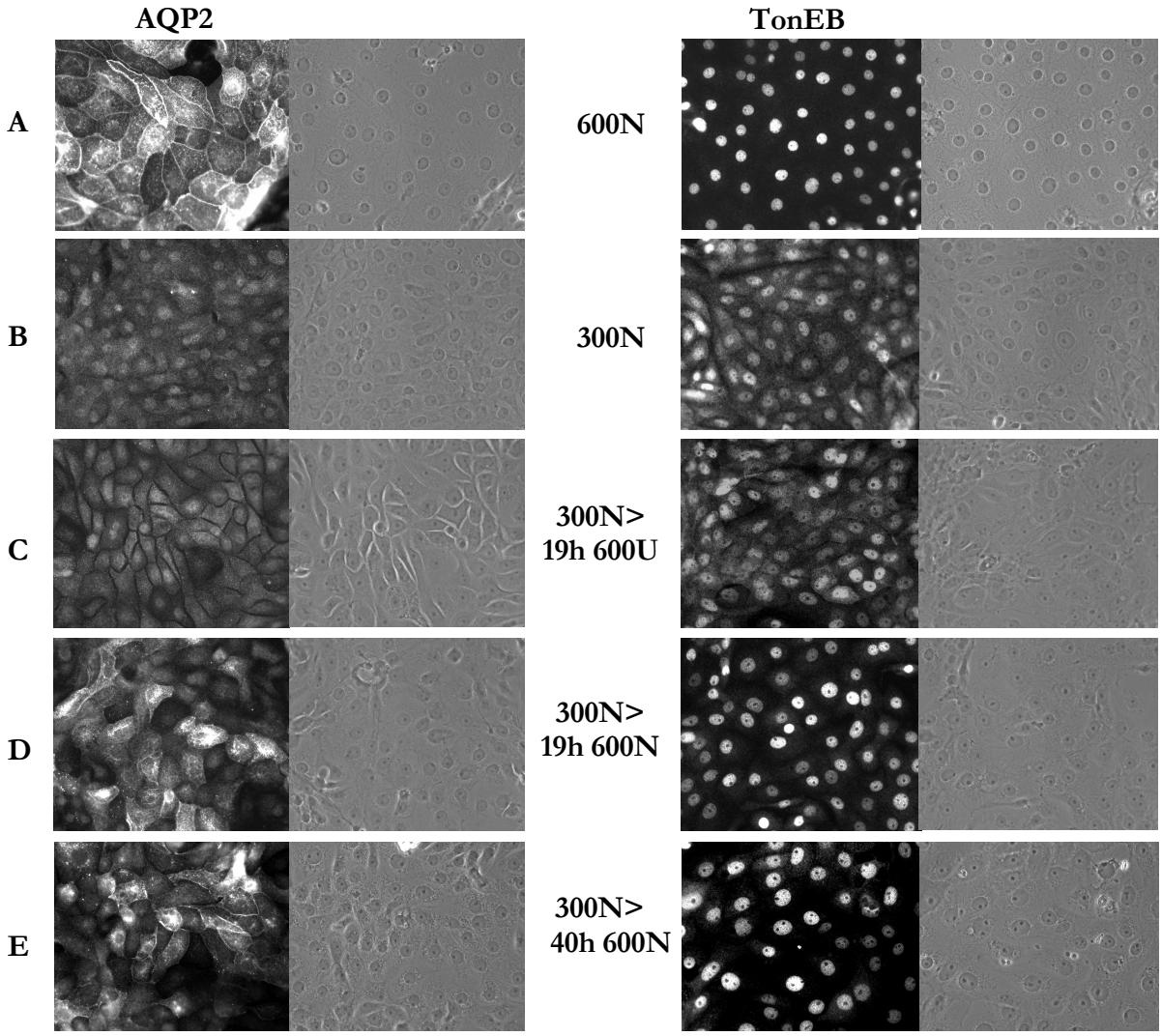

Fig. 21. Nuclear localization of TonEBP is induced by hypertonicity - not by hyperosmotic urea
IMCD cells were seeded in 600N medium, which was changed 24 h later to 300N medium (panels B-E) except for positive controls in panels A. 72h later, 300N medium was replaced by medium elevated to 600 mosmol/l (except for negative controls in panels B) by urea (600U, panels C), or an equimolar combination of sodium and urea (600N, panels D and E), for the consecutive 19 h (D) or 40 h (E) prior to fixation. All media contained 500 μM DBcAMP. *Left panels.* Visualization of AQP2 (*left side*) in IMCD cells (*right side*, light micrographs). *Right panels:* Visualization of TonEBP (*left side*) in IMCD cells (*right side*, light micrographs). AQP2 was detected with specific rabbit anti-AQP2 antiserum. TonEBP was detected with a specific rabbit anti-TonEBP antiserum (kindly provided by Drs. López-Rodríguez and Rao). Cy3-conjugated goat anti-rabbit antibodies were used as secondary antibodies.

The immunofluorescence data (Fig. 21) shows that the elevation of medium osmolality from 300 mosmol/l to 600 mosmol/l (600N) for 19 h induced a translocation of TonEBP from the cytosol to the nucleus. Elevation of medium osmolality by the membrane-permeating

compound urea (600U) failed to induce translocation of TonEBP to the nucleus and consecutive induction of AQP2 expression in IMCD cells. This indicates that sodium-derived tonicity is the signal for TonEBP translocation. According to the hypothesis that TonEBP participates in the regulation of AQP2 by tonicity, the finding that elevated urea alone does not increase nuclear abundance of TonEBP is in good agreement with the low expression level of AQP2 observed for IMCD cells cultured under these conditions (confer to Western blot data; Fig. 12) despite the high level of overall TonEBP expression observed for IMCD cells cultured in 600U medium (see Fig. 18). Elevation of medium tonicity from 300 to 600 mosmol/l solely by sodium could not be analysed, due to the induction of because the steep increase in medium tonicity in the absence of urea triggered apoptosis in IMCD cells adapted to 300N medium. After 19 h, only very few cells with an abnormal morphology remained attached to the collagene type IV coated glass support (not shown).

3.8 Investigations of the effect of compounds reported to interfere with the TonE/TonEBP pathway on the expression of AQP2

3.8.1 Proteasome inhibition abrogates up-regulation of AQP2 expression in response to hypertonic challenge

It was reported that the induction of mRNA of the hypertonicity-triggered genes BGT-1 and SMIT was markedly reduced in MDCK cells exposed to hypertonic medium due to addition of 200 mmol raffinose in the presence of 1 μM MG-132, a cell permeable, reversible inhibitor which reduces the degradation of ubiquitin-conjugated proteins by the 26S complex of the proteasome. In this experimental setting, immunofluorescence studies demonstrated that proteasome inhibition reduces nuclear TonEBP abundance and the expression of TonE-regulated genes ([2]Woo et al., 2000), but the underlying mechanisms are yet unclear. Therefore, the effect of MG-132 on the hypertonicity-elicited increase in AQP2 protein expression was studied.

