Heliyon 6 (2020) e03301

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Modification of TRPV4 activity by acetaminophen

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ARTICLE INFO

Keywords:
Electrophysiology
Molecular neuroscience
Cell culture
Membrane
Physiology
Nervous system
Ca^{2+}
Acetaminophen
Paracetamol
TRPV4

ABSTRACT

N-Acetyl-p-aminophenol (APAP/acetaminophen) is a widely used analgesic/antipyretic with weaker inhibitory effects on cyclooxygenase compared to those of non-steroidal anti-inflammatory drugs. The effect of APAP is mediated by its metabolites, *N*-arachidonoyl-phenolamine and *N*-acetyl-*p*-benzoquinone imine, which activate transient receptor potential (TRP) channels, including TRP vanilloid 1 (TRPV1) and TRP ankyrin 1 (TRPA1) or cannabinoid receptor type 1. However, the exact molecular mechanism underlying the cellular actions of APAP remains unclear. Recently, we observed that APAP promotes cell migration through TRPV4; in this study, we examined the effect of APAP on Ca^{2+}-channel activity of TRPV4.

In the rat cell line PC12 expressing TRPV4, GSK1016790A (GSK), a TRPV4 agonist, stimulated an increase in $[Ca^{2+}]_i$; these effects were abrogated by HC-067047 treatment. This GSK-induced Ca^{2+} entry through TRPV4 was inhibited by APAP in a dose-dependent manner, whereas APAP alone did not affect $[Ca^{2+}]_i$. The specificity of the effect of APAP on TRPV4 was further confirmed using HeLa cells, which lack endogenous TRPV4 but stably express exogenous TRPV4 (HeLa-mTRPV4). GSK-induced $[Ca^{2+}]_i$ elevation was only observed in HeLa-mTRPV4 cells compared to that in the control HeLa cells, indicating the specific action of GSK on TRPV4. APAP dose-dependently suppressed this GSK-induced Ca^{2+} entry in HeLa-mTRPV4. However, it is unlikely that the metabolites of APAP were involved in these effects as the reaction in this study was rapid.

The results suggest that APAP suppresses the newly identified target TRPV4 without being metabolized and exerts antipyretic/analgesic and/or other effects on TRPV4-related phenomena in the body. The effect of APAP on TRPV4 was opposite to that on TRPV1 or TRPA1, as the latter is activated by APAP.

1. Introduction

Acetaminophen [paracetamol, *N*-acetyl-p-aminophenol (APAP)] is one of the most frequently used antipyretic analgesics. The other widely used analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), exert their effects by inhibiting cyclooxygenase (COX), but also have related adverse effects that are characteristic of NSAIDs, whereas APAP shows a low risk of COX-related adverse effects. In contrast, although various molecules have been implicated in the mechanism of APAP's antipyretic and analgesic effects, detailed mechanisms remain unclear. It has been suggested that APAP is metabolized *in vivo* to *N*-arachidonoylaminophenol (AM404) and/or *N*-acetyl-*p*-benzoquinone imine (NAPQI), which then activate transient receptor potential vanilloid 1 (TRPV1) and/or transient receptor potential ankyrin 1 (TRPA1) [1, 2, 3, 4, 5, 6].

TRP channels are non-selective cation channel type receptors present in the cell membrane and consist of 7 subfamilies including TRPV and TRPA in humans [7]. Some of these channels are stimulated by changes in temperature, acid, alkali, osmotic pressure, and pressure, and play important roles in sensing various physical and chemical stimuli such as visual, taste, olfactory, auditory, tactile, and warming [8, 9]. One such TRP superfamily member, TRPV4, is a Ca^{2+}-permeable channel that has been functionally identified as an osmotic sensor [10, 11]. TRPV4 is widely expressed in the body including in the brain, skin, bone, lung, bladder epithelium, and vascular endothelium and is activated by relatively low temperatures, hypo-osmotic stimulation, mechanical stimulation, acid, arachidonic acid metabolites, and endogenous cannabinoids, among other factors [8, 9]. TRPV4 has also been implicated in a wide variety of disorders, including various musculoskeletal diseases and bladder dysfunction, respiratory function abnormality, and skin dryness [12, 13].

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https://doi.org/10.1016/j.heliyon.2020.e03301
Received 1 March 2019; Received in revised form 15 December 2019; Accepted 22 January 2020

Recently, we reported that APAP promotes cell migration while investigating the cellular functions modified by APAP [14]. The involvement of previously known targets of APAP such as TRPV1 and TRPA1 in cell migration was pharmacologically examined. Unexpectedly, the results suggested that APAP exerts its function through TRPV4, which has not been reported as a target of APAP. Therefore, in this study, we examined the effect of APAP on the TRPV4 Ca^{2+} channel activity. Experiments using cell lines that endogenously express TRPV4 and cell lines stably expressing exogenous TRPV4 revealed that APAP inhibits the Ca^{2+} channel activity of TRPV4.

