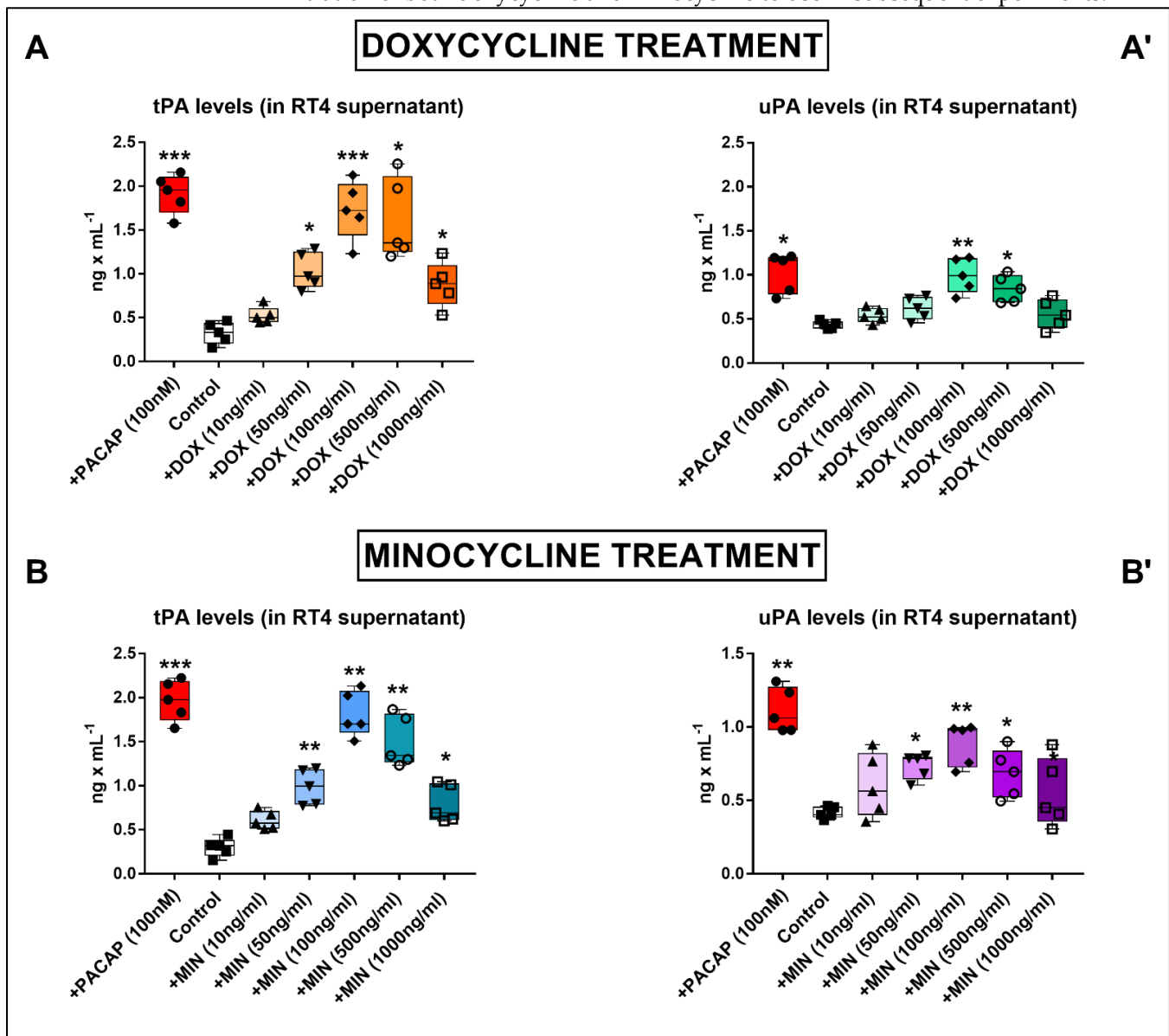


Fig. 1. Dose-response effect of doxycycline and minocycline on tPA and uPA secretion in RT4 cells. (A and B) Levels of secreted tPA or uPA in the supernatant of RT4 cells treated with either doxycycline (A and A') or minocycline (B and B'). RT4 cells were treated with the indicated treatments for 24 h. Cell supernatants were harvested and the concentration of tPA and uPA were determined by ELISA. Data shown is the result of two independent experiments using five different batches of cells (n=5). $*p<0.05$, $**p<0.01$ or $***p<0.001$, as determined by ANOVA followed by Dunnett's *post-hoc* test.