IMCD were seeded in 600N medium and grown for 7 days. Except controls, cells were treated with 10 nM and 100 nM MG-132 48 h before preparation for Western blot analysis. To assess whether MG-132 inhibits the up-regulation of AQP2 in response to hypertonic challenge (see also Fig.15), IMCD cells were routinely seeded in 600N medium that was exchanged 24 h later to hypotonic medium (300N) for 72 h to induce AQP2 down-regulation. Subsequently, up-regulation of AQP2 protein expression was induced by challenging the cells with hypertonicity (600N medium) for 48 h prior to lysis with or without MG-132.

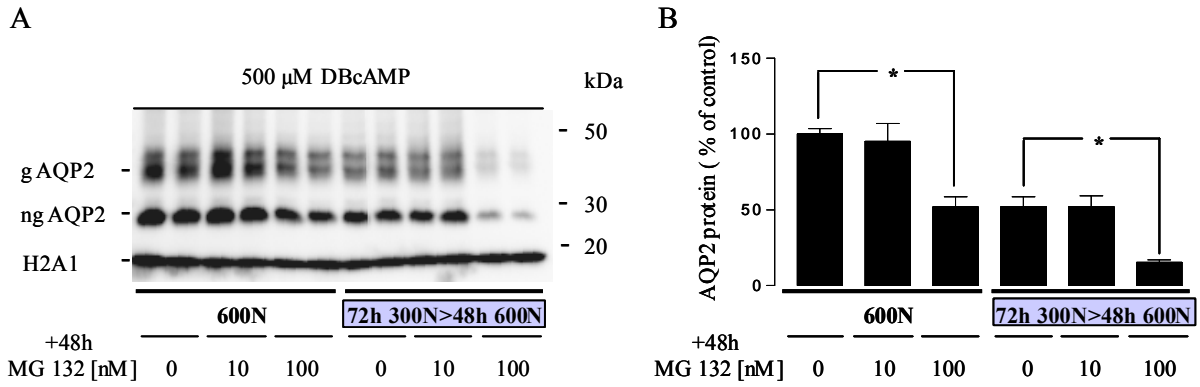

Fig. 22. Inhibition of proteasome function impairs AQP2 expression and completely abrogates the up-regulation of AQP2 in response to hypertonicity. IMCD cells were seeded in 600N medium and grown for 6 days in 24-well plates in the presence of 500 μM DBcAMP. Cells were left untreated (control, 600N) or treated with 10 and 100 nM MG-132 48 h before cell lysis as indicated. To examine the influence of proteasome inhibition on the hypertonicity-induced up-regulation of AQP2, 24 h after seeding cells were challenged with 300N medium for 72 h, and subsequently exposed to 600N medium for 48 h without, with 10 nM, or 100 nM MG-132 as indicated. A: AQP2 (g, glycosylated; ng, non-glycosylated) and histone H2A1 (as indicated) were detected by immunoblotting with anti-AQP2 antiserum. Per lane of an SDS-PAGE gel, total homogenate (protein) from 1 cm^2 confluent cell monolayer was loaded. B: Densitometrical analysis of AQP2 protein levels (glycosylated and non-glycosylated) detected in total homogenates. Values are means +/- SE (n=4). *P < 0.05.

Fig. 22 illustrates that treatment with 100 nM MG-132 for 48 h resulted in a ~50 % decrease in AQP2 expression in IMCD cells continuously cultured in 600N medium. Moreover, the up-regulation of AQP2 in response to 48 h of hypertonicity was completely abolished in the presence of 100 nM MG-132. Lower concentrations had no effect on AQP2 protein expression. Lactacystin, an irreversible inhibitor of proteasome function, yielded comparable results. When applied for 3 h, MG-132 [10 μM] and lactacystin [10 μM] caused a modest increase in AQP2 expression in IMCD cells continuously grown in 600N medium (~ 110 % of controls, n=4; data not shown). This indicated that the proteasome participates in the regulation of AQP2 degradation. Both inhibitors lead to reduced cell survival when used at a concentration of 10 μM for 24 h or more.

3.8.2 Rottlerin impairs AQP2-upregulation in response to hypertonic challenge

It was reported that rottlerin, a potent PKCδ inhibitor, represses the expression of TonE-regulated genes, an effect not achieved by global PKC down-regulation with long-term phorbol ester treatment. This indicated that rottlerin acts on TonEBP-dependent transcription presumably by a PKCδ-independent mechanism (Zhao et. al 2002). Therefore, the effects of rottlerin treatment on AQP2 protein expression in IMCD cells challenged with hypertonicity (see Fig. 23) or kept continuously in 600N medium (Fig. 24) was investigated.

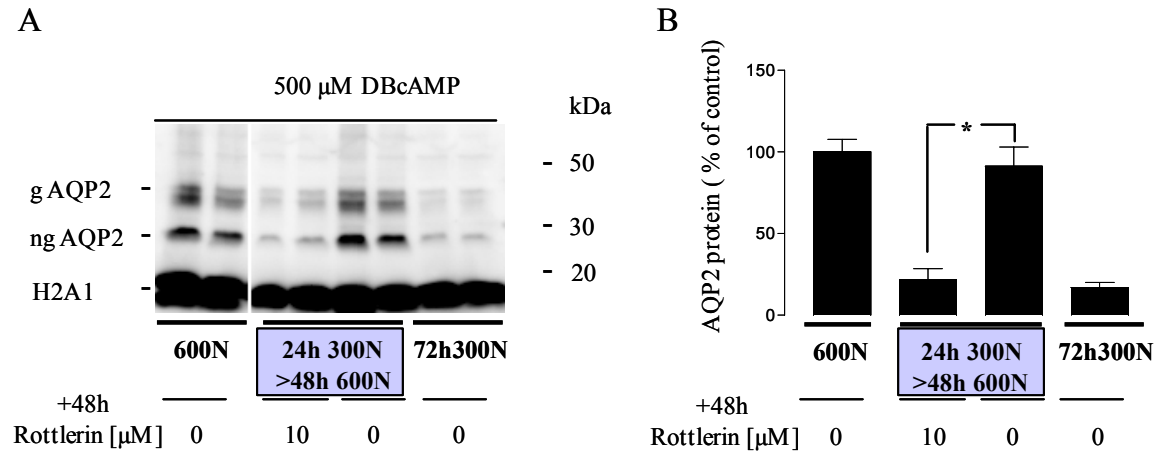

Fig. 23. Rottlerin abolishes the hypertonicity-elicited recovery of AQP2 expression levels after prolonged exposure to hypotonicity. IMCD cells were seeded in 600N medium and grown for 6 days in 24-well plates in the presence of 500 µM DBcAMP (600N, control). To investigate the influence of rottlerin on the hypertonicity-induced up-regulation of AQP2, IMCD cells were challenged with 300N medium for 24 h and subsequently exposed to 600N medium for 48 h with, or without 10 µM rottlerin (as indicated). For comparison, IMCD cells were challenged for 72 h with 300N medium. A: AQP2 (g, glycosylated; ng, non-glycosylated) and histone H2A1 were detected by immunoblotting with anti-AQP2 antiserum. Per lane of an SDS-PAGE gel, total homogenate (protein) from 1 cm^2 confluent cell monolayer was loaded. B: Densitometrical analysis of AQP2 protein levels (glycosylated and non-glycosylated) detected in total homogenates. Values are means +/- SE (n=4). *P < 0.05.