2. Materials and methods

2.1. Reagents

GSK1016790A (GSK), HC-067047 (HC), and AM404 were all purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan) and APAP was purchased from Showa Yakushin Kako (Tokyo, Japan). Anti-TRPV4 antibody was purchased from Abcam (Cambridge, UK), anti-β-actin antibody was from FUJIFILM Wako Pure Chemical, and horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG antibodies were from Cell Signaling Technology (Danvers, MA).

2.2. Cell culture

The rat adrenal pheochromocytoma-derived cell line PC12, mouse calvaria-derived cell line MC3T3-E1, human cervical carcinoma HeLa cells, and other human-derived cell lines including Ca9-22, SAS, HaCaT, HSC-2, and HEK293 were obtained from RIKEN BioResource Research Center (Ibaraki, Japan) and maintained in the growth medium according to the instructions.

2.3. Establishment of exogenous TRPV4 stably expressing cell line

A human cell line, HeLa, which does not express detectable levels of endogenous TRPV4, was first transfected with the mouse TRPV4 gene (mTRPV4/pcDNA3.1; generous gift from Prof. Makoto Tominaga) [15]. Next, a single-cell cloning was performed by the limiting dilution method with G418 supplementation. Three of the 12 clones expressed high levels of mTRPV4 and showed comparable responses to the TRPV4 agonist. One of the three clones was used as HeLa-mTRPV4 in this study.

2.4. Reverse transcription (RT)-PCR

The total RNA from each cell line or rat brain was prepared using ISOGEN II (Nippon Gene, Tokyo, Japan) or NucleoSpin RNA (Takara Bio, Shiga, Japan), followed by the reverse transcription reaction using the ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). Then, PCR was performed using Quick Taq HS DyeMix (TOYOBO). The expression of each gene was confirmed by agarose gel electrophoresis of the PCR products. The primers used for PCR are as follows: Mouse and rat β-actin (forward; 5′-TGTCCACCTTCCAGCAGATGT-3′, reverse; 5′-AGCTCAGTAACAGTCC GCCTAG-3’) , mouse and rat TRPV1 (forward; 5′-TCCTTGAGTTGCCA-GAGTATGC-3′, reverse; 5′-ATGGAGCAAGTGCCTTGGAG-3′), mouse and rat TRPV2 (forward; 5′-ATAGAGCAGGAAGCTGTGGTAG-3′, reverse; 5′-GTATCGTCCACCCTCCACCT-3′), mouse and rat TRPV4 (forward; 5′-TTCTTCTCTCTACGACCTCTCC-3′, reverse; 5′-ACAGTTCGTTAATGG GCTCTAC-3′), mouse and rat TRPA1 (forward; 5′-ACAATGACGGATG-CACACCT-3′, reverse; 5′-TCCCATGGAGATCCCCTTCA-3′), human β-actin (forward; 5′-CATGTACGTTGCTATCCAGGC-3′, reverse; 5′-CTCCTTAATGTCACGCACGAT-3′), human TRPV4 (forward; 5′-TCCTC CCTGGCCAATCTGTT-3′, reverse; 5′-CAGCAGATCGATGGGGTTGG-3′), human TRPV1 (forward; 5′-GCCTGGAGCTGTTCAAGTTC-3′, reverse; 5′-TCTCCTGTGCGATCTTGTTG-3′), rat FAAH (forward; 5′-TGAATGAGGG-CATGCCATCG-3′, reverse; 5′-TTCCACGGGTTCATGGTCTG-3′), human FAAH (forward; 5′-TGAATGAAGGGGTGCCGGC-3′, reverse; 5′-TTCCACGGGTTCATGGTCTG-3′). This study was approved by and performed in accordance with the guidelines of the institutional animal research ethics committee of the Kyushu Dental University (Approval number, 07–047).

2.5. Measurement of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)

Cells seeded into a glass-bottom dish (Matsunami Glass, Osaka, Japan) were incubated in a serum-free medium containing 1 mg/mL of Fura-2-acetoxymethyl ester (AM) (Dojindo Laboratories, Kumamoto, Japan). After the loading of Fura-2, the cells were washed with Hank's balanced salt solution (HBSS; #14065056, Thermo Fisher Scientific, Waltham, MA, USA). The fluorescence was measured at 500 nm following excitation at 340 nm and 380 nm and was recorded for each single cell using a fluorescence image analysis system (AQUACOSMOS, Hamamatsu, Shizuoka, Japan). The change in $[Ca^{2+}]_i$ was calculated as the ratio of fluorescence intensity following excitation at each wavelength. Each reagent was added directly to the dish with a pipette tip or refluxing HBSS using a peristaltic pump (ATTO, Tokyo, Japan) at 1 mL/min while switching to HBSS containing the appropriate reagent.

2.6. Western blot analysis

An equal number of cells were washed with ice-cold phosphate-buffered saline and solubilized in Laemmli's sample buffer (1% sodium dodecyl sulfate, 1% β-mercaptoethanol, 6% glycerol, 0.002% bromophenol blue, and 0.2 M Tris-HCl) and then boiled for 10 min. The lysates were subjected to western blot analysis. The proteins of interest were visualized using the enhanced chemiluminescent substrate reagent Immobilon (Merck-Millipore, Billerica, MA, USA) and images were obtained using LAS-3000mini (FUJIFILM).

