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Research article

Contrast bath-induced neurite outgrowth in PC12m3 cells via the p38 mitogen-activated protein kinase pathway

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ABSTRACT

We investigated the differentiation and activation of p38 MAPK induced by contrast bath in drug-hypersensitive PC12m3 mutant cells. The rate of neurite outgrowth in PC12m3 cells induced by contrast bath was much higher than that induced by warming or cooling alone or that induced by two warmings with an interval of room temperature, indicating that contrast bath has a synergistic effect. The results of an experiment using a p38 MAPK inhibitor, SB203580, showed that neurite outgrowth of PC12m3 cells induced by contrast bath is p38 MAPK-dependent. Moreover, p38 MAPK activity induced by contrast bath was greater than that induced by warming or cooling alone, indicating that the synergistic effect of a contrast bath on neurite outgrowth depends on the activity of p38 MAPK. Since calcium ions are involved in the activations of P38 MAPK, we investigated the effect of the TRP ion channel inhibitor (Capsazepine) that inhibits calcium influx in the cells. Neurite outgrowth induced by contrast bath treatment was greatly suppressed by the addition of Capsazepine.

These findings suggest that calcium dependent activation of the p38 MAPK pathway induced by contrast bath is responsible for the neurite outgrowth of PC12m3 cells.

1. Introduction

Contrast bath is often used in rehabilitation for improvement of blood flow impairment and recovery of muscle fatigue. The use of contrast bath is a treatment method aimed at improving peripheral circulatory disturbance by immersing the affected part of the body alternately in hot water and cold water. The intensity of stimulation is adjusted by the temperature of the hot water, temperature of the cold water, immersion time and number of immersions. The treatment usually starts and ends with immersion in hot water. The effects of the treatment include improvement in vascular tone, increase in resistance to cold, pain relief and recovery of muscle fatigue [1]. It has been reported that contrast bath was an effective intervention for reducing the level of neuropathy pain in patients with diabetes mellitus [2]. Pain may be reduced by both heat and cold stimuli [3]. However, the molecular mechanisms by which a contrast bath acts on neural cells are not clear.

To clarify the mechanisms by which contrast bath acts on cells, we investigated the differentiation and activation of p38 MAPK induced by contrast bath in drug-hypersensitive PC12m3 mutant cells. We obtained PC12m3 cells from a variant cell line of PC12 cells that spontaneously showed impaired NGF-induced neurite outgrowth by culturing for 2 weeks under an acidic condition of Cl [4]. PC12m3 cells showed poor neurite outgrowth despite normal sustained activation of ERK by NGF treatment. However, neurite outgrowth of PC12m3 cells was stimulated by various stressful stimuli such as heat shock, osmotic shock, and microwave irradiation in the presence of NGF [4, 5, 6]. PC12m3 cells also exhibited sustained activation of p38 MAPK in response to various stimulants [6, 7, 8, 9, 10, 11]. Since PC12m3 cells have mutations somewhere in the p38 MAPK pathway, various stresses cause high p38 MAPK activity. It has been reported that high p38 MAPK activity causes neuronal differentiation of PC12m3 cells [7, 8]. On the other hand, since PC12 cells have weak p38 MAPK activity due to stress, in the presence of stress, there is little neuronal differentiation of PC12 cells due to stress. As described above, PC12m3 cells are the only cells capable of detecting neuronal differentiation by stress, and we therefore used PC12m3 cells for the experiment with a contrast bath in this study.

In this study, we found that contrast bath stimulation induced enhancement of neurite outgrowth in PC12m3 cells. Furthermore, p38

