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Effect of non-steroidal anti-inflammatory drugs and new fenamate analogues on TRPC4 and TRPC5 channels

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ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used anti-inflammatory therapeutic agents, among which the fenamate analogues play important roles in regulating intracellular Ca^{2+} transient and ion channels. However, the effect of NSAIDs on TRPC4 and TRPC5 is still unknown. To understand the structure–activity of fenamate analogues on TRPC channels, we have synthesized a series of fenamate analogues and investigated their effects on TRPC4 and TRPC5 channels.

Human TRPC4 and TRPC5 cDNAs in tetracycline-regulated vectors were transfected into HEK293 T-REx cells. The whole cell current and Ca^{2+} movement were recorded by patch clamp and calcium imaging, respectively.

Flufenamic acid (FFA), mefenamic acid (MFA), niflumic acid (NFA) and diclofenac sodium (DFS) showed inhibition on TRPC4 and TRPC5 channels in a concentration-dependent manner. The potency was FFA > MFA > NFA > DFS. Modification of 2-phenylamino ring by substitution of the trifluoromethyl group in FFA with –F, $–CH_3$, $–OCH_3$, $–OCH_2CH_3$, –COOH, and $–NO_2$ led to the changes in their channel blocking activity. However, 2-(2′-methoxy-5′-methylphenyl)aminobenzoic acid stimulated TRPC4 and TRPC5 channels. Selective COX1-3 inhibitors (aspirin, celecoxib, acetaminophen, and indomethacin) had no effect on the channels. Longer perfusion (>5 min) with FFA (100 μM) and MFA (100 μM) caused a potentiation of TRPC4 and TRPC5 currents after their initial blocking effects that appeared to be partially mediated by the mitochondrial Ca^{2+} release.

Our results suggest that fenamate analogues are direct modulators of TRPC4 and TRPC5 channels. The substitution pattern and conformation of the 2-phenylamino ring could alter their blocking activity, which is important for understanding fenamate pharmacology and new drug development targeting the TRPC channels.

1. Introduction

The transient receptor potential (TRP) channel family is a class of Ca^{2+}-permeable channels. To date, 28 mammalian TRP channels have been identified and divided into six subfamilies on the basis of amino acid sequence homology: TRPC (“canonical”), TRPM (“melastatin”), TRPV (“vanilloid”), TRPA (“ankyrin”), TRPML (“mucolipin”), and TRPP (or PKD) (“polycystin”). The canonical TRP subfamily (TRPC) has seven members that can be grouped into three subgroups: TRPC1/4/5, TRPC3/6/7, and TRPC2 (a pseudogene in human) [1]. Human TRPC channels seem to be ubiqutously distributed and mediate the Ca^{2+} influx evoked by G-protein coupled receptor activation or/and Ca^{2+} store depletion which are involved in many cellular functions, such as cell proliferation, apoptosis, secretion, smooth muscle contraction and migration [2–4]. TRPCs have been demonstrated as the potential therapeutic targets for neointimal growth, segmental glomerulosclerosis, overexposure to mercury, and rheumatoid arthritis [2–7]. Therefore, the identification of regulators of these channels is important for understanding their physiological properties in native cells that may lead to new therapeutic agents.

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of fever, pain and inflammation. Some like aspirin have the potential for the prevention of ischemic heart diseases, but some NSAIDs may increase the cardiovascular risk, such as the fenamate analogue diclofenac [8–11]. The cardiovascular risk associated with NSAIDs seems to be not determined by the selectivity of COX-2 [8]. In addition, the risk of some types of cancer was reduced by aspirin [12,13] and non-aspirin NSAIDs [14]. These observations suggest that mechanisms other than COX-2 inhibition may exist. Indeed, several possible mechanisms have been proposed for the action of NSAIDs including anti-proliferative

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effects by regulating several target genes [12], stabilization of DNA mismatch repair [15], and regulation of cytosolic and mitochondrial Ca^{2+} homeostasis [12,16].