2.7. Statistical analyses

Unless indicated otherwise, quantified data are presented as the means ± SEM and statistical analysis of data was performed by one-way analysis of variance (ANOVA) among means, followed by posthoc comparisons to determine the significant differences between groups using the Tukey-Kramer HSD (honestly significant difference) test. JMP 15 software (SAS Institute, Cary, NC, USA) was used for statistical analysis.

3. Results

3.1. TRPV4-dependent $[Ca^{2+}]_i$ mobilization in PC12 cells

Previously, using the mouse osteoblast-like cell line MC3T3-E1, we reported that APAP promotes cell migration which may be mediated through TRPV4 [14]. In this study, we examined the effect of APAP on the channel activity of TRPV4 using MC3T3-E1 cells. However, the response of MC3T3-E1 to the TRPV4 agonist GSK was extremely weak and unstable (data not shown). Therefore, we examined whether the rat PC12 cells, well-known to differentiate into neuron-like cells, could be used, as TRPV4 has been reported to be involved in the neurite outgrowth of PC12 cells [16]. As shown in Figure 1A, the mRNA expression of TRPV2 and TRPV4, but not TRPV1 and TRPA1, was detected in PC12 cells. This was appropriate to this study owing to the absence of TRPV1 and TRPA1 expression, both of which are known targets of APAP [1, 5].

Next, we tested whether TRPV4 was activated in PC12 cells. PC12 cells were labeled with the Ca^{2+} fluorescent indicator Fura-2-AM and stimulated with various concentrations of the TRPV4 agonist GSK1016790A (GSK). As shown in Figure 1B, a strong increase in the fluorescence ratio at 340/380 nm was observed in a concentration-dependent manner, indicating that TRPV4 mobilized $[Ca^{2+}]_i$ in PC12 cells. Furthermore, this GSK-induced $[Ca^{2+}]_i$ increase was abrogated by a specific inhibitor of TRPV4, HC-067407 (HC), in a concentration-dependent manner (Figure 1C). These results indicated that the