The data presented in Fig. 23 shows that the expression of AQP2 of IMCD cells exposed to 300N medium for 24 h completely recovered within 48 h of exposure to 600N medium. In the presence of 10 µM rottlerin, the recovery of AQP2 protein expression caused by hypertonicity was abolished, i.e. AQP2 expression was comparable to that of IMCD cells kept in hypotonic medium for 72 h prior to lysis.

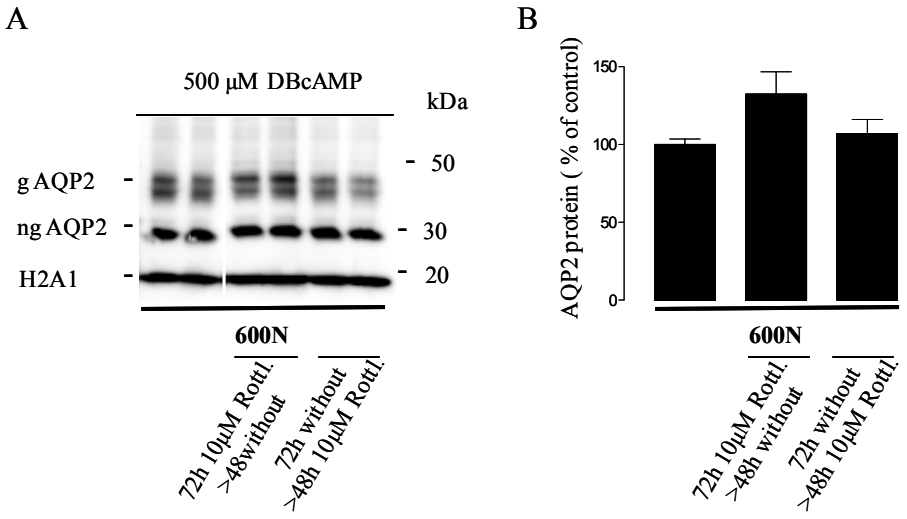

Fig. 24. Rottlerin does not affect AQP2 expression levels of IMCD cells continuously cultured under hypertonic conditions. IMCD cells were seeded in 600N medium and grown for 6 days in 24-well plates in the presence of 500 µM DBcAMP (600N, control). 24 h after seeding, cells were either exposed for 72 h to 10 µM rottlerin that was withdrawn for the consecutive 48 h, or left untreated for 72 h and exposed to 10 µM rottlerin for 48 h prior to lysis as indicated. A: AQP2 (g, glycosylated; ng, non-glycosylated) and histone H2A1 (as indicated) were detected by immunoblotting with anti-AQP2 antiserum. Per lane of an SDS-PAGE gel, total homogenate (protein) from 1 cm^2 confluent cell monolayer was loaded. B: Densitometrical analysis of AQP2 protein levels (glycosylated and non-glycosylated) detected in total homogenates. Values are means +/- SE (n=4). Statistical evaluation showed that there was no significant difference in AQP2 expression between treatments.

No statistically significant effect of a treatment with 10 µM rottlerin for 48 h was detectable in IMCD cells continuously cultured in 600N medium. The same applied to the AQP2 expression of IMCD continuously cultured in 600N medium and treated for 72 h with 10 µM rottlerin which was ommitted from the medium 48 h prior to preparation (Fig. 24). In contrast, rottlerin treatment completely abolished hypertonicity-elicited increase in AQP2 expression (Fig. 23).

3.8.3 MEK/p38 inhibition reduces the detrimental effect of hypotonic challenge on AQP2 expression

Each of the three main families of mitogen-activated serin/threonine protein kinases (MAPK), namely p38, the extracellular signal regulated kinases (ERKs) and the jun-NH_2-terminal kinases JNKs) have been implicated in a variety of cellular responses to changes in osmolality. Activity of p38, the mammalian homologue of the HOG1 kinase involved in the osmoregulation of Saccaromyces cerevisae, has been reported to be essential for the osmotically driven expression of BGT-1 and HSP70 mRNAs in MDCK cells (Sheikh-Hamad et al., 1998). In HepG2 cells, the hypertonic induction of AR and BGT1 mRNAs was decreased by inhibition of p38 and also by inhibition of the ERK activating MAPKK MEK1 (Nadkarni et al., 1999). In contrast, in renal papillary cells, p38 kinase activity was not found necessary for regulation of AR via the ORE/TonE (Kultz et al., 1997). Moreover, it was reported that MAPKs are regulated in vivo in the rat renal papilla by water loading and restriction (Wojtaszek et al., 1998). No difference in MAPK expression and activity was observed comparing mIMCD3 cells adapted to 300 mosmol/l or 600 mosmol/l ([1]Capasso et al., 2001). It was therefore tested, whether the osmotic regulation of AQP2 in IMCD cells shown here is affected by inhibition of either p38 with SB 203580 or MEK1 with PD 0908059.

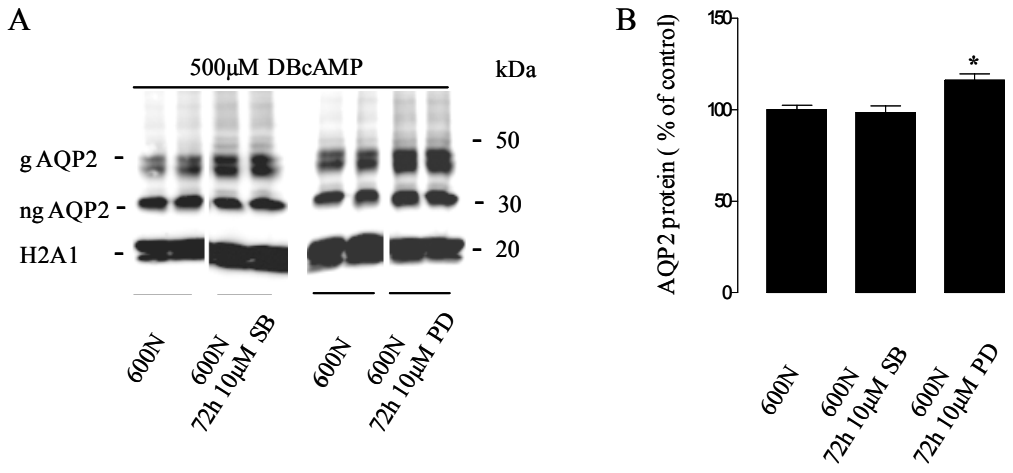

Fig. 25. Effect of p38 and MEK1 inhibition on AQP2 protein expression in IMCD cells continuously cultured in hypertonic medium. IMCD cells were seeded in 600N medium and grown for 6 days in 24-well plates in the presence of 500 µM DBcAMP (600N, control). 72 h before lysis, cells were either treated (except controls) with 10 µM SB 203580, a specific inhibitor of p38 kinase or with 10 µM PD 098059, a specific inhibitor of MEK1. A: AQP2 (g, glycosylated; ng, non-glycosylated) and histone H2A1 (as indicated) were detected by immunoblotting with anti-AQP2 antiserum. Per lane of an

SDS-PAGE gel, total homogenate (protein) from 1 cm² confluent cell monolayer was loaded. B: Densitometrical analysis of AQP2 protein levels (glycosylated and non-glycosylated) detected in total homogenates. Values are means +/- SE (n=4). *P<0.05 vs. control. Statistical evaluation revealed a small, but significant elevation of AQP2 protein expression in cells treated for 72 h with PD 098059.