The relevance of NSAIDs to Ca^{2+} signalling has been recognized through the inhibition of production of prostaglandins and leukotrienes from arachidonic acid (AA) by cyclooxygenase (COX) inhibition, and AA and its metabolites themselves can influence Ca^{2+} influx through a number of Ca^{2+}-permeable channels [17–19]. In addition, AA release from membrane phospholipids by phospholipase A2 (PLA2) is also Ca^{2+}-related, since the group IVA PLA2 (also known as cytosol PLA2α) activation is Ca^{2+}-dependent [20]. These indirect effects on Ca^{2+} signal through COX inhibition could be a class effect of NSAIDs. On the other hand, the PLA2 inhibitor N-(p-amylcinnamoyl)anthranilic acid (ACA) inhibited TRPM2 channels, but PLA2 inhibitors without the skeleton of anthranilic acid had no effect on the channel [21], suggesting the parent structure of anthranilic acid is essential for the channel blocking effect. The fenamate NSAIDs are anthranilic acid derivatives with structural similarity to ACA [22], and their effects on a variety of channels have been demonstrated including the Ca^{2+}-activated Cl^- channels, the voltage-dependent Na^+ or Ca^{2+} channels, and the TRP channels [23], for example, flufenamic acid (FFA) inhibited TRPM2, TRPM4, TRPM5, TRPC3, and TRPC5 [24,25], but activated TRPC6 [26–28] and TRPA1 [29]. These observations indicated that NSAIDs can influence intracellular Ca^{2+} level or Ca^{2+}-permeable channels in both direct and indirect ways, and the compounds with the skeleton of anthranilic acid are critical for the direct effect. Therefore, we hypothesized that non-fenamate NSAIDs may exert their effects mainly through PLA2/COX pathway or other mechanisms, while the fenamate NSAIDs could modulate Ca^{2+}-permeable channel activity directly. Structure modification on the fenamate skeleton could directly alter the potency of the channel blocking effect.

To test our hypothesis, we examined the effects of fenamate analogues, non-fenamate NSAIDs, and selective COX1-3 inhibitors on TRPC4 and TRPC5 channels. The subgroup of TRPC4/5 was used in this study, because the current for TRPC4 and TRPC5 is robust and stable after channel activation and therefore suitable for screening new drugs to address the unknown effect of NSAIDs on TRPC4/5 channels. To understand the structure–activity relationship of fenamates, we synthesized fenamate analogues by the 2-phenylamino ring modification and compared their effects on the TRPC channels. Moreover, as several reports have suggested that NSAIDs may interfere with mitochondria Ca^{2+} release [30,31], the involvement of intracellular Ca^{2+} homeostasis in the TRPC channel activity regulated by NSAIDs was also investigated.

2. Materials and methods

2.1. Cell culture and transfection

Human TRPC4α (GenBank accession number NM_016179) and TRPC5 (AF054568) in a tetracycline-regulatory vector were transfected into HEK-293 T-REx cells (Invitrogen, Paisley, UK). TRPC4 was tagged with enhanced yellow fluorescent protein (EYFP) at the N-terminus. The expression was induced by 1 μg ml^{-1} tetracycline for 48–72 h before recording. The non-induced cells without addition of tetracycline were used as control. Cells were grown in DMEM-F12 medium (Invitrogen, Paisley, UK) containing 10% foetal calf serum (FCS), 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cells were maintained at 37 °C under 95% air and 5% CO_2 and seeded on coverslips prior to experiments.

2.2. Smooth muscle cell isolation and culture

Eight-week-old male rats were killed by inhalation of CO_2 in accordance with the Schedule 1 in the Code of Practice of UK Animals Scientific Procedures Act 1986. The thoracic aorta was dissected out and the adventitia was carefully removed. The endothelium was removed by gently rubbing the luminal surface with a curved forceps. The smooth muscle layer was cut into 0.5 mm^2 segments and cultured in DMEM-F12 including 10% FBS and antibiotics for cell expansion. The cultured smooth muscle cells at passage 4–5 were used for calcium imaging experiment.