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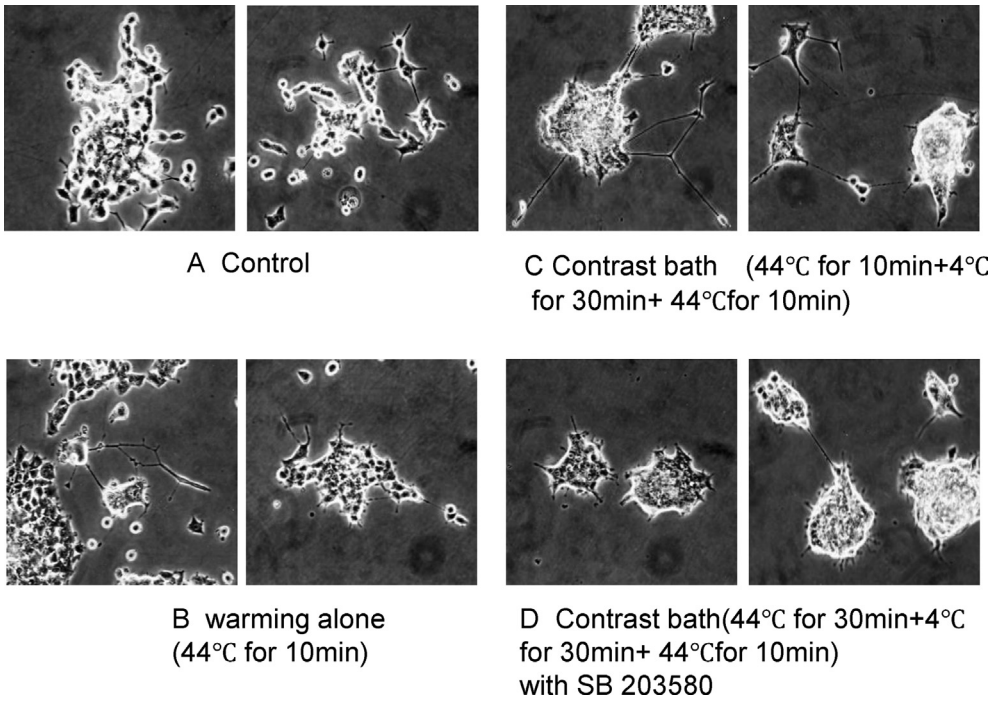

Fig. 1. Stimulation of neurite outgrowth in PC12m3 cells in the presence of NGF (30 ng/mg) by warming alone, a contrast bath or contrast bath treatment with SB 203580, a specific inhibitor of p38 MAPK. Phase-contrast photomicrographs (× 200) of PC12m3 cells were taken 7 days after treatment with warming alone (44 °C for 10 min) (B), contrast bath (44 °C for 30 min + 4 °C for 30 min + 44°Cfor 10 min) (C), or contrast bath with SB 203580(D). An untreated experimental control (presence of NGF alone) was also used (A).

MAPK was strongly activated in PC12m3 cells treated by contrast bath stimulation.

2. Materials and methods

2.1. Cell culture

PC12m3 mutant cells were used for this experiment. We are negotiating to sell the used PC12m3 cells used in our study via a cell depository company. However, if the negotiation fails, we plan to deposit the cell line in the cell bank. PC12m3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.35% glucose, 10% horse serum, 5% fetal bovine serum (FBS), and 100 units/ml kanamycin. All cells were grown at 37 °C in 5% CO_2.

2.2. Reagents

NGF (2.5 S) was purchased from Takara (Osaka, Japan). The p38 MAPK inhibitor SB203580 was purchased from Sigma (St. Louis, MO). Capsazepine was purchased from Fujifilm Wako (Osaka, Japan).

2.3. Warming, cooling or contrast bath treatment of cells

For each treatment, the screw cap of the flask was tightened. For warming treatment, the flasks were placed in an incubator warmed to 44 °C for 10 min. For cooling treatment, they were soaked horizontally about 1 cm horizontally on a fine ice packed tightly in a cooler box. As measured by a spot thermometer, the temperature of the medium in the flask was stable at about 4° after 3 min. For contrast bath treatment, in the above manner after warming in the incubator for 10 min, it was immediately cooled for 30 min on ice and immediately warmed again for 10 min.