3.2. Effects of doxycycline or minocycline treatment on RT4 cell viability

To investigate if doxycycline or minocycline were toxic to cells, we treated RT4 cells with increasing concentrations of either doxycycline or minocycline and assessed viability using the MTT assay. At the concentrations tested, neither doxycycline nor minocycline were toxic to RT4 cells, as cells displayed normal growth rate up to 100ng/ml concentration (**** $p < 0.0001$ Vs Ctrl for both doxycycline and minocycline), to then plateau at 500ng/ml (**** $p < 0.0001$ Vs Ctrl for both) and finally record a slight decline at the highest concentration tested (1000ng/ml, ** $p < 0.01$ Vs Ctrl for both, Fig. 2a and b).

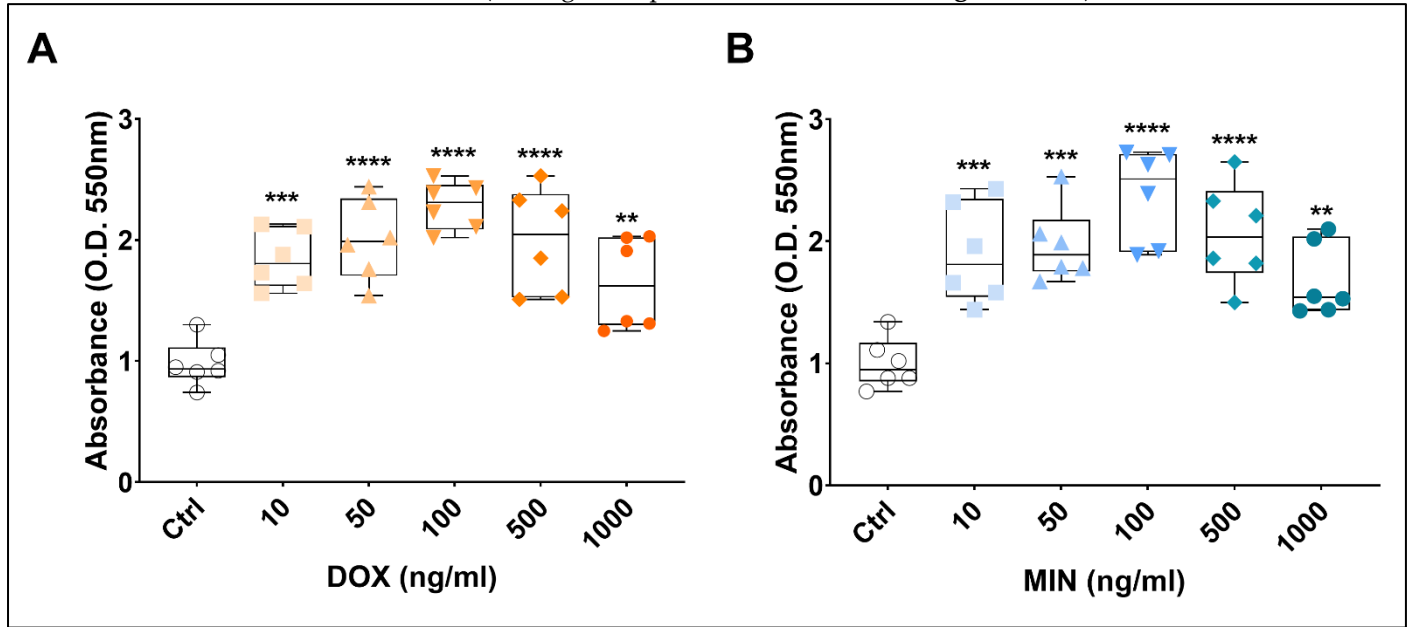


Figure 2. Cell viability of RT4 cells exposed to increasing concentrations of doxycycline or minocycline. Analysis of cell viability (MTT assay). RT4 cells were grown under normal conditions and exposed to increasing concentrations (0, 50, 100, 500 or 1000ng/ml) of (A) doxycycline or (B) minocycline for 24 h. Values are reported as mean optical densities (OD) \pm S.E.M. from two experiments, each using five batches of cells. ** $p < 0.01$, *** $p < 0.001$ or **** $p < 0.0001$, as determined by ANOVA followed by Dunnett's *post-hoc* test.

3.3. Enzymatic activity of tPA and uPA in RT4 cells exposed to doxycycline or minocycline

Our next step was to establish if doxycycline or minocycline stimulated tPA and uPA enzymatic activity in RT4 cells using zymography. We used exogenous PACAP as our positive control [22]. RT4 cells were treated with either PACAP (100nM), doxycycline or minocycline (100ng/ml) for 24 h.