2.3. Electrophysiological recordings

Whole-cell clamp was performed at room temperature (23–26 °C) [5]. Briefly, signal was amplified with an Axoclamp 2B or Axopatch B200 patch clamp amplifier and controlled with pClamp software 10. A 1-s ramp voltage protocol from −100 mV to +100 mV was applied at a frequency of 0.2 Hz from a holding potential of 0 mV. Signals were sampled at 10 kHz and filtered at 1 kHz. The glass microelectrode with a resistance of 3–5 MΩ was used. The 200 nM Ca^{2+} buffered pipette solution contained 115 CsCl, 10 EGTA, 2 $MgCl_2$, 10 HEPES, and 5.7 $CaCl_2$ in mM, pH was adjusted to 7.2 with CsOH and osmolarity was adjusted to ~290 mOsm with mannitol, and the calculated free Ca^{2+} was 200 nM using EQCAL (Biosoft, Cambridge, UK). The standard bath solution contained (mM): 130 NaCl, 5 KCl, 8 D-glucose, 10 HEPES, 1.2 $MgCl_2$ and 1.5 $CaCl_2$. The pH was adjusted to 7.4 with NaOH.

2.4. Ca^{2+} measurements

The rat aortic smooth muscle cells were preincubated with 2 μM fura-PE3 AM at 37 °C for 30 min in Ca^{2+}-free bath solution, followed by a 20-min wash period in the standard bath solution at room temperature. Fura-PE3 fluorescence was monitored with an inverted epifluorescence microscope (Nikon Ti-E, Japan). A xenon arc lamp provided excitation light, the wavelength of which was selected by a Nikon imaging system controlled by software Element 3.0. Emission was collected via 510 nm filter for fura-PE3 AM and a cooled Orca-R2 CCD camera (Hamamatsu, Japan). Images were analyzed using regions of interest (ROIs), which selected parts of the image frame corresponding to individual smooth muscle cells. The ratio of Ca^{2+} dye fluorescence (F_{340}/F_{380}) was measured by NIS-Element Ca^{2+} imaging software. Agents were applied to smooth muscle cells using a continuous bath perfusion system with a flow rate of 4 ml min^{-1}. All the experiments were performed at room temperature.

2.5. Reagents and drugs

All general salts and reagents were purchased from Sigma–Aldrich (Poole, UK). FFA, mefenamic acid (MFA), niflumic acid (NFA), diclofenac sodium (DFS), aspirin (ASP), acetaminophen (APAP), indomethacin (IND), gadolinium chloride (Gd^{3+}), 2-aminoethoxydiphenyl borate (2-APB), cyclosporine A, and foetal calf serum were purchased from Sigma–Aldrich. Celecoxib (CXB) was purchased from Cayman Chemical (Ann Arbor, MI, USA) and Fura-PE3 AM from Invitrogen (Paisley, UK). Fura-PE3 AM (5 mM) and 2-APB (100 mM) were made up as stock solutions in 100% dimethyl sulphoxide (DMSO). Ten fenamate derivatives (Fig. 2, table entries 1–10) were synthesized by the copper-catalysed coupling of either 2-chloro- or 2-bromobenzoic acid with the appropriate aniline derivative [32]. In the case of entry 5 [2-(4′-carboxyphenylamino)benzoic acid] ethyl 4-aminobenzoate was used but the ethyl ester group suffered in situ hydrolysis. Products gave satisfactory ^1H,^{13}C-NMR and mass spectra and their purity was estimated to be >95%.

2.6. Statistics

Data are expressed as mean ± S.E.M. where *n* is the cell number for electrophysiological recordings and Ca^{2+} imaging. Data sets were compared using paired *t*-test for the results before and after treatment, or the ANOVA Dunnett's post hoc analysis for comparing more than two groups with significance indicated if $P < 0.05$.