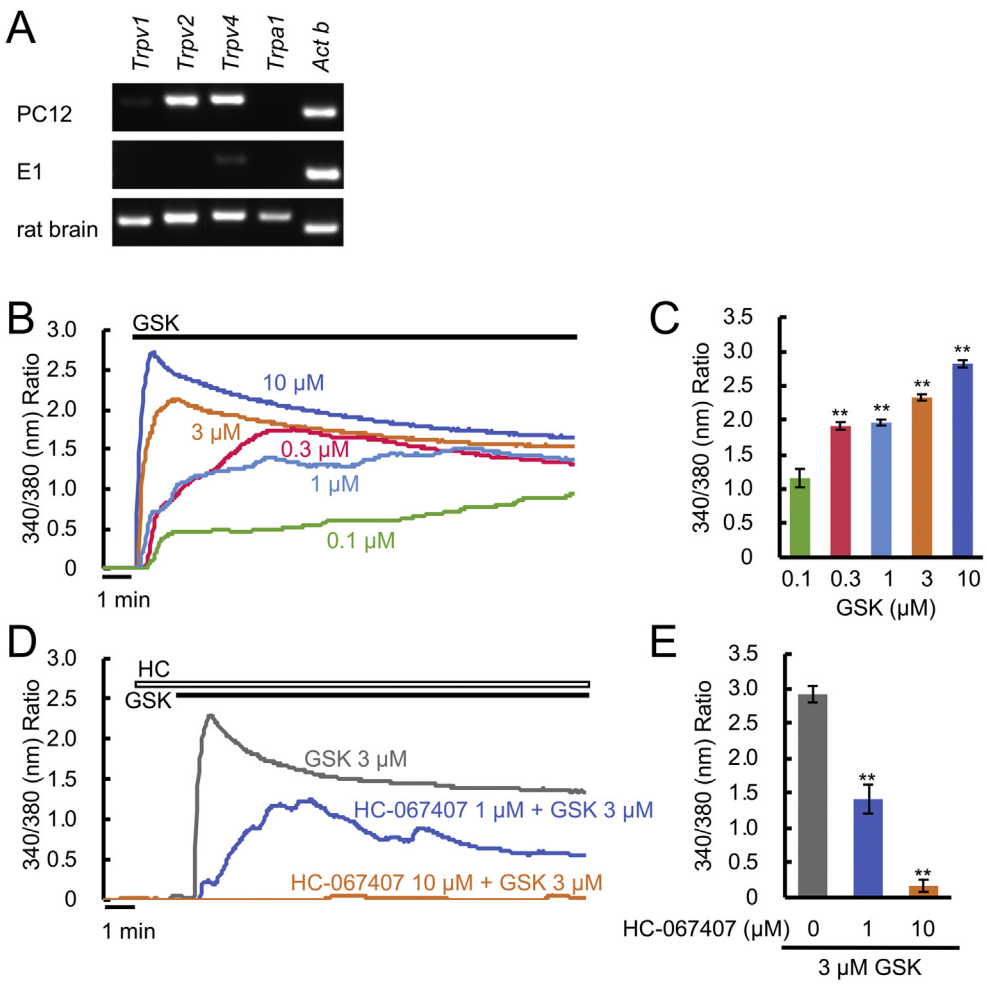

Figure 1. TRPV4-dependent $[Ca^{2+}]_i$ change in PC12 cells (A) Gene expression of TRP channels. Total RNA prepared from PC12, MC3T3-E1 (E1), and rat brain were subjected to reverse transcription followed by PCR using primers to assess mRNA expression of the indicated genes. Typical images of PCR products separated by agarose gel are shown. Intact images are shown in the supplementary Fig. S1 (B) PC12 cells were loaded with Fura-2-AM and stimulated with the TRPV4 agonist GSK1016790A (GSK). The change in the ratio of 510-nm fluorescence intensity excited at 340/380 nm was recorded and traces of the mean values from cells treated with the indicated concentrations of GSK are presented (C) Peak amplitudes in the GSK-induced $[Ca^{2+}]_i$ increase were measured and summarized in the bar graph. Each bar represents the means ± SEM of three independent experiments with approximately 10–20 cells in each experiment. $^{**}P < 0.01$ versus the corresponding value for cells stimulated with 0.1 μM GSK (D) Fura-2-loaded PC12 cells were first incubated with the indicated concentrations of HC-067047 (HC) or the vehicle alone, followed by stimulation with 3 μM GSK. Traces of the mean values from the cells are presented (E) Peak amplitudes in the GSK-induced $[Ca^{2+}]_i$ rise are summarized as in (C). $^{**}P < 0.01$ versus the corresponding value for cells treated with 3 μM GSK anole (0 μM HC-067407).

channel activity of TRPV4 can be observed as a change in $[Ca^{2+}]_i$ induced by GSK stimulation of PC12 cells.

3.2. Effect of APAP and AM404 on $[Ca^{2+}]_i$ in PC12 cells

It has been reported that APAP is metabolized into AM404 which then activates TRPV1 and TRPA1 *in vivo*. Hence, we evaluated whether APAP and/or AM404 activated TRPV4. As shown in Figure 2A, APAP and AM404, up to 1 mM and 100 μM, respectively, did not alter the $[Ca^{2+}]_i$ in PC12 cells. The results indicated that neither APAP nor AM404 alone affects the Ca^{2+} channel activity of TRPV4.

3.3. Effect of APAP on $[Ca^{2+}]_i$ increase by GSK in PC12 cells

Next, we investigated the effect of APAP on $[Ca^{2+}]_i$ elevation mediated through TRPV4 in PC12 cells. As shown in Figures 2B and 2C, APAP suppressed the elevation in $[Ca^{2+}]_i$ stimulated by 3 μM GSK in a dose-dependent manner (0.1–10 μM). Considering the short contact time with the cells, these results suggested that APAP suppressed the $[Ca^{2+}]_i$ increase without being metabolized.

3.4. Effect of AM404 on $[Ca^{2+}]_i$ increase by GSK in PC12 cells

As AM404 was shown to be metabolized from APAP *in vivo* and activated TRPV1 and TRPA1, we also examined whether AM404 influenced the TRPV4-dependent $[Ca^{2+}]_i$ increase. As shown in Figures 2D and 2E, AM404 suppressed $[Ca^{2+}]_i$ elevation stimulated by 3 μM GSK in a dose-dependent manner (10–100 μM). Although higher concentrations of AM404 were not examined, as AM404 cannot be dissolved at concentrations higher than 100 μM, the inhibitory effect of less than 100 μM AM404 was comparable to that of APAP, i.e., the GSK-induced $[Ca^{2+}]_i$ elevation was suppressed by approximately 20% of the control by 100 μM APAP and 30% of the control by 100 μM AM404 (Figures 2C and 2E).

3.5. Effect of APAP on cells expressing exogenous TRPV4

Owing to the possibility that the APAP effect on Ca^{2+} channel activity in PC12 cells was mediated through other channels but not through TRPV4, we next investigated the effect of APAP using cells devoid of endogenous TRPV4 but expressing exogenously transfected TRPV4. As shown in Figure 3A, only HeLa cells failed to express detectable levels of endogenous TRPV4 among the human cell lines examined. However, all cells, including HeLa cells, expressed comparable levels of TRPV1. Thus, we used HeLa cells to generate a cell line stably expressing exogenously transfected mouse TRPV4 (HeLa-mTRPV4 cells). The expression of both TRPV4 mRNA and protein in the established cell line is shown in Figure 3B.