Quantification of both tPA and uPA lytic bands on gels (white bands corresponding to 70kDa [tPA] and 50kDa [uPA], Fig. 3a) confirmed that exogenous PACAP increases the activity of both plasminogen activators (** $p < 0.01$ Vs Control). Both doxycycline and minocycline treatments significantly increased both tPA (** $p < 0.01$ and *** $p < 0.001$ Vs Control, respectively, Fig. 3b and c) and uPA activity (* $p < 0.05$ Vs Control for both, Fig. 3b and c).

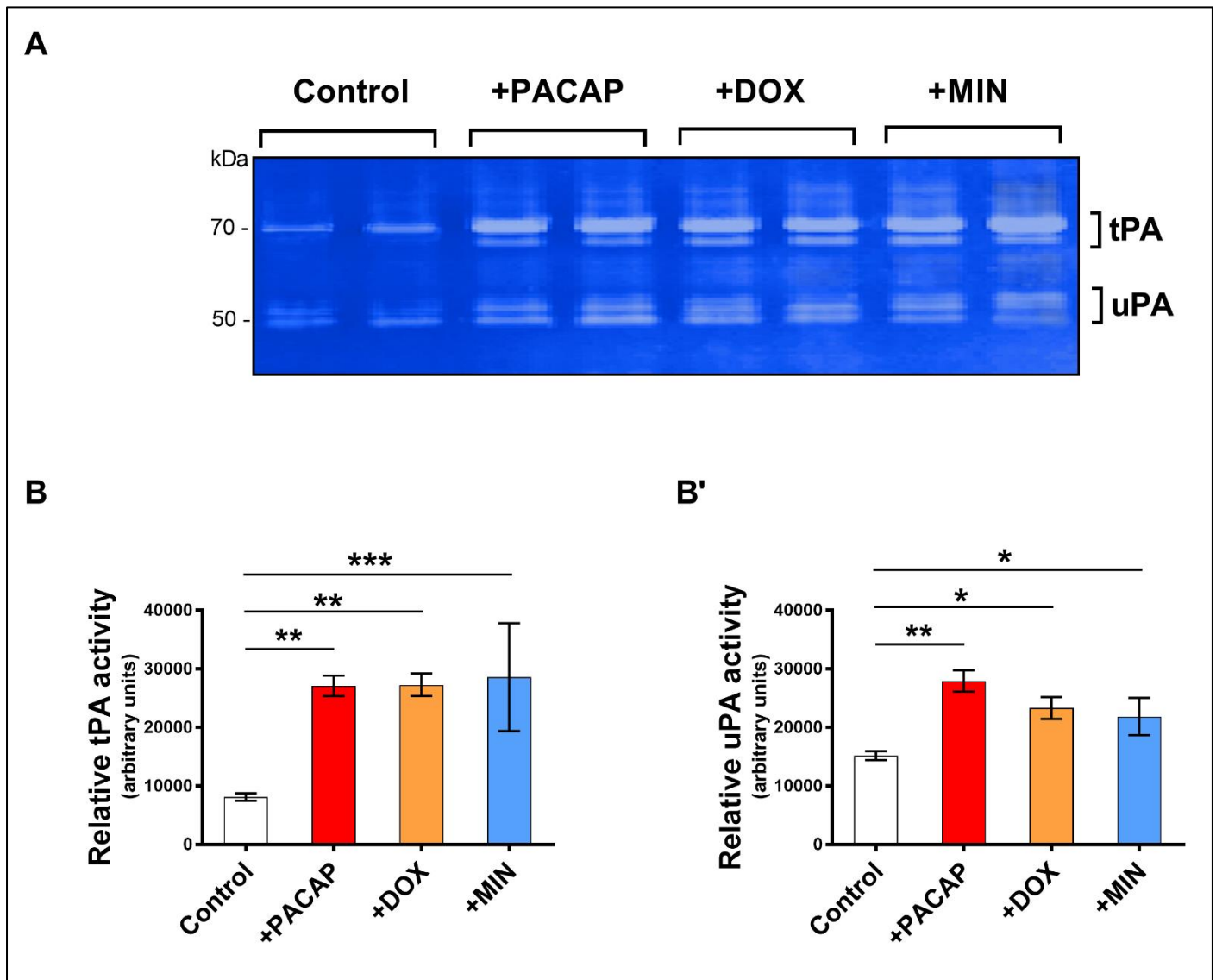


Figure 3. Zymographic assessment of tPA and uPA activity of RT4 cells in response to PACAP, doxycycline or minocycline. (A) Representative zymogram and densitometric analyses showing the relative levels of (B) tPA and (B') after treatment with PACAP, doxycycline or minocycline for 24 h. Values reported (arbitrary units) are the mean \pm S.E.M. of two separate experiments, each run using distinct batches of cells ($n=4$ /group), measured using ImageJ software. * $p<0.05$, ** $p<0.01$ or *** $p<0.001$, as determined by ANOVA followed by Dunnett's *post-hoc* test.