2.4. Determination of neurite outgrowth

A single-cell suspension of PC12m3 cells was obtained by trituration in DMEM. For experiments on neuritogenesis, the cells were plated in 25 cm^2 flasks at a density of 2–5x10^5 cells per dish of serum-containing DMEM and then treated with NGF or without NGF, and exposed to warming (44 °C) for 10 min (warming alone), cooling (4 °C) for 30 min (cooling alone), warming (44 °C) for 10 min + cooling (4 °C) for 30 min (warming and cooling), warming (44 °C) for 10 min + room temperature (25 °C) for 30 min + warming (44 °C) for 10 min (two warmings with a room temperature) or warming (44 °C) for 10 min + cooling (4 °C) for 30 min + warming (44 °C) for 10 min (contrast bath). In order to examine the effect of a p38 inhibitor, PC12m3 cells were preincubated with or without SB 203580, a specific inhibitor of p38 MAPK, for 1 hour at 37 °C before contrast bath stimulation. After 7 days of incubation, the lengths of neurites were measured, and the numbers of neurites were counted. Cells possessing at least one neurite with a length of at least 1.5-fold greater than the diameter of the cell body were counted as previously described [2]. Each value shown is the mean ± S. D. for 100–200 cells sampled from three independent experiments.

2.5. Detection of activated p38 MAPK, ERK and JNK

Activation of activated p38 MAPK, ERK and JNK were determined as described previously [2]. Briefly, PC12m3 cells were plated at a density of 1 × 10^6 cells/25 cm^2 in a flask of serum-containing medium and cultured for 3 days. Then the culture medium was replaced by 0.5% FBS-containing medium, and the cells were cultured for a further 48 h. PC12m3 cells were then exposed to warming alone, cooling alone or contrast bath stimulation. p38 MAPK, ERK and JNK activity in cell lysates were then assayed. The cells were lysed in a lysing buffer. Aliquots of the lysates (10–15μg) from each sample were fractionated on SDS-10% polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The blots were probed with an antibody specific for phospho-p38 MAPK, total p38 MAPK, phospho-ERK, or phospho-JNK (New Englang BioLabs, Beverly, MA) at a dilution of 1:1000 in a blocking buffer for 12 h at 4 degrees centigrade. The blots were probed with a secondary antibody, horseradish peroxidase-linked anti-rabbit IgG (New Englang BioLabs, Beverly, MA), at a dilution of 1:2000 in a blocking buffer for 60 min at room temperature. The blots were stained for 1 min using the nucleic acid chemiluminescence reagent (LumiGLO chemiluminescent reagent, Kirkegaard and Perry Laboratories) and exposed to x-ray film.