As shown in Figure 3C, an increased $[Ca^{2+}]_i$ stimulated by 100 nM GSK was observed only in HeLa-mTRPV4 cells, but not in control HeLa cells. This GSK-induced $[Ca^{2+}]_i$ elevation was completely blocked in the presence of HC, the specific TRPV4 inhibitor (Figure 3D), confirming that the $[Ca^{2+}]_i$ increase observed in GSK-treated HeLa-mTRPV4 cells was mediated through TRPV4. These data indicated that HeLa-mTRPV4 was suitable for evaluating the effect of APAP on channel activity specific to TRPV4. Additionally, we generated HeLa cells stably expressing enhanced green fluorescent protein together with HeLa-mTRPV4 and

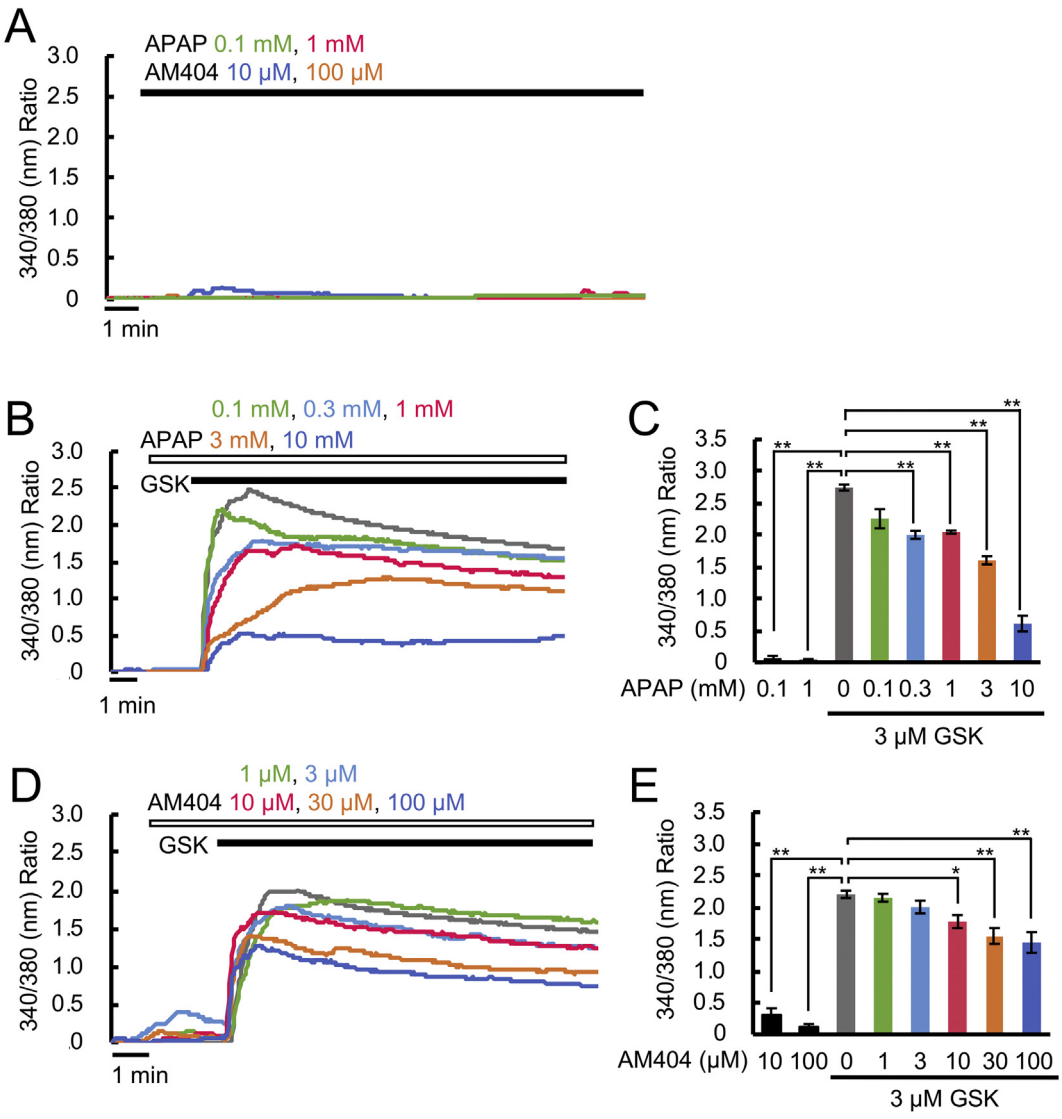

Figure 2. Effect of APAP and AM 404 on $[Ca^{2+}]_i$ of PC12 cells (A) PC12 cells labeled with Fura-2-AM were treated with APAP (0.1 or 1 mM) or AM404 (10 or 100 μM) and the change in the ratio of 510-nm fluorescence intensity excited at 340/380 nm was recorded over time. Traces of the mean values from cells treated with the indicated concentrations of either APAP or AM404 are shown (B) Fura-2-loaded PC12 cells were first incubated with various concentrations of APAP, followed by adding 3 μM GSK1016790A (GSK). Traces of the mean values from the cells are shown (C) Summary of peak amplitudes in the GSK-induced increase in $[Ca^{2+}]_i$. Each bar represents the means ± SEM of three independent experiments with approximately 10–20 cells in each experiment. $^{**}P < 0.01$ versus the corresponding value for cells treated with 3 μM GSK in the absence of APAP (0 mM APAP) (D) Fura-2-loaded PC12 cells were first incubated with various concentrations of AM404, followed by adding 3 μM GSK. Traces of mean values from the cells treated with different concentrations of AM404 are shown (E) Summary of peak amplitudes in the GSK-induced increase in $[Ca^{2+}]_i$. Each bar represents the means ± SEM of three independent experiments with approximately 10–20 cells in each experiment. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the corresponding value for cells treated with 3 μM GSK in the absence of AM404 (0 μM AM404).

examined the increase in $[Ca^{2+}]_i$ induced by GSK. The results confirmed that there were no differences between enhanced green fluorescent protein-expressing HeLa cells and wild-type HeLa cells (data not shown); thus wild-type HeLa cells were used as the control in this study.