3. Results

3.1. Effect of fenamates on TRPC4 and TRPC5 channels

To examine the effect of fenamates on TRPC4 and TRPC5 channels, the whole-cell current was recorded in HEK-293 cells stably transfected with human TRPC4 or TRPC5. The expression

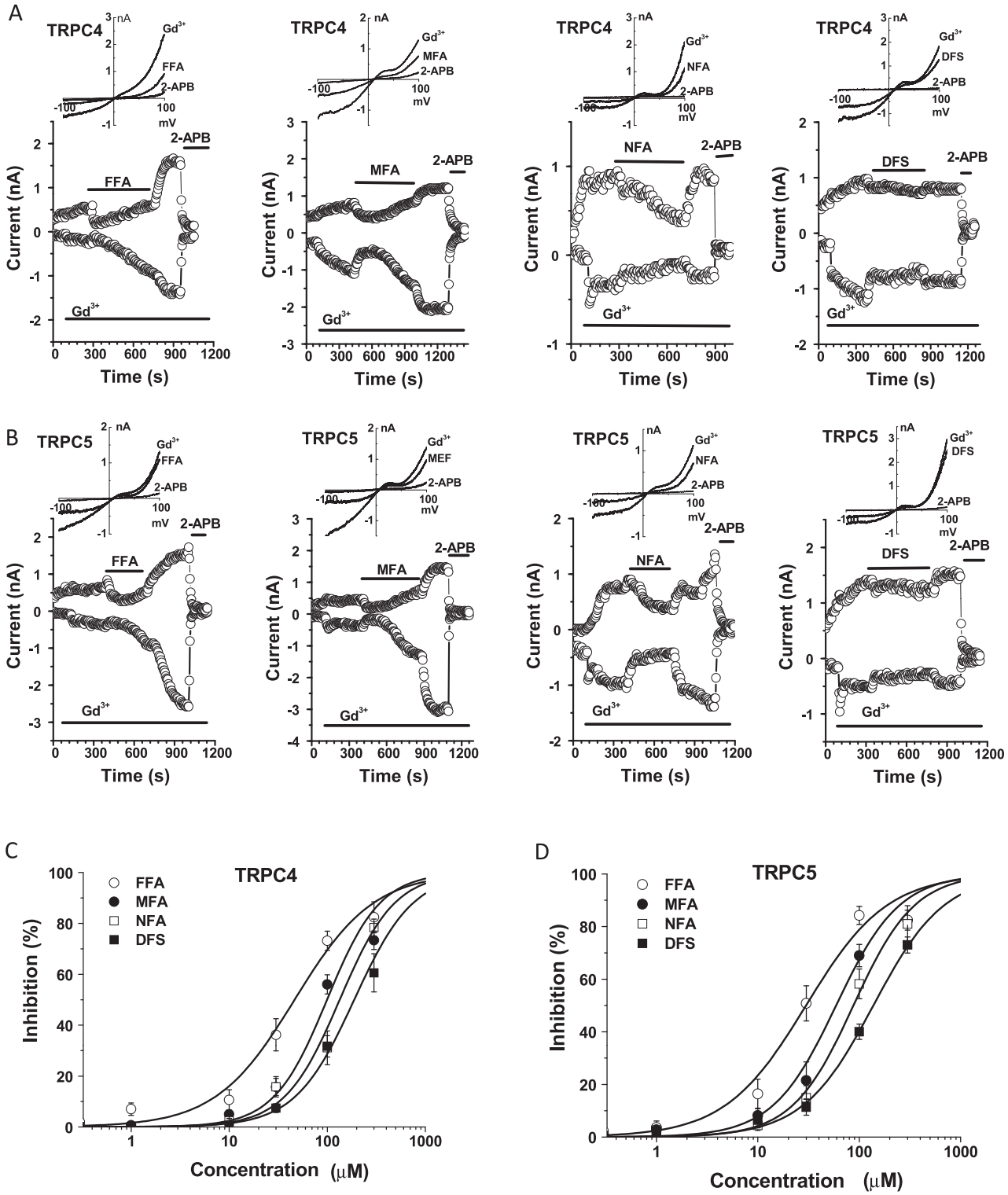
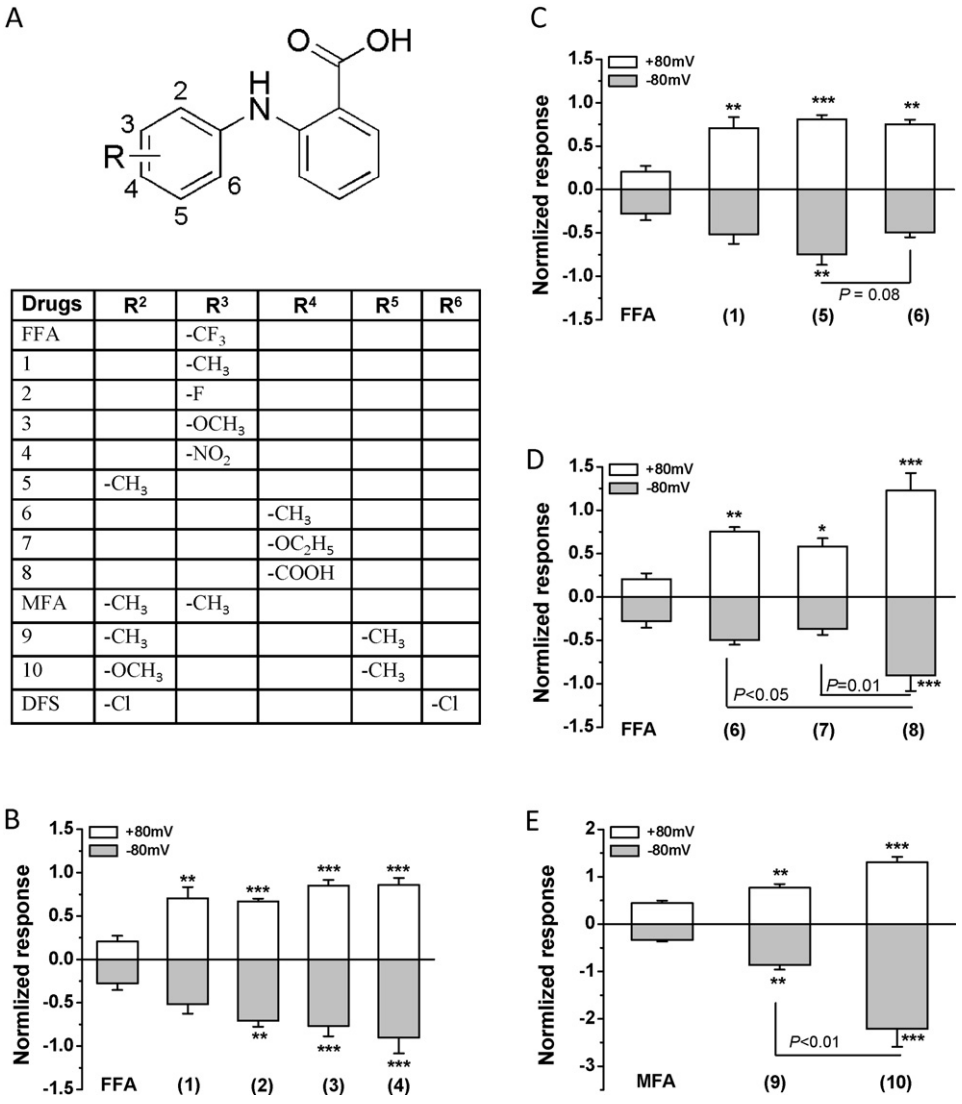