The data presented in Fig. 25 shows that prolonged inhibition (72 h) of p38 did not affect the expression of AQP2 protein in IMCD cells continuously kept in 600N medium (isotonic conditions), whereas inhibition of MEK1 caused a small increase in AQP2 protein expression. No increase in AQP2 expression was observed within shorter time periods of treatment with PD 098059 or SB 203580 or both in IMCD cells cultured in 600N medium (n=8, not shown).

To asses whether these kinases play a role in the hypertonic induction of AQP2 expression, IMCD cells were seeded in 600N medium which was exchanged 24 h later by 300N medium for the following 72 h to induce AQP2 down-regulation. Subsequently cells were re-exposed to 600N medium (hypertonic conditions) to induce AQP2 up-regulation, or treated additionally with either 10 μM SB 0203580 or 10 μM PD 098059 or with a combination of both inhibitors.

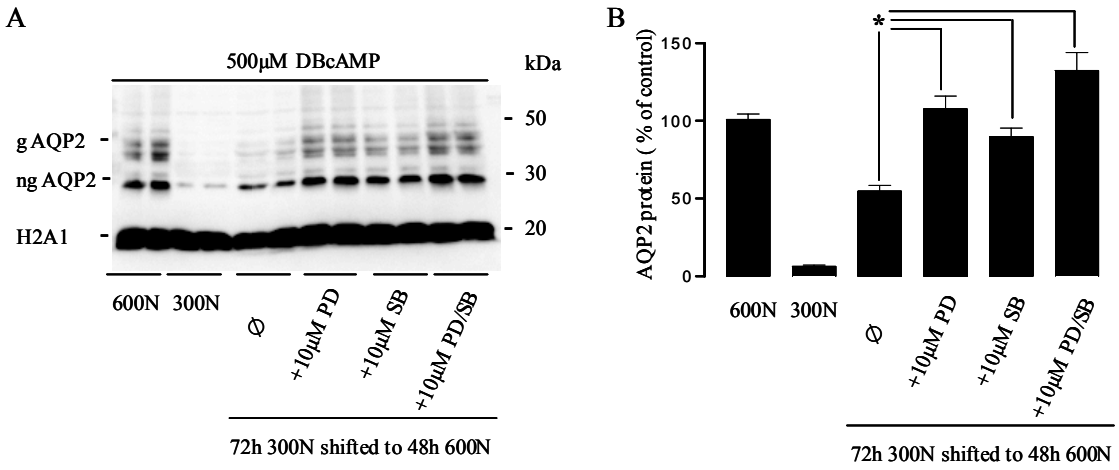

Fig. 26. The hypertonicity-triggered up-regulation of AQP2 protein expression is increased by inhibition of p38 and/or MEK1 in IMCD cells. IMCD cells were seeded in 600N medium and grown for 6 days in 24-well plates in the presence of 500 μM DBcAMP. 24 h after seeding, medium was exchanged with 300N medium (except for controls, 600N). 72 h later, cells were either left untreated (300N), or were exposed for the consecutive 48 h to 600N medium without (∅) or with either PD 098059, SB 0203580, or both. A: AQP2 (g, glycosylated; ng, non-glycosylated) and histone H2A1 (as indicated) were detected by immunoblotting with anti-AQP2 antiserum. Per lane of an SDS-PAGE gel, total homogenate (protein) from 1 cm² confluent cell monolayer was loaded. B: Densitometrical analysis of AQP2 protein levels (glycosylated and non-glycosylated) detected in total homogenates. Values are means +/- SE (6<n<11), *P<0.001 vs. ∅.

The Western blot depicted in Fig. 26 A shows that the hypertonicity-induced up-regulation of AQP2 protein expression in IMCD cells beforehand adapted to hypotonic medium (300N medium) is further promoted in the presence of SB 0203580 and/or PD 098059. The graphical representation summarizing a series of experiments (Fig. 26 B) illustrates that AQP2 expression reached ~50 % of control levels (600N) when IMCD cells kept for 72 h in 300N medium were exposed for 48 h to 600N medium. In this experimental setting, concomitant treatment with SB 0203580 and/or PD 098059 yielded AQP2 expression levels comparable to controls. The increase in AQP2 protein levels elicited by the MAPK inhibitors was statistically significant. Moreover, AQP2 expression did not differ significantly from controls (continuously kept in 600N) when cells were treated with either SB 0203580 or PD 098059. Both inhibitors in combination lead to AQP2 expression levels significantly higher than controls.

Consequently, it was next tested whether these inhibitors affect the down-regulation of AQP2 protein expression in response to hypotonicity (see Fig. 27; turn page).