Next, we examined whether APAP suppressed the Ca^{2+} channel activity of TRPV4 using HeLa-mTRPV4 cells. As shown in Figures 3E and 3F, the increase in $[Ca^{2+}]_i$ induced by 100 nM GSK was inhibited by APAP in a dose-dependent manner. These results strongly suggest that APAP suppressed the Ca^{2+} channel activity of TRPV4.

3.6. Expression of the enzyme required for converting APAP to AM404

To examine the possibility that the APAP effect on TRPV4 was mediated by the metabolite AM404, we examined whether fatty acid amide hydrolase (FAAH), an enzyme required for converting APAP to AM404, was expressed in PC12 and HeLa-mTRPV4 cells. As shown in Figure 4, PC12 cells expressed FAAH mRNA at a level comparable to that in the rat brain. However, the expression of human FAAH mRNA was not detected in HeLa-mTRPV4 cells, whereas it was observed in another human cell line, SAS.

4. Discussion

In this study, we observed that APAP inhibited TRPV4-mediated Ca^{2+} entry into PC12 and HeLa-mTRPV4 cells, in addition to MC3T3-E1 cells in which we observed a functional relationship between APAP and TRPV4. In initial experiments using MC3T3-E1 cells, GSK-stimulated changes in $[Ca^{2+}]_i$, detected by labeling the cells with Fura-2-AM, were extremely weak, and approximately 300-fold higher concentrations of both the agonist (GSK) and antagonist (HC) for TRPV4 were required compared to the concentrations generally used in similar studies (data not shown). Therefore, we used PC12 cells which responded to