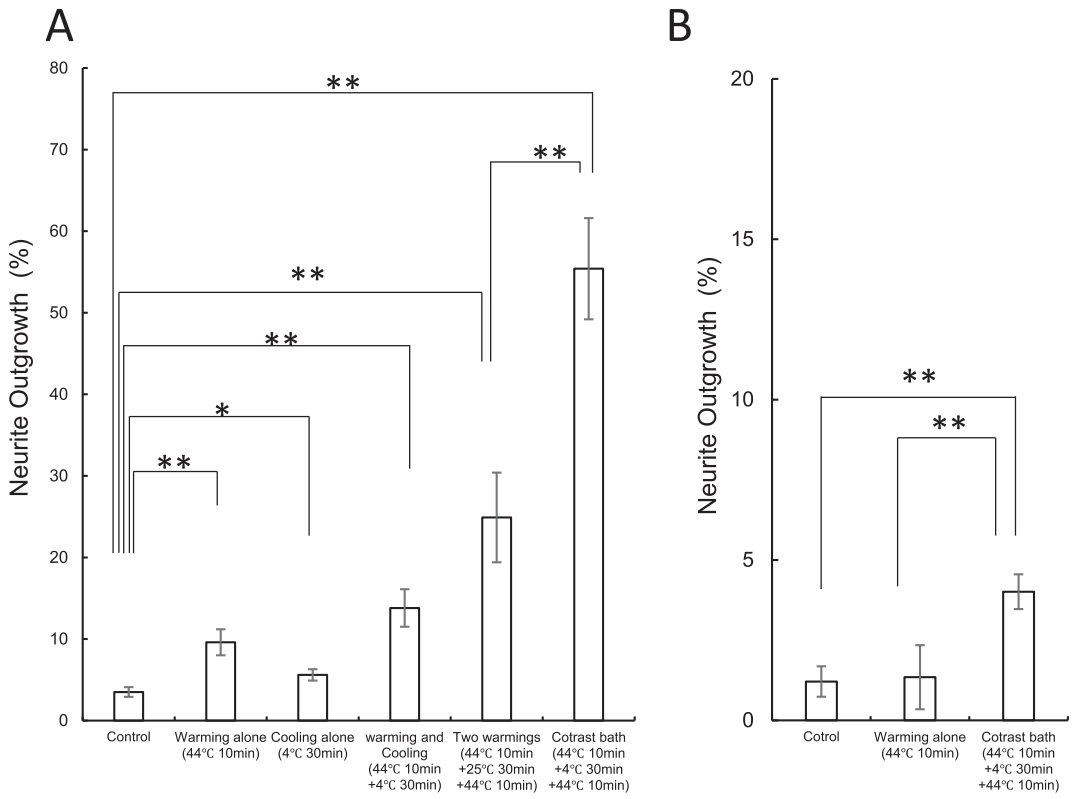

Fig. 2. Percentage of neurite outgrowth in PC12m3 cells induced by stimulation with warming alone, cooling alone, warming and cooling, two warmings with an interval of room temperature, contrast bath and in untreated control cells with NGF (A) and without NGF (B). The percentages of cells with neurites were determined after 7 days. Each value is the mean ± S.E.M. for 100 cells sampled from three independent experiments.

2.6. Survival experiments on PC12m3 cells with warming and contrast bath treatment

Single-cell suspensions of PC12m3 cells were obtained by trituration in DMEM. For cell survival studies, the cells were exposed to a temperature of 44 °C for various periods ranging from 10 minutes to 30 minutes and incubated in serum-containing DMEM medium for 10 days until colony staining, and then the colonies were counted. For the contrast bath experiments, PC12m3 cells were exposed to warming (44 °C) for 10 min + cooling (4 °C) for 30 min + warming (44 °C) for 10 min. Each value is the mean ± S. D. for cells sampled from three independent experiments.

2.7. Statistical analysis

The levels of activated kinase bands were quantified by densitometric scanning and NIH Image software analysis. The statistical significance of differences between values ($p < 0.05$) was determined by analysis of variance (ANOVA).

3. Results

3.1. Contrast bath-induced neurite outgrowth and effects of a p38 MAPK inhibitor

When cultures of PC12m3 cells with NGF were exposed to warming alone or cooling alone stimulation, neurite outgrowth was slightly but significantly induced ($p < 0.05$) (Figs. 1 and 2A). The rate of neurite outgrowth was about 10-fold greater in a warming and cooling experiment than that induced by NGF alone (control) (Fig. 2A). The rate of outgrowth induced by two warmings with an interval of room temperature was approximately 2-fold greater than that induced by warming alone (Fig. 2A). The rate of outgrowth induced by contrast bath stimulation was 2-fold greater than that induced by two warmings with an interval of room temperature (Fig. 2A). It has been shown that activation of the p38 MAPK pathway is required for NGF-induced neurite outgrowth of PC12 cells. Incubation of PC12m3 cells with SB203580, a

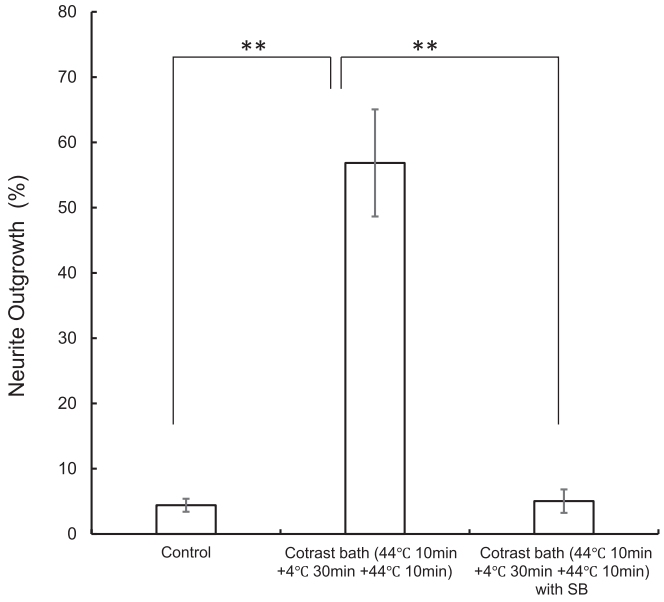

Fig. 3. Percentage of neurite outgrowth in PC12m3 cells induced by stimulation with contrast bath and contrast bath with SB 203580 (2 μM) and in untreated control cells in the presence of NGF. The percentages of cells with neurites were determined after 7 days. Each value is the mean ± S.E.M. for 100 cells sampled from three independent experiments.