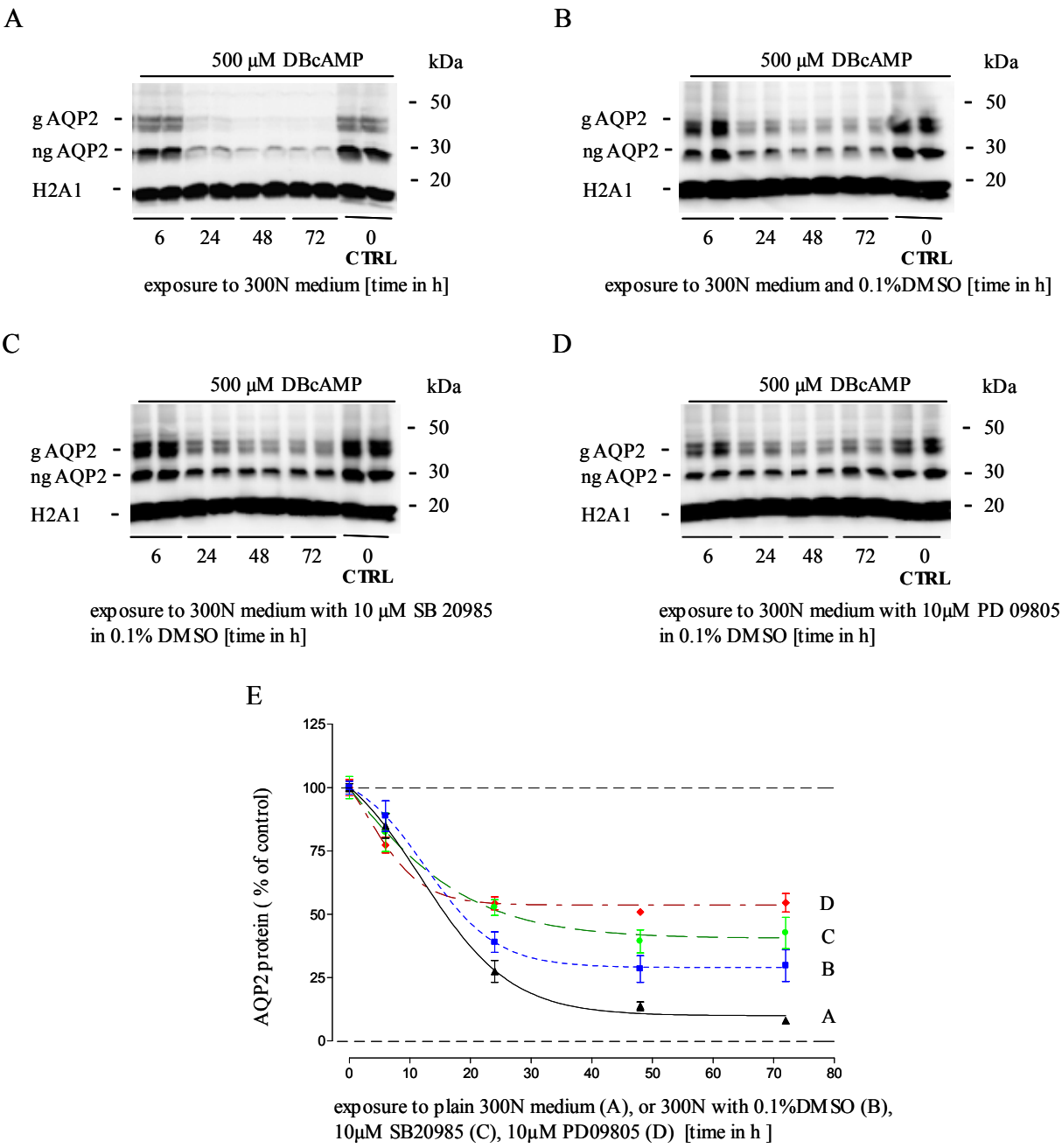

Fig. 27. Kinetics of AQP2 down- regulation in response to hypotonic challenge in the presence of 10 µM SB 0203580 or PD 098059. IMCD cells were cultured for a total of 168 h (7days) in 24-well plates in the presence of 500 µM DBcAMP. A: Cells were seeded in 600N medium. Down-regulation of AQP2 was induced by changing the culture medium to 300N (except controls; 0-h time point) for 72, 48, 24, 6, or 3 h before cell lysis (168 h after seeding) and Western blot analysis. B: cells were treated as in A, but medium contained 0.1 % v/v DMSO. C: cells were treated as in A, but medium contained 10 µM SB 0203580 in DMSO (0.1 % v/v). D: cells were treated as in A, but medium contained 10 µM PD 098059 in DMSO (0.1 % v/v). AQP2 (g, glycosylated; ng, non-glycosylated) and histone H2A1 (as indicated) were detected by immunoblotting with anti-AQP2 antiserum. Per lane of an SDS-PAGE gel, total homogenate (protein) from 1 cm^2 confluent cell monolayer was loaded. E: Graphical representation summarizing a series of the above shown experiments. Values are means +/- SE (12>n>4). Graphs

were obtained by performing non-linear regression analysis [Graph Pad Prism; R^2 (unweighted) = 0.9992 (A), 0.9999 (B), 0.9994 (C), 0.9996 (D)].

Fig. 27 shows that both compounds diminished the extent of AQP2 down-regulation with time of hypotonic treatment as evident after 24 h. Interestingly, DMSO, the solvent used for both inhibitors, also diminished the inhibitory effect of hypotonicity on AQP2 expression, but to a lesser degree. Inhibition of the ERK activator MEK1 by PD 098059 appeared more effective in reducing the negative effect of hypotonicity on AQP2 protein expression than the p38 inhibitor SB 0203580, although AQP2 protein levels detected at the time points were not significantly different between treatments. Compared to cells exposed to hypotonicity together with DMSO, AQP2 protein levels were significantly higher in cells treated with PD 098059 for 48 h and 72 h. The levels of AQP2 protein in IMCD cells treated with SB 0203580 were not significantly different from those observed in cells treated with DMSO.

To assess the effect of a more prolonged treatment with SB 0203580 or PD 098059, the previous experiment was repeated but inhibitors were applied with hypotinicity for 120 h prior to preparation for Western blot analysis (see Fig. 28).

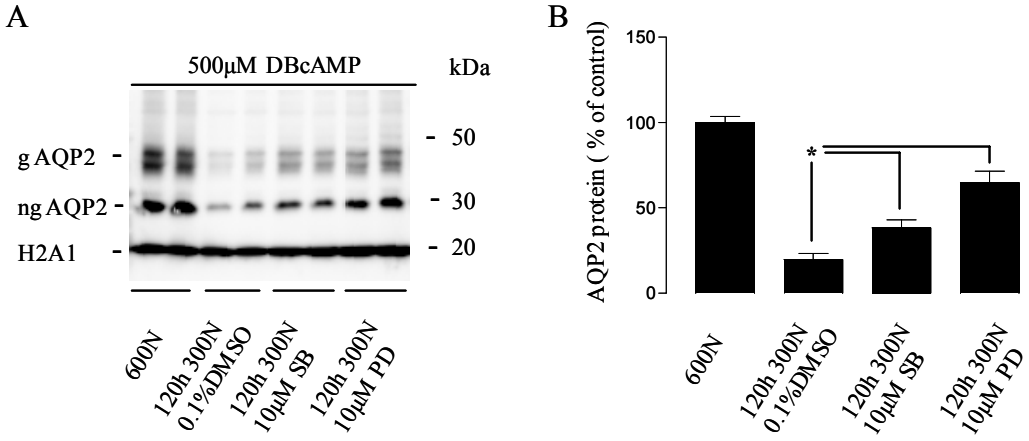

Fig. 28. Effect of a 120-h treatment with DMSO, SB 0203580 or PD 098059 on AQP2 regulation in IMCD cells exposed to hypotonicity. IMCD cells were cultured for a total of 144 h (6 days) in 24-well plates in the presence of 500 µM DBcAMP. A: Cells were seeded in 600N medium. Down-regulation of AQP2 was induced by changing the culture medium to 300N (except controls) for 120 h before cell lysis and Western blot analysis. Cells were treated with either 0.1 % DMSO, 10 µM SB 0203580 in DMSO (0.1 % v/v), or 10 µM PD 098059 in DMSO (0.1 % v/v) at the onset of hypotonicity. AQP2 (g, glycosylated; ng, non-glycosylated) and histone H2A1 (as indicated) were detected by immunoblotting with anti-AQP2 antiserum. Per lane of an SDS-PAGE gel, total homogenate (protein) from 1 cm^2 confluent cell monolayer was loaded. B: Graphical representation summarizing the results of a series of the above shown experiments. Values are means +/- SE (n=4), *P>0.001 vs. 120 h 300N with 0.1 % DMSO.