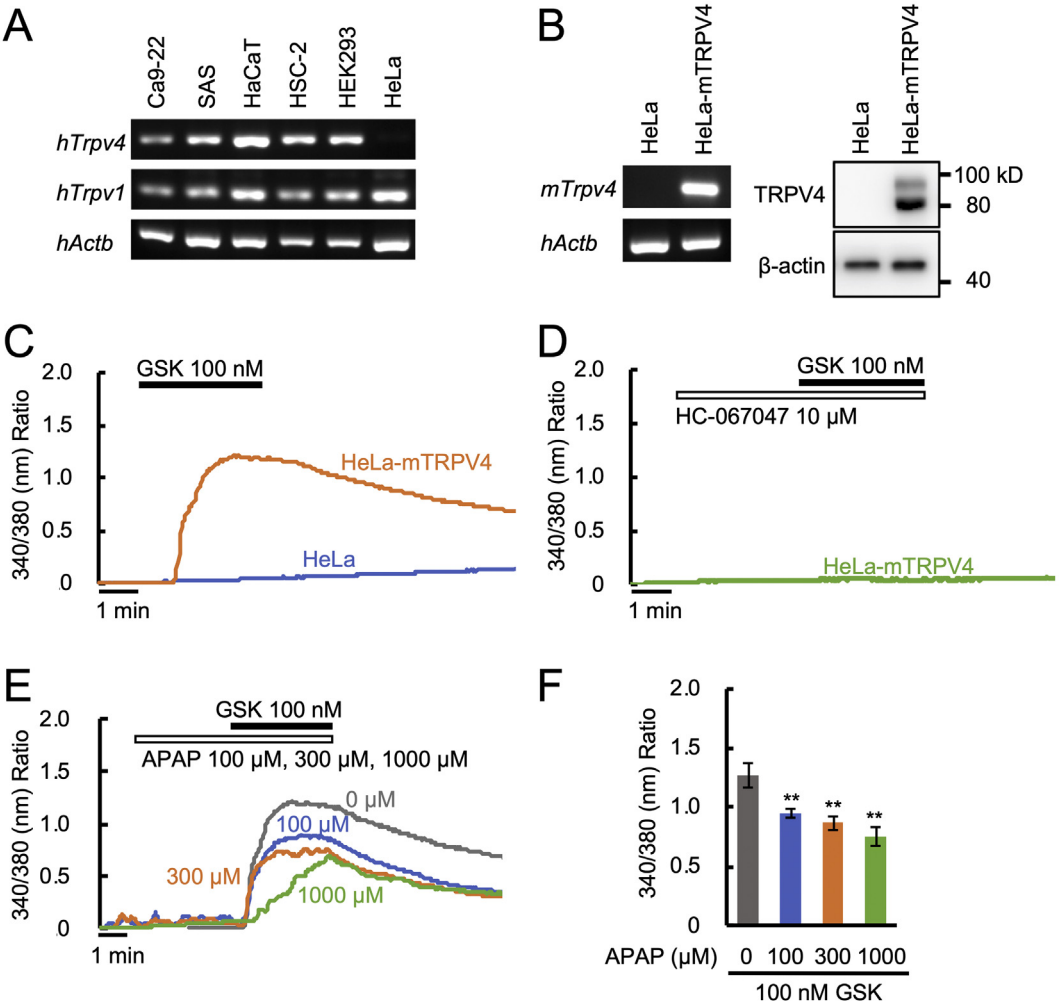

Figure 3. Effect of APAP on $[Ca^{2+}]_i$ in HeLa cells stably expressing exogenous TRPV4 (A) Total RNA prepared from Ca9-22, SAS, HaCaT, HSC-2, HEK293, and HeLa cells were subjected to reverse transcription followed by PCR using primers to assess mRNA expression of the indicated genes. Typical images of PCR products separated by agarose gel are shown. Intact images are shown in Fig. S2 (B) Total RNA prepared from wild-type HeLa (HeLa) and stable HeLa cell line expressing exogenous mouse TRPV4 (HeLa-mTRPV4) were subjected to reverse transcription followed by PCR using primers to assess the mRNA expression of the indicated genes. Typical images of PCR products separated on the agarose gel are shown in the left panels. Intact images are shown in Fig. S3. Expression of mouse TRPV4 mRNA was detected in HeLa-TRPV4 cells, but not in wild-type HeLa cells. The total cell lysate was prepared from an equal number of the control HeLa and HeLa-mTRPV4 cells. The lysates were subjected to western blot analysis using antibodies against the indicated proteins. Typical images of immunoblots are shown in the right panels. The full area of the selected blots is shown in Fig. S4 of the supplementary file (C) Fura-2-loaded HeLa-mTRPV4 cells were stimulated with the indicated concentrations of GSK1016790A (GSK), but not HeLa cells. Traces of mean values from the cells treated with GSK are shown (D) Fura-2-loaded HeLa-mTRPV4 cells were first incubated with 10 μM HC-067407, followed by adding 100 nM GSK. Typical traces of mean values from the cells are shown (E) Fura-2-loaded HeLa-mTRPV4 cells were first incubated with various concentrations of APAP, followed by adding 100 nM GSK1016790A (GSK). Traces of mean values from the cells are shown (F) Summary of peak amplitudes in the GSK-induced increase in $[Ca^{2+}]_i$. Each bar represents the means ± SEM of at least three experiments. $^{**}P < 0.01$ versus the corresponding value for cells treated with 100 nM GSK alone (0 μM APAP).

micromolar levels of GSK, although these concentrations were still more than 10-fold higher compared to the typical concentrations used. The reason why such a high concentration of the agonist was required to activate TRPV4 remains unknown. One possibility is that the experiments were conducted at approximately 25 °C, and the channel activity of TRPV4 is largely temperature-dependent [17, 18]. Nevertheless, it was confirmed that the effect of GSK on $[Ca^{2+}]_i$ increase in PC12 cells was mediated through TRPV4, using a complete blockage of the GSK-stimulated $[Ca^{2+}]_i$ increase by HC. Therefore, it is strongly suggested that the inhibitory effect of APAP on $[Ca^{2+}]_i$ increase induced by GSK was also mediated through TRPV4. However, we performed additional experiments to further eliminate the possibility that the high concentrations of GSK and HC used in PC12 cells may have nonspecifically affected other Ca^{2+} channels rather than TRPV4, i.e., a HeLa-derived cell line (HeLa-mTRPV4) lacking the endogenous expression of TRPV4 but stably expressing exogenous TRPV4 was established and used to confirm that the effect of APAP on the GSK-induced increase in $[Ca^{2+}]_i$ was mediated through TRPV4.

Notably, the effects of APAP on TRPV4 differed from the previously reported action on TRPV1 and TRPA1 [6,19,20] in at least two ways. First, the effect on TRPV4 was suppressive, while both TRPV1 and TRPA1 were activated by APAP *in vivo* [1, 5, 6]. Second, APAP exerted its effect without being metabolized, while the effects of APAP on both TRPV1 and TRPA1 were exerted by its metabolites including AM404 and NAPQI, respectively [1, 2, 5]. Although the effect of AM404 on TRPV4 activity was not examined at concentrations higher than 100 μM owing to the insolubility of these compounds, AM404 demonstrated comparable dose-dependency in suppressing TRPV4-mediated Ca^{2+} entry at the examined concentrations. However, the contact time with the cells during the $[Ca^{2+}]_i$ measurement would not be enough for APAP to be converted to the required amount of AM404 to suppress TRPV4. Moreover, although PC12 cells expressed FAAH, a key enzyme converting APAP to