3.4. tPA-like immunoreactivity in RT4 cells exposed to PACAP, doxycycline or minocycline

We have previously demonstrated that treatment with PACAP or the PAC1 agonist maxadilan did not affect the distribution but only the expression of tPA in RT4 cells [22]. As such, we performed immunocytochemistry to determine if, similarly to PACAP, doxycycline or minocycline treatment increased the expression but not the cellular distribution of either plasminogen activators. Sections where the primary antibody was omitted were used as negative controls (Fig. 4a). Cells were either left untreated (Fig. 4b) or exposed to PACAP (Fig. 4c), doxycycline (Fig. 4d) or minocycline (Fig. 4e) for 24 h.

tPA-like immunoreactivity (IR) was moderate (about 25% of cell surface area) and mainly cytoplasmic in untreated cells (Fig. 4b). As expected, administration of PACAP significantly increased the expression of tPA-IR (*** $p<0.001$ Vs Control, >40% cell surface area, Fig. 4c). Similarly to PACAP, both doxycycline and minocycline reliably increased

the percentage of tPA⁺ IR compared with controls (** $p < 0.01$ Vs control, about 35% of cell surface area, Fig. 4d and e).

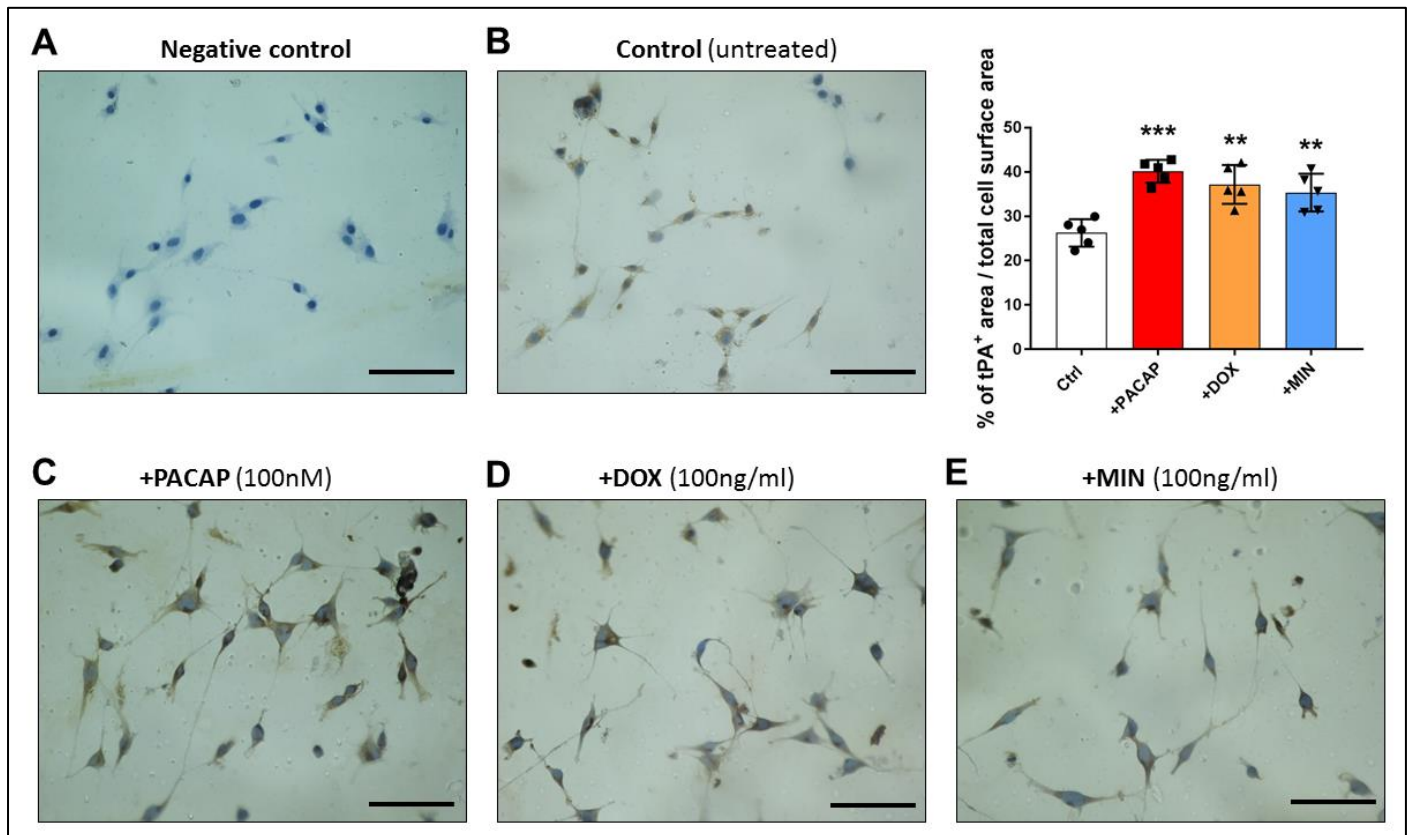


Figure 4. Immunocytochemistry of tPA in RT4 cells exposed to PACAP, doxycycline or minocycline. Representative cytochemistry images showing tPA immunoreactivity in (a) negative control (no primary Ab), (b) untreated, (c) PACAP treated, (d) doxycycline