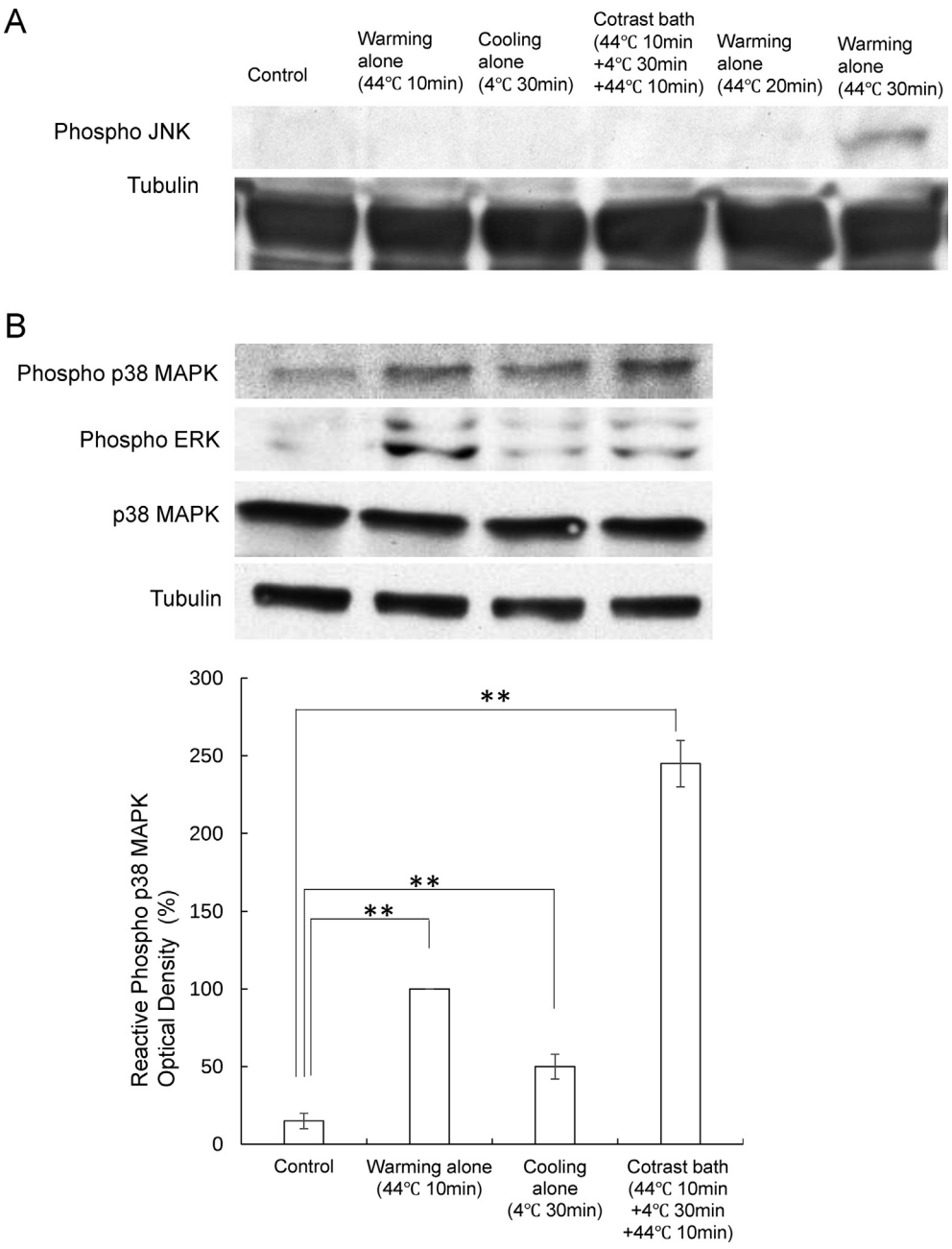

Fig. 4. Activity of JNK (A), ERK and p38 MAPK (B) in PC12m3 cells induced by stimulation with warming alone, cooling alone, and contrast bath and in untreated control cells. These were determined by Western blotting. Regarding warming alone, JNK detection was performed as an additional positive control at 44 °C for 10 minutes, 20 minutes (A). Level of p38 MAPK activity after the blot had been scanned using a densitometric apparatus is shown. Each column shows reactive optical density of phospho p38 MAPK. Data are presented as means and error bars (±SEM). **P < 0.01 means statistically significant. Full, non-adjusted image of each image of blots was provided as supplementary Fig. 1.

specific inhibitor of p38 MAPK, resulted in marked inhibition ($p < 0.05$) of the contrast bath stimulation-induced neurite outgrowth (Figs. 1 and 3).

The rate of outgrowth induced by contrast bath stimulation without NGF was 3-fold greater than that induced by warming alone or control ($p < 0.05$) (Fig. 2B).

3.2. Contrast bath-induced activation of p38 MAPK

Since activation of p38 MAPK has been shown to play an important role in neuronal differentiation in PC12 cells [6, 7, 8, 9, 10, 11], we examined whether the ability of contrast bath stimulation to induce neurite outgrowth of PC12m3 cells is a reflection of its effect on p38 MAPK activity. When cells were exposed to warming alone or cooling alone stimulation, the extent of the stimulation-induced phosphorylation of p38 MAPK was significantly greater in stimulated PC12m3 cells than in nontreated control cells. The level of p38 MAPK activity with contrast bath stimulation was greatly increased compared to that with warming alone or cooling alone stimulation in PC12m3 cells (Fig. 4B).