As shown in Fig. 28, in the presence of either SB 0203580 or PD 098059 the magnitude of AQP2 down-regulation in response to prolonged hypertonicity (120 h) was clearly decreased compared to DMSO controls. The stabilizing effect of DMSO on AQP2 protein expression observed after 72 h of hypotonic treatment appeared attenuated after 120 h of hypotonic treatment (29.8 % +/-6.2 vs. 19.9 %+/-3.5; see Fig. 27). AQP2 expression of cells treated with SB 0203580 was significantly lower than of PD 098059 treated cells ($P<0.05$) as determined by ANOVA.

3.8.4 Influence of proteasome inhibition, rottlerin-treatment, and inhibition of MEK1/p38 on IMCD cell morphology, cellular localization and expression of AQP2, TonEBP and the actin cytoskeleton

To evaluate the effects of proteasome inhibition, rottlerin treatment and MEK1/p38 inhibition on the cellular localization of TonEBP and the expression of AQP2, immunofluorescence studies were performed. Concomitantly, it was tested whether these compounds affect the distribution of actin in IMCD cells because it was observed that hypotonic challenge elicits an increase in actin stress fiber formation in IMCD cells (compare Figs. 29 and 31, upper panels). Inhibitor studies were performed for 40 h prior to preparation on IMCD cells continuously grown in 600N medium for 6 days (isotonic conditions; Fig. 29), cells exposed for 3 days to 300N medium and challenged with 600N medium for 40 h (hypertonic conditions; Fig. 30), as well as cells kept for 4 days in 600N medium and challenged for 40 h with 300N medium (hypotonic conditions; Fig. 31). For visualization of the Cy-3 fluorophore, coupled to either goat-anti-rabbit secondary antibodies or to phalloidin, a 12-bitled Zeiss Sensi-cam mounted on a Zeiss axiovert fluorescense microscope was used. The various cell batches were prepared and visualized identical. Thus, the data shown provides not only information about the subcellular localization of AQP2, TonEBP and actin, but aditionally gives rough information about the expression levels of these proteins (turn page).

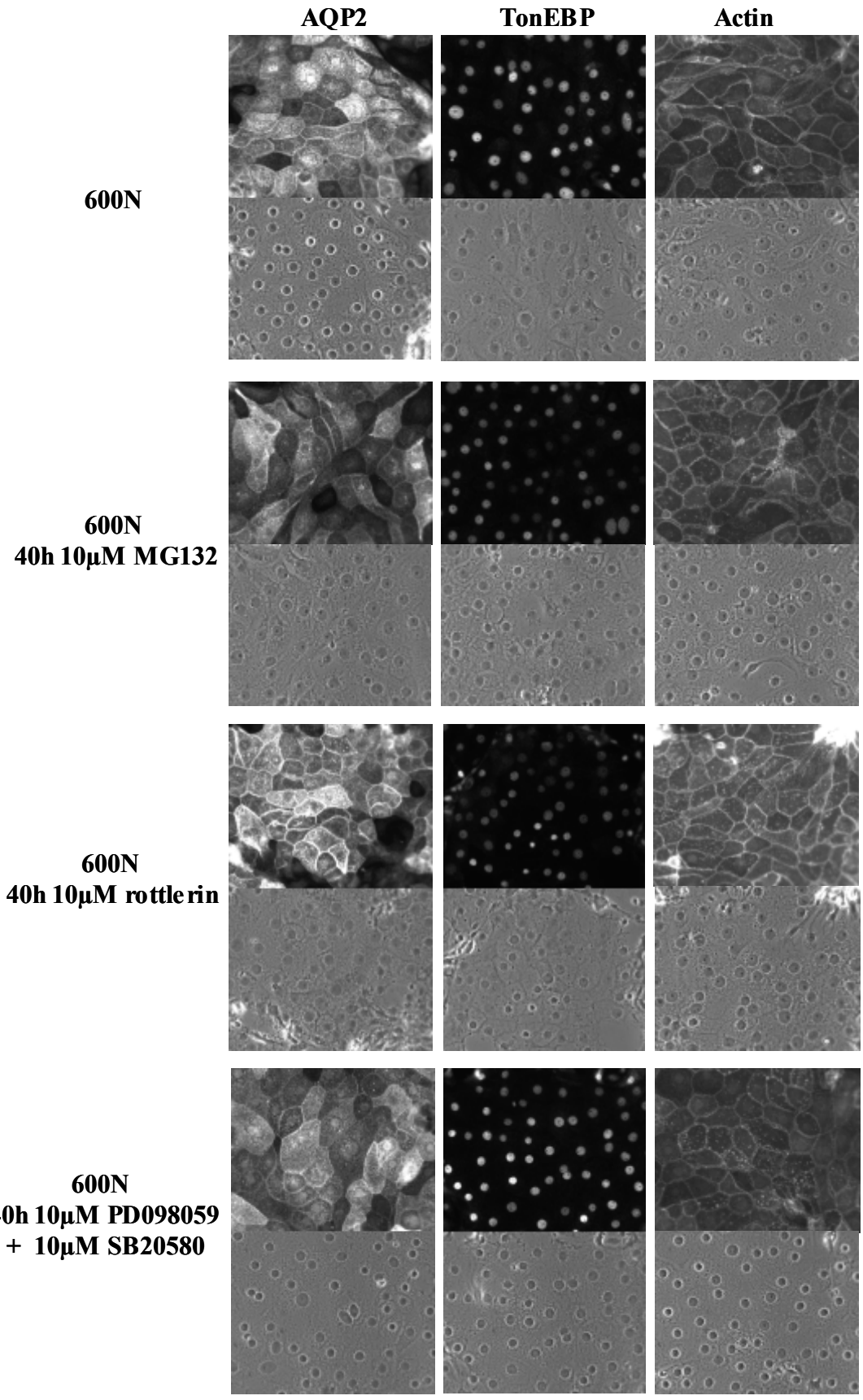

Fig. 29. Effect of proteasome inhibition, rottlerin treatment and MEK1/p38 inhibition on AQP2, TonEBP-, and actin distribution in IMCD cells continuously grown in 600N medium (isotonic conditions). Cells were seeded and grown in 600N medium for 6 days in the continuous presence of 500 µM DBcAMP. Inhibitors (in the concentrations indicated) or vehicle (0.1 % DMSO, for controls) were

applied 40 h prior to preparation as indicated. Shown is (from left to right, *upper part of panels*) AQP2 fluorescence, TonEBP fluorescence and actin fluorescence. *Lower part of panels:* Light micrographs corresponding to the preparations shown above. Cells were exposed for 40 h prior to preparation to either DMSO (600N, control; *upper panels*), MG-132 (*panels 2nd to top*), rottlerin (*panels 2nd to bottom*), or SB0203580 and PD098059 (*bottom panels*). AQP2 was detected with specific rabbit anti-AQP2 antiserum (1:500). TonEBP was detected with a specific rabbit anti-TonEBP antiserum (kindly provided by Drs. López-Rodriguez and Rao; 1:500). Actin was detected using Cy-3 coupled phalloidin (1:500). Cy3-conjugated goat anti-rabbit antibodies were used as secondary antibodies (1:600). All Cy-3 fluorescence images were exposure matched (900 ms) and processed identically.