treated and (e) minocycline treated RT4 cells. Values are reported as percent tPA positive cell surface area normalized to total cell surface area \pm S.E.M. ** $p < 0.01$, *** $p < 0.001$ or **** $p < 0.0001$, as determined by ANOVA followed by Dunnett's *post-hoc* test. Magnification = 20 \times . Scale bar = 60 μ m.

3.5. Pre-treatment with PACAP-38 blocks doxycycline- and minocycline-induced tPA activity

To assess if doxycycline- and minocycline-induced tPA and uPA activities were related to PAC1 receptor activation, RT4 cells pre-treated (or not) with PACAP6-38 (a PAC1 receptor antagonist, 10 μ M) were treated cells with PACAP (100nM), doxycycline (100ng/ml) or minocycline (100ng/ml) for 24h and assess via zymography.

As shown, lytic areas on gels showed a significant increase in tPA and uPA activities after treatment with PACAP (** $p < 0.01$ Vs Control for both tPA and uPA), doxycycline (* $p < 0.05$ for both tPA and uPA) and minocycline (** $p < 0.01$ for tPA and * $p < 0.05$ for uPA) (Fig. 5a, b and b'). Pre-treatment with PACAP6-38 completely prevented PACAP-induced tPA or uPA activities (\$ $p < 0.01$ Vs PACAP-treated cells). In cells treated with doxycycline or minocycline, PACAP6-38 also significantly reduced tPA (\$ $p < 0.05$ Vs doxycycline or minocycline treated cells, respectively) and uPA (\$ $p < 0.05$ Vs doxycycline-treated and \$ $p < 0.01$ Vs minocycline treated cells) (Fig. 5a, b and b').