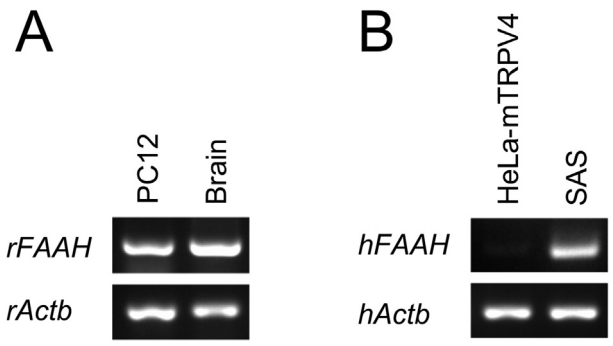

Figure 4. Expression of FAAH in PC12 and HeLa-mTRPV4 cells (A) Total RNA prepared from PC12 cells and rat brain was subjected to reverse transcription (RT) followed by PCR using primers for assessing mRNA expression of rat FAAH (rFAAH) or β-actin (rActb). Typical images of the PCR products separated on agarose gel are shown and the source images of agarose gel are shown in Supplementary Fig. S5 (B) RT-PCR was performed to detect mRNA expression of human FAAH (hFAAH) and β-actin (hActb) in HeLa-mTRPV4 cells and in SAS cells as a positive control expressing hFAAH. Typical images of the PCR products are shown and the source images of agarose gel are shown in Supplementary Fig. S5.

AM404, HeLa-mTRPV4 cells did not express detectable levels of FAAH mRNA, supporting that the inhibition of TRPV4 Ca^{2+} channel activity observed in the study was mediated through APAP itself. AM404, also known as *N*-arachidonoylaminophenol, is converted from APAP by conjugation with arachidonic acid and migrates into the cell to act on the intracellular region of the TRP channel [1]. In contrast, APAP may be transported through the cellular membrane into the cell in its original form. However, it is also postulated that APAP is first metabolized to *p*-aminophenol mainly in the liver *in vivo* and then taken up by cells [1]. Thus, the direct target site of APAP remains unclear, including whether the site is outside or inside of the cellular membrane. Hence, predicting the molecular mechanism of how APAP influences the Ca^{2+} channel activity of TRPV4 is complex.

The GSK-stimulated increase in $[Ca^{2+}]_i$ was decreased to approximately 30% of the control in the presence of 10 mM APAP against 3 μM GSK in PC12 cells and approximately 60% or 70% of the control in the presence of 1000 or 100 μM APAP, respectively, against 100 nM GSK in HeLa-mTRPV4 cells. Although the concentrations of GSK used in this study were very high as mentioned above, 100 μM (15 μg/mL) APAP is within the clinically used dose, as the peak concentrations of APAP in the blood range from 8 to 32 μg/mL after the recommended doses are orally administered [21], suggesting that APAP affects TRPV4 *in vivo*.

The involvement of TRPV1 and TRPA1 in the analgesic and antipyretic actions of APAP, mediated through its metabolites AM404 and NAPQI, have widely been examined in studies using mice with genetic deficiencies in the APAP-metabolizing enzymes, TRPV1, and TRPA1 [2,5,6]. In contrast, TRPV4 has not been reported as a target molecule of APAP. However, some studies have supported this possibility. First, TRPV4 is expressed in the peripheral nerve endings of sensory ganglia, A fiber, and C fiber [22]. Second, the susceptibility to acid and pressure noxious stimuli were decreased in genetically deficient TRPV4 mice [22]. Third, the sensitization of TRPV4 by prostaglandin E2 was suggested to be involved in the hyperalgesic response to heat, hypotonic pressure, and pressure stimulation at the site of inflammation [22]. Therefore, TRPV4 may also be involved in the clinical effects of APAP.

It would be interesting not only to clarify the involvement of TRPV4 in the analgesic/antipyretic action of APAP, but also to investigate the effect of APAP on skin barrier function, bladder sensation, the cardiovascular system, and bone remodeling, which are thought to involve TRPV4 based on studies using TRPV4 gene-deficient mice [12, 13, 23]. To clarify the molecular mechanism of how APAP suppresses Ca^{2+} entry into cells through TRPV4, studies aimed at identifying the important sites on TRPV4 targeted by APAP, using site-directed point mutagenesis or molecules that interact with TRPV4 and control the activity or trafficking of the channel, are required while considering both the direct and indirect effects of APAP on TRPV4. Further studies using electrophysiological recordings could elucidate a mechanistic insight into how APAP inhibits TRPV4. These results will provide insight into the mechanism of the antipyretic/analgesic action of APAP.

In conclusion, APAP suppressed the $[Ca^{2+}]_i$ response mediated by TRPV4. Owing to this inhibitory effect that was observed at the concentration range of APAP close to that used clinically, the results suggest that TRPV4 is a new target of APAP. These results contribute to the application of APAP for treating diseases related to TRPV4 and the development of more appropriate methods using APAP. Indeed, because TRPV4 is implicated in the induction of pulmonary edema associated with heart failure and is related to the homeostasis of cardiovascular functions, the development of a TRPV4 inhibitor for preventing and treating pulmonary edema has been suggested [24].

Declarations

Author contribution statement

Fumio Nakagawa: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sen Higashi, Eika Ando: Performed the experiments.

Tomoko Ohsumi: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Seiji Watanabe: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Hiroshi Takeuchi: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Funding statement

This work was supported by a grant-in-aid from Kyushu Dental University Internal Grants (to HT).

Competing interest statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2020.e03301.

Acknowledgements

We are grateful to Professor Makoto Tominaga (National Institute for Physiological Sciences) for providing the plasmid to express mouse TRPV4.

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