It has been found that ERK induced activity by NGF does not affect neurite outgrowth in PC12m3 cells. We examined ERK activity of PC12m3 cells exposed to contrast bath stimulation. High ERK activity was observed only with warm treatment at 44 °C for 10 min, and ERK activity was lower with the contrast bath treatment than with thermal treatment at 44 °C for 10 min (Fig. 4B). When PC12m3 cells were subjected to warm treatment at 44 °C for 20 min or 30 min, JNK, a protein that causes apoptosis, was activated; however, JNK was not activated by contrast bath treatment (Fig. 4A).

3.3. Survival experiments on PC12m3 cells with warming and contrast bath treatment

We performed survival experiments and found that cell death did not occur in the warming and cooling treatments with the contrast bath. The

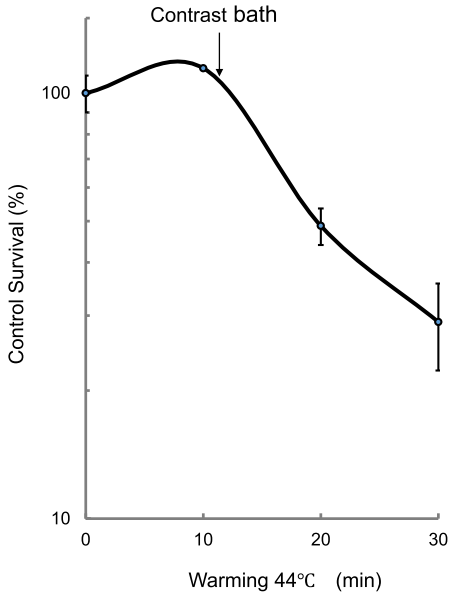

Fig. 5. Survival experiments on PC12m3 cells with warming and contrast bath treatment. the cells were exposed to a temperature of 44 °C for various periods ranging from 10 minutes to 30 minutes. For the contrast bath, PC12m3 cells were exposed to warming (44 °C) for 10 min + cooling (4 °C) for 30 min + warming (44 °C) for 10 min. After that they were incubated in serum-containing DMEM medium for 10 days until colony staining, and then the colonies were counted. Each value is the mean ± S. D. for cells sampled from three independent experiments.

results of contrast bath experiment combined with the results of the survival experiment by warming treatment carried out at the same time as the contrast bath experiment are shown in Fig. 5. The results showed that warming treatment at 44 °C for 20 and 30 min had a strong toxic effect, whereas contrast bath treatment had no toxic effect on PC12m3 cells.