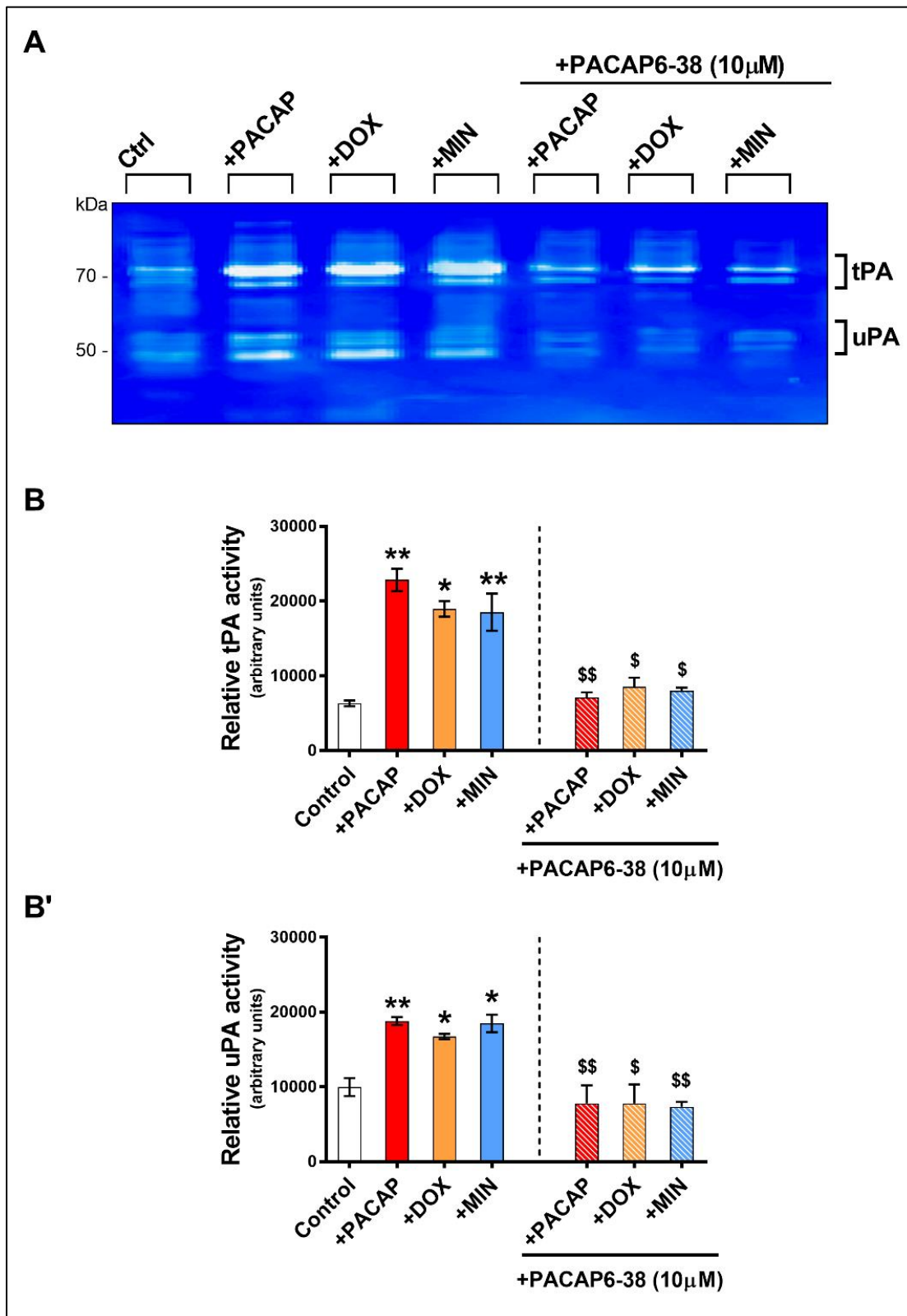


Figure 5. Zymographic assessment of tPA and uPA activity of RT4 cells in response to PACAP, doxycycline or minocycline with and without PACAP6-38 pretreatment. (A) Representative zymogram and densitometric analyses showing the relative levels of tPA (B) and uPA (B'). Cells were treated with either PACAP, doxycycline or minocycline only or pre-treated for 1h with PACAP6-38 and then assessed after 24h. Values reported (arbitrary units) are the mean \pm S.E.M. of two separate experiments, each run using four separate batches of cells ($n=4$ /group), measured using ImageJ software. * $p<0.05$ or ** $p<0.01$ Vs Control; \$ $p<0.05$ or \$\$ $p<0.01$ Vs corresponding drug-treatment groups, as determined by ANOVA followed by Dunnett's *post-hoc* test.

3.6. Pre-treatment with PACAP-38 blocks doxycycline- and minocycline-induced tPA and uPA protein expression levels

To evaluate whether PACAP6-38 induced reduction in plasminogen activators activities were also associated with a reduction in protein expression, we performed Western blot analyses in RT4 cells treated as per zymographic analyses.

Significant increases in tPA protein expression were observed both in PACAP- (* $p < 0.05$ Vs Ctrl), doxycycline- or minocycline-treated cells (* $p < 0.05$ for both, **Fig. 6a** and **a'**).

Pre-treatment with PACAP6-38 prevented the induction of tPA expression by PACAP (\$ $p < 0.01$ Vs PACAP-treated) but also by doxycycline (\$\$\$ $p < 0.001$ Vs doxycycline-treated) and minocycline (\$\$ $p < 0.01$ Vs minocycline-treated) treated RT4 cells. Similarly, PACAP or tetracyclines'-induced uPA protein expression levels were prevented by PACAP6-38 pre-treatment. Specifically, PACAP6-38 completely rescued the induction of uPA protein levels caused by PACAP or doxycycline treatment (\$\$ $p < 0.01$ Vs corresponding drug treatment, **Fig. 6b** and **b'**). However, we observed only a partial reduction of uPA protein expression in RT4 cells that were pre-treated with PACAP6-38 and then exposed to minocycline (\$ $p < 0.05$ Vs minocycline-treated, **Fig. 6b** and **b'**).