Fig. 29 depicts the influence of prolonged proteasome inhibition, rottlerin treatment and p38/MEK1 inhibition on the morphological appearance, AQP2- and TonEBP distribution, and the actin cytoskeleton in IMCD cells kept continuously in 600N medium. In the absence of any inhibitor, IMCD cells cultivated in 600N medium exhibited a strong AQP2 staining in the cytoplasm as well as in the plasma membrane. TonEBP signals were strong and confined to the nuclei. It is to note, that TonEBP signals varied in between cells of one batch. Phalloidin staining revealed that actin was concentrated mainly at cell-cell boundaries with a rare appearance of stress fibers within cells. Proteasome inhibition did neither alter cell morphology nor appearance of the actin cytoskeleton, but reduced the labeling for AQP2 and TonEBP. The decrease in AQP2 expression suggested by immunofluorescence data is in good agreement with data obtained by Western blot experiments (see Fig. 22). IMCD cells treated with rottlerin exhibited a small reduction in TonEBP signal intensity, but cell morphology as well as distribution and expression of AQP2 and actin remained unaltered compared to controls.

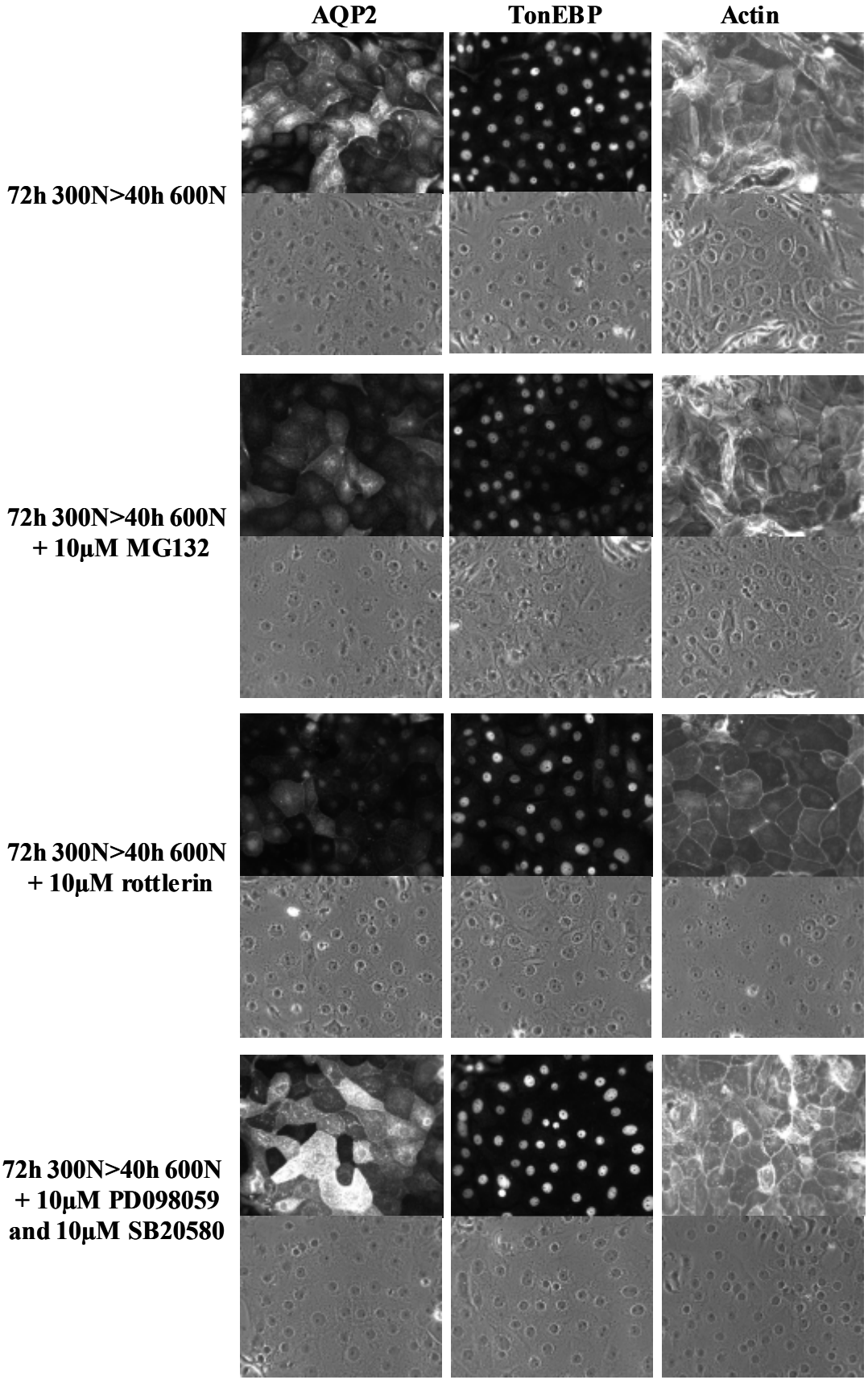

Fig. 30. Effect of proteasome inhibition, rottlerin treatment and MEK1/p38 inhibition on AQP2, TonEBP, and actin distribution in IMCD cells challenged with hypertonicity. Cells were seeded in 600N medium and transferred 24 h later to 300N medium for another 72 h and subsequently challenged

with hypertonicity (600N medium) for 40 h prior to preparation. All media contained 500 μM DBcAMP. Inhibitors (in the concentrations indicated) or vehicle (0.1 % DMSO, for controls) were applied with hypertonicity. Shown is (from left to right, *upper part of panels*) AQP2 fluorescence, TonEBP fluorescence and actin fluorescence. *Lower part of panels:* Light micrographs corresponding to the preparations shown above. Cells were exposed for 40 h prior to preparation to either DMSO (600N, control; *upper panels*), MG-132 (*panels 2nd to top*), rottlerin (*panels 2nd to bottom*), or SB0203580 and PD098059 (*bottom panels*). AQP2 was detected with specific rabbit anti-AQP2 antiserum (1:500). TonEBP was detected with a specific rabbit anti-TonEBP antiserum (kindly provided by Drs. López-Rodriguez and Rao; 1:500). Actin was detected using Cy-3 coupled phalloidin (1:500). Cy3-conjugated goat anti-rabbit antibodies were used as secondary antibodies (1:600). All Cy-3 fluorescence images were exposure matched (900 ms) and processed identically.