3.4. Effects of a TRP ion channel inhibitor on contrast bath-induced neurite outgrowth

We investigated how the TRP ion channel is involved in neurite outgrowth induced by contrast bath treatment. Neurite outgrowth induced by contrast bath treatment using PC12m 3 cells was greatly suppressed by the addition of 10 mM of Capsazepine, a TRP ion channel inhibitor (Fig. 6). This suggests that p38 MAPK activity was suppressed because Capsazepine inhibited calcium influx in the cells by activated TRP ion channels.

4. Discussion

Gon et al. [12] examined the effect of contrast bath treatment via the p38 mitogen-activated protein kinase pathway using human bronchial epithelial cells. They found that there was p38 MAPK activity but no activity of interleukin-8 (IL-8) in the case of only cooling but that both p38 MAPK and IL-8 were activated by cooling and rewarming. In the present study, both p38 MAPK activity and neurite outgrowth activity were observed with only cooling and with contrast bath stimulation. The difference between the results obtained by Gon et al. and our results is thought to be due to the difference in the cells used. Generally, when p38 MAPK is activated by heat treatment or cold treatment, it causes apoptosis of cells. However, in our study, we found that p38 MAPK activated by heat or cold treatment did not cause apoptosis but in fact induced cell differentiation and thus played a role in cell survival. The reason is that the PC12m3 cells that we used in our study are mutant cells with a mutation in p38 MAPK signaling.

It was shown that stimulation of PC12m3 cells by a contrast bath greatly enhanced their neurite outgrowth. The effect of contrast bath treatment on neurite outgrowth was greater than the effect of warming alone, cooling alone or two warmings with an interval of room temperature, indicating that contrast bath treatment has a synergistic effect. The

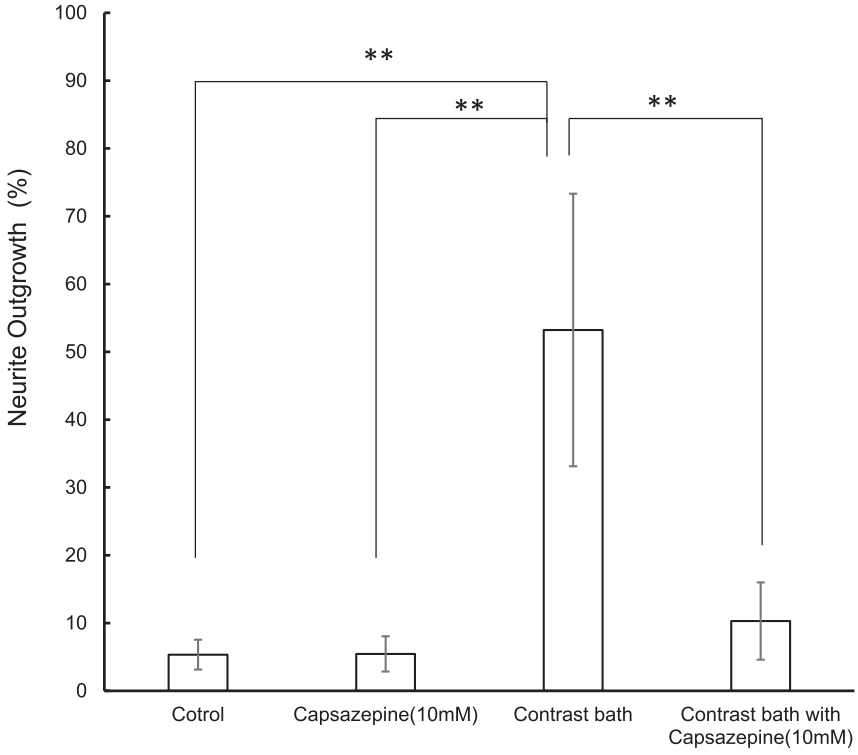

Fig. 6. Percentage of neurite outgrowth in PC12m3 cells induced by stimulation with contrast bath and contrast bath with 10 mM of Capsazepine, a TRP ion channel inhibitor and in untreated control cells in the presence of NGF. The percentages of cells with neurites were determined after seven days. Each value is the mean ± S.E.M. for 100 cells sampled from three independent experiments.