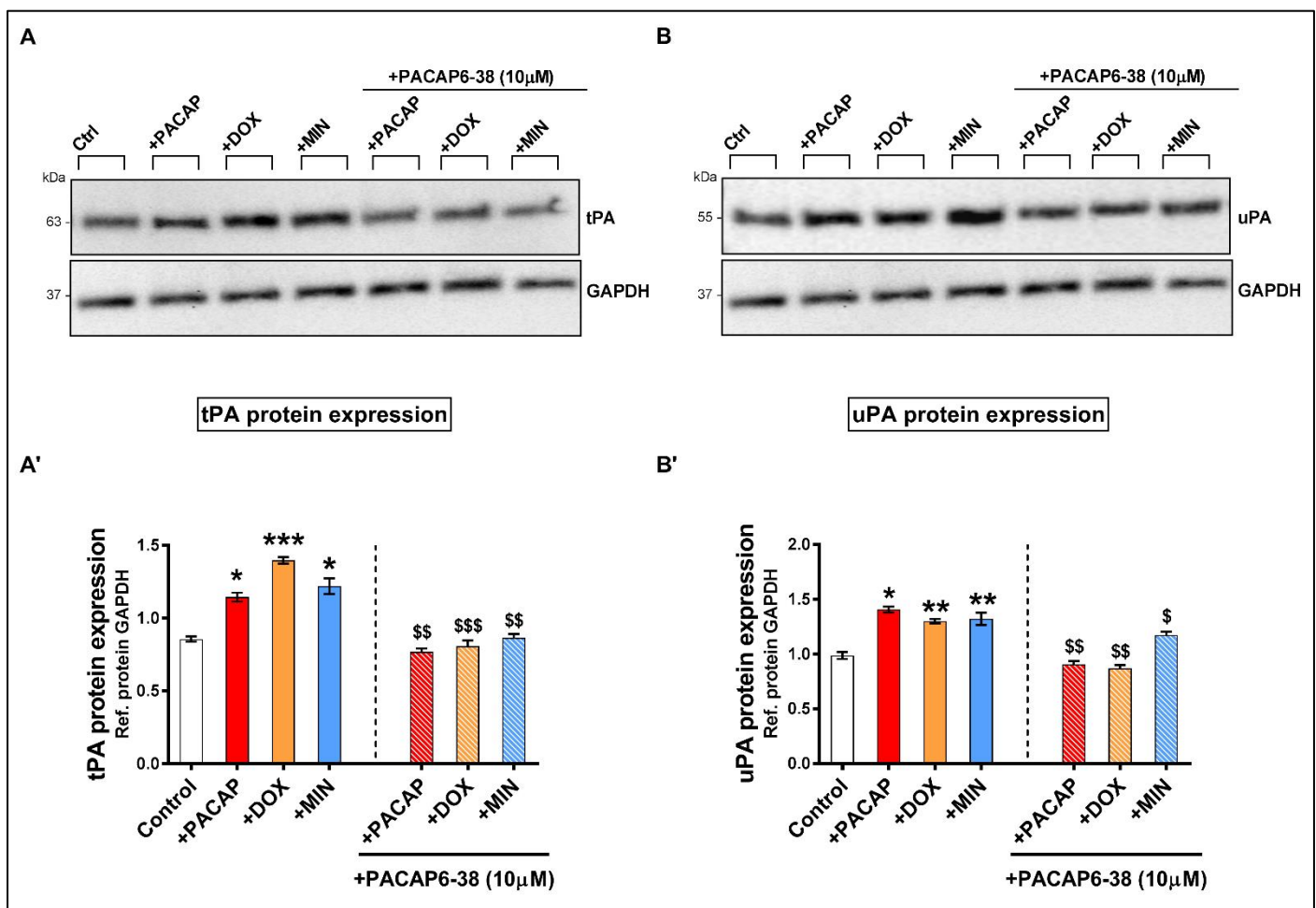


Figure 6. Protein expression of tPA and uPA in treated RT4 cells. Western blots and densitometric analyses of tPA (a & a') and uPA (b & b') in RT4 cells exposed to PACAP6-38 for 1h then treated with PACAP, doxycycline or minocycline for 24h. Total proteins were normalized to GAPDH, the loading control. Data represents means of $n = 4$ samples for each group. Results are expressed as mean \pm S.E.M. * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$, as determined by ANOVA followed by Dunnett's post-hoc test.

4. Discussion

In the present study, we report for the first time that doxycycline and minocycline induce both the expression and activity of the two plasminogen activators, tPA and uPA, in RT4 Schwann-cell like cultures. Furthermore, we linked this induction to PAC1 receptor activity, as pre-treatment with the PAC1 receptor antagonist, PACAP6-38, mitigated this response. As such, this study provided additional evidence to the novel finding that these tetracyclines may act as PAMs of the PAC1 receptor, first documented by Song and colleagues (2019) [23].

RT4 Schwann cells were utilized in this study. These immortalized Schwann cell lines share a similar transcriptional profile and biology with primary Schwann cells [32], making these cell lines a viable alternative to study PNS myelin-producing glia.