Compared to IMCD cells continuously cultured in 600N medium (isotonic conditions), the effects of the inhibitors used were much more prominent in IMCD challenged for 40 h with hypertonicity after AQP2 down-regulation had been induced beforehand by prolonged exposure to hypotonicity (see Fig. 30). When inhibitors were omitted (Fig. 30; upper panels), IMCD cells exhibited a robust AQP2 expression, in many cells with the strongest AQP2 appearance close to the nucleus, i.e. most probably within the endoplasmic reticulum, suggestive for enhanced AQP2 protein synthesis. TonEBP signals were strong and confined to the nuclei of IMCD cells. The actin cytoskeleton of hypertonically challenged IMCD cells appeared altered compared to controls (continuously cultured in 600N medium; see Fig. 29), since more stress fibers were observed and overall staining for actin was increased. Enhanced staining for actin and increased stress fiber abundance was also observed in hypertonically challenged IMCD cells with impaired proteasome function due to application of MG-132. These cells exhibited a clearly reduced AQP2 expression and diminished nuclear TonEBP signals (panels 2nd to top). No effect on cell morphology was evident. When rottlerin was present at the onset of hypertonicity (panels 2nd to bottom), recovery of AQP2 expression appeared abolished, and the actin cytoskeleton exhibited a clear decrease in stress fiber abundance compared to controls (upper panels). In contrast, the signal intensity and distribution of TonEBP was not affected by rottlerin-treatment. When p38 and MEK1 function were inhibited with the onset of hypertonicity, AQP2 signal intensities exceeded that of controls. A similar increase was evident for the nuclei confined TonEBP signals. The amount of actin stress fibers appeared unaltered by p38/MEK1 inhibition.

The immunofluorescence data presented in Fig. 30 revealed that the increase in AQP2 protein elicited by hypertonicity (see also Fig. 26) was accompanied by increased nuclear TonEBP abundance. The Western blot data shown in Fig. 27 and 28, indicated that p38/MEK1 inhibition attenuated the detrimental influence of hypotonicity on the AQP2 protein expression in IMCD cells. It was therefore investigated whether p38/MEK1 inhibition would interfere with the hypotonicity elicited diminution of nuclear TonEBP signals (see Fig. 31).

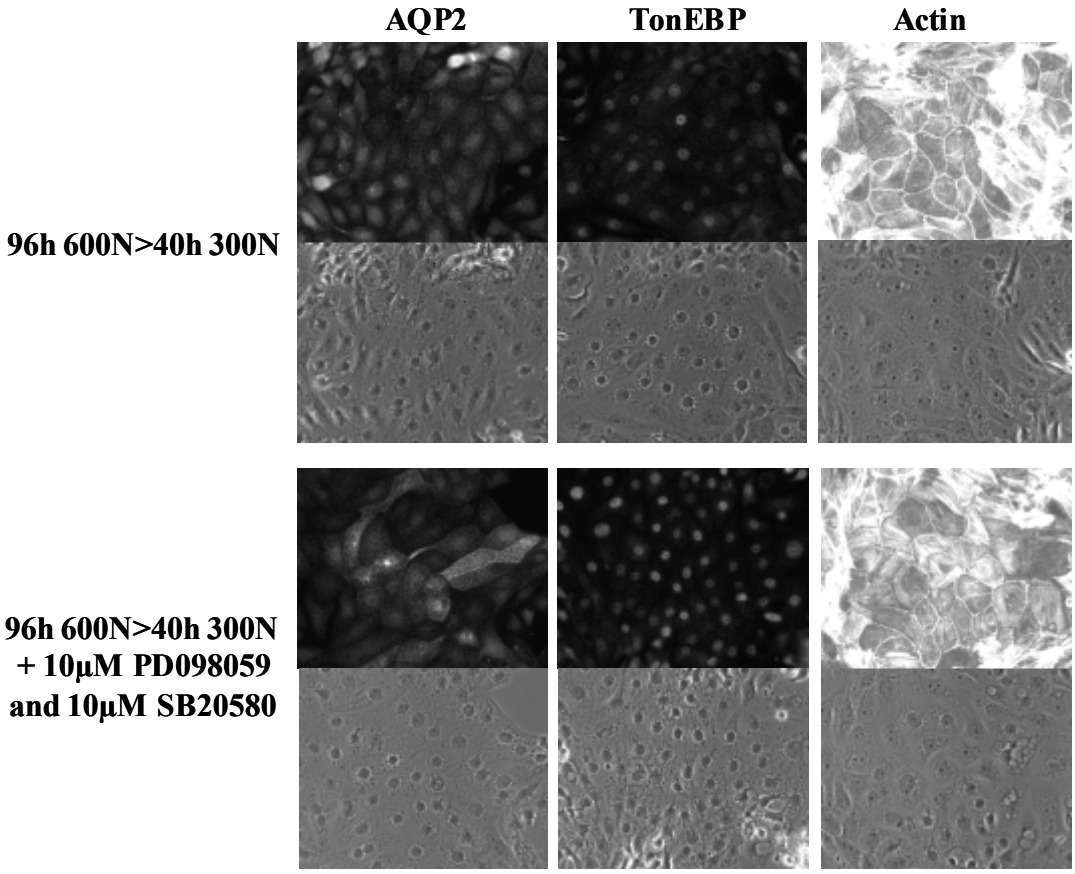

Fig. 31. Effect of MEK1/p38 inhibition on AQP2-, TonEBP-, and actin distribution in IMCD cells challenged with hypotonicity. Cells were seeded and grown in 600N medium for 4 days in the continuous presence of 500 µM DBcAMP. 40 h prior to preparation, cells were exposed to hypotonicity in form of 300N medium. Inhibitors (in the concentrations indicated) or vehicle (0.1 % DMSO, for controls) were applied with hypotonicity (as indicated). Shown is (from left to right, *upper part of panels*) AQP2 fluorescence, TonEBP fluorescence and actin fluorescence. *Lower part of panels:* Light micrographs of the preparations shown above. Cells were exposed for 40 h prior to preparation to either DMSO (*upper panels*), or SB0203580 and PD098059 (*bottom panels*). AQP2 was detected with specific rabbit anti-AQP2 antiserum (1:500). TonEBP was detected with a specific rabbit anti-TonEBP antiserum (kindly provided by Drs. López-Rodriguez and Rao; 1:500). Actin was detected using Cy-3 coupled phalloidin (1:500). Cy3-conjugated goat anti-rabbit antibodies were used as secondary antibodies (1:600). All Cy-3 fluorescence images were exposure matched (900 ms) and processed identically.

The results presented in Fig. 31 show that hypotonically challenged IMCD cells exhibited a faint overall staining for AQP2 and TonEBP, but a major increase in actin stess fiber formation. TonEBP singnals were clearly enhanced in the nuclei of cells treated with p38 and MEK1 inhibitors. Concomitant with enhanced nuclear TonEBP signals, AQP2 expression was substantially increased. No effect of p38/MEK1 inhibition was evident on the actin cytoskeleton.