results of an experiment using a p38 MAPK inhibitor showed that neurite outgrowth of PC12m3 cells induced by contrast bath treatment is p38 MAPK-dependent (Fig. 3). Moreover, it was shown that p38 MAPK activity was greater when cells were treated by contrast bath than when cells were treated by warming or cooling alone (Fig. 4B), indicating that the synergistic effect of contrast bath treatment on neurite outgrowth depends on the activity of p38 MAPK. When neurite outgrowth induction was carried out with only the contrast bath without NGF, the rate of neurite outgrowth was low but it was certainly induced (Fig. 2B). This result suggested that contrast bath treatment causes neurite outgrowth only by the activity of p38 MAPK. Application of NGF to PC12 cells stops the growth of almost 100% of the cells, and buds of the nerves appear after 1–2 days. Thereafter, neurite outgrowth is induced by up to 10 times the diameter of the cell in a culture containing 10% HS + 5% FBS after one week. On the other hand, in PC12m3 cells, the proliferation stops in about half of the cells with contrast bath treatment and the cells differentiate into neuronal cells. When PC12m 3 cells are subjected to hyperosmolarity treatment, 100% of the cells differentiate into neural-like cells. When EGF is allowed to act on PC12 cells, brief activation of ERK via activation of EGFR and Ras occurs, leading to proliferation of the cells. On the other hand, when NGF acts on PC12 cells, ERK is activated for a long time via activation of TK and Rap1, which activates transcription factors such as Elk1 and CREB to differentiate the cells [13]. On the other hand, in the case of PC12m3 cells, various stress stimuli activate p38 MAPK for a long time, which activates CREB to differentiate cells [8].

The mechanism by which contrast hydrotherapy acts on p38 MAPK activity and neurite outgrowth is not clear. However, one possibility is that phosphorylation is reactivated by low temperature treatment by the contrast bath for saturation of p38 MAPK phosphorylation due to continued warming. In our previous study, we found that when cells were continuously exposed to heat (44 °C) for 20 min, the extent of heat stimulation-induced phosphorylation of p38 MAPK was greatly reduced compared with that when cells were exposed to heat (44 °C) for 10 min [14].

Another possibility is that cold stimulation itself contributed to p38 MAPK phosphorylation in a contrast bath. The receptors that are stimulated by cold treatment and by heat treatment are known. The receptor that is stimulated by cold treatment is TRPM8, which is one of the transient receptor potential (TRP) ion channels. TRPM8 is activated by menthol, and it is the first TRP ion channel that was found to be activated by a low temperature. This discovery contributed to the establishment of the general theory for temperature-dependent activation of TRP ion channels [15]. The receptor that is activated by heat is TRPV1, which is also one of the TRP ion channels. TRPV1 is activated by capsaicin, which is the spicy ingredient of chilli peppers. Both TRPM8 and TRPV1 are activated by contrast bath treatment, but the relationship between activation of these receptors and activity of p38 MAPK is not known. It has been reported that the intracellular level of calcium is increased in cells in which TRP ion channels are activated [16, 17]. In our previous study, we found that neurite outgrowth of PC12m3 cells was induced when the cells were treated with a calcium ionophore [4]. It has also been reported that calcium ions are involved in the activation of P38 MAPK [16]. Neurite outgrowth induced by contrast bath treatment was suppressed by the activation of a TRP ion channel inhibitor in PC12m3 cells (Fig. 6).

Thus, the results suggest that the induction of neurite outgrowth in PC12m3 cells by contrast treatment bath via the p38 mitogen-activated protein kinase pathway is due to the activity of p38 MAPK induced by calcium influx in the cells by activated TRP ion channels.

Declarations

Author contribution statement

Hirotoshi Motoda: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Fukumi Hiragami, Kenji kawamura, Shigeki Inoue, Yutaka Gomita: Analyzed and interpreted the data.

Yoshio Kano: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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