Our rationale to conduct this study was based on previous observations, in which we identified that both PACAP and the PAC1 receptors are endogenously expressed in RT4 Schwann cell lines [21]. Using these cells, we have shown that exogenous administration of PACAP up-regulates the expression and activity of tPA via the PAC1 receptor and that conditions able to increase the endogenous PACAP/PAC1 signaling (i.e. serum starvation) can also increase the activity of plasminogen activators [22]. In addition, we have also determined that inflammatory factors to mimic the local microenvironment of nerve injury (lipopolysaccharide, aka LPS) can up-regulate PACAP expression and concurrently down-regulate a direct repressor of plasminogen activators expression (miR-340) in RT4 cells [5], corroborating the idea that the crosstalk between the PACAP neuropeptide system and the plasminogen activator system may be a critical step in the cascade of events that are initiated within Schwann cells following nerve injury. These findings align with other studies showing that these cell-types undergo important biochemical alterations after a nerve injury that allow cells to secrete factors that promote neuronal survival and axonal regeneration [33].

In the RT4 cell line, PACAP up-regulates the expression of multiple myelin markers [34], an effect that suggests a broader involvement of the peptide in the nerve regeneration process after injury, summarized by two stages: (1) in which PACAP stimulates Schwann cells to secrete factors that promote debris clearance and axonal regrowth and (2) a second stage where PACAP promotes the accumulation of pro-myelinating factors by Schwann cells that will aid in remyelination once neurite regrowth is completed. This theory has been partly confirmed by recent evidence *in vivo*, where PACAP was found to promote myelin regeneration following sciatic nerve injury [6]. PACAP is significantly increased in peripheral neurons post-injury, and it is detectable at higher levels one month after nerve damage, suggesting it plays a key role in both axon regeneration and the late remyelination process [35]. Moreover, axon regeneration following injury is severely disrupted in PACAP knockout mice [20]. More recently, transcriptional profiling of the skin of patients undergoing surgery for carpal tunnel syndrome revealed that *ADCYAP1* (the gene encoding for PACAP) was the most robustly up-regulated and its expression was associated with nerve recovery. Additionally, when human induced pluripotent stem cell-derived sensory neurons were treated with PACAP, it enhanced axonal outgrowth in a dose dependent manner [36]. Together, these studies suggest a prominent role of PACAP induced PAC1 activity in mediating neuronal survival, axon regeneration and remyelination following peripheral nerve injury.

Targeting the PAC1 receptor has proven to be challenging [15]. Most studies have aimed to use the neuropeptide PACAP to stimulate PAC1 activity; nonetheless, as with most peptides, PACAP has limited bioavailability and is subject to rapid enzymatic degradation [37]. Despite being able to cross the blood brain barrier, albeit by passive diffusion or through the specific peptide transporter system 6 (PTS6), PACAP has an extremely short half-life [37]. In C57BL/6 mice, it was determined that the half-life of PACAP38 administered intravenously was less than two minutes [38]. Dipeptidyl peptidase IV (DPP IV) is the main enzyme involved in the degradation of PACAP, with mice lacking DPP IV showing significantly slower clearance of the peptide [38, 39]. Despite these challenges,

PACAP has a safe and wide therapeutic index and low doses of PACAP are still able to exert neuroprotection and other beneficial activities.

The recent discovery of doxycycline and minocycline as putative PAMs of the PAC1 receptor may provide an excellent opportunity to target the PAC1 receptor therapeutically. The two antibiotics have well-established neuroprotective and anti-inflammatory functions in the CNS [26, 40] and some evidence suggests that, at least minocycline, may aid in nerve regeneration in bio-artificial nerve grafts that are free from Wallerian degeneration [41]. However, a few studies have shown that antibiotic treatment can inhibit Wallerian regeneration [42], an essential process of nerve rejuvenation, whereas others don't [43]. Therefore, it is not clear if the balance between beneficial and detrimental outcomes of tetracycline's treatment can ultimately result in an improved nerve recovery or not. Our data suggests that the up-regulation of plasminogen activators in RT4 Schwann cells implicates doxycycline and minocycline as agents that can improve extracellular matrix/debris clearance and consequently, promote axonal regrowth. However, additional work is required to confirm such beneficial activities using primary Schwann cells, neuronal/glia co-cultures and/or *in vivo*, as the repurposing of this class of antibiotics could indeed become a game changer in the treatment of nerve injury.

5. Conclusions

In conclusion, we have confirmed that pharmacological blockade of the PAC1 receptors (as well as VPAC receptor subtypes) by PACAP6-38 prevents tetracycline-induced activation of plasminogen activators by RT4 Schwann cells. Whilst the study was not aimed at identifying the existence of a PAC1 binding site for doxycycline or minocycline, the biochemical evidence provided supports their the novel function as PAMs of the PAC1-receptor in RT4 Schwann cells.

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