Fig. 1. Effect of fenamates on TRPC4 and TRPC5 channels. Whole cell currents in the HEK-293 T-REx cells transfected with TRPC4 and TRPC5 were recorded by whole-cell patch clamp. (A) The time course (lower) and *IV* curve (upper) for the effect of fenamates (FFA, MFA, NFA, and DFS) at 100 μM on TRPC4 channel. Gd^{3+} (100 μM) was used as a channel activator, and 2-APB (100 μM) was used at the end of fenamate perfusion. The current amplitude was measured at ±80 mV. (B) Similar to (A), but TRPC5 current was recorded. (C) Concentration–response curves for FFA, MFA, NFA, and DFS on TRPC4 channel ($n = 6$ for each group). (D) Concentration–response curves for TRPC5 ($n = 6$ for each group).

of TRPC4 and TRPC5 was induced by tetracycline and the current was characterized by the Gd^{3+} activation with a unique "N" shaped current–voltage (*IV*) relationship, and by fully blockade by 100 μM 2-APB (Fig. 1). FFA, MFA, NFA and DFS significantly inhibited the TRPC4 and TRPC5 currents in a concentration-dependent manner. The IC_{50} for FFA, MFA, NFA and DFS on TRPC4 was 55 ± 5 μM, 84 ± 8 μM, 102 ± 9 μM, and 138 ± 7 μM, respectively; and the IC_{50} for TRPC5 was 37 ± 5 μM, 80 ± 5 μM, 80 ± 9 μM, and 170 ± 9 μM, respectively. These data suggested that fenamates are inhibitors on TRPC4 and TRPC5 channels. The potency of inhibition was FFA > MFA > NFA > DFS.

3.2. Synthesis of fenamate analogues and the effect on TRPC4

Ten analogues were synthesized based on the 2-phenylamino-benzoic acid skeleton replacing the trifluoromethyl group of FFA with –F, $–CH_3$, $–OCH_3$, $–OCH_2CH_3$, –COOH, and $–NO_2$ substituents at various positions in the 2-phenylamino ring (Fig. 2A). The effect of these analogues at 100 μM on the TRPC4 current was compared with FFA by whole-cell patch recording. The substitution with $–CH_3$ (**1**), single –F atom (**2**), $–OCH_3$ (**3**) and $–NO_2$ (**4**) reduced the potency of inhibition on TRPC4 channel, and the percentage of inhibition was 67%, 40%, 32%, and 14% of the FFA inhibition (100%), respectively (Fig. 2B). The position of a single methyl substituent (analogue **1**, **5**, and **6**) also affected the potency (Fig. 2C). The substitution of $–CH_3$ at R^4 with $–OCH_2CH_3$ (**7**) increased the blocking activity with a similar potency on the inward current, however the introduction of –COOH (**8**) to the ring in the same position showed the loss of blocking effect (Fig. 2D), suggesting that the polarity of modifying group at R^4 could be important. In addition, we observed the effect of modifications with two substituents in the ring (Fig. 2E). MFA and analogue (**9**) showed a significant difference in their inhibitory activity. However replacement of the 2-methyl in (**9**) with a methoxy group (**10**) gave an analogue which potentiated the channel activity of TRPC4. The introduction of negative charges, such as –COOH (**8**) and Cl– (DFS), decreased the potency of blocking effect. Moreover, the modification on the benzoic acid by C to N substitution (i.e., NFA) showed a significant decrease in the blocking activity comparing to FFA (31 ± 7% vs. 73 ± 4% at the concentration of 100 μM) (see Fig. 1C).



Drugs	R^2	R^3	R^4	R^5	R^6
FFA		$-CF_3$			
1		$-CH_3$			
2		-F			
3		$-OCH_3$			
4		$-NO_2$			
5	$-CH_3$				
6			$-CH_3$		
7			$-OC_2H_5$		
8			-COOH		
MFA	$-CH_3$	$-CH_3$			
9	$-CH_3$			$-CH_3$	
10	$-OCH_3$			$-CH_3$	
DFS	-Cl				-Cl

Fig. 2. Fenamate analogues and effect on TRPC4 channel. (A) The structures of fenamate analogues. (B) Mean ± S.E.M. data for the effect on TRPC4 current by the modification at R^3 position. FFA group (100 μM) was used as control for comparison between the groups. (C) Effect of the position of methyl substituent on the channel blocking activity. (D) Effect of the modification at R^4 position. (E) Location of two methyl substituents on the TRPC4 channel activity. MFA group (100 μM) was used as control for statistical comparison between the groups. The current measured at ±80 mV after exposure to each compound for 5 min was normalized by the amplitude blocked by 2-APB (100 μM). ANOVA test was used and $n = 6$ for each group. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ for the comparisons with FFA group in (B, C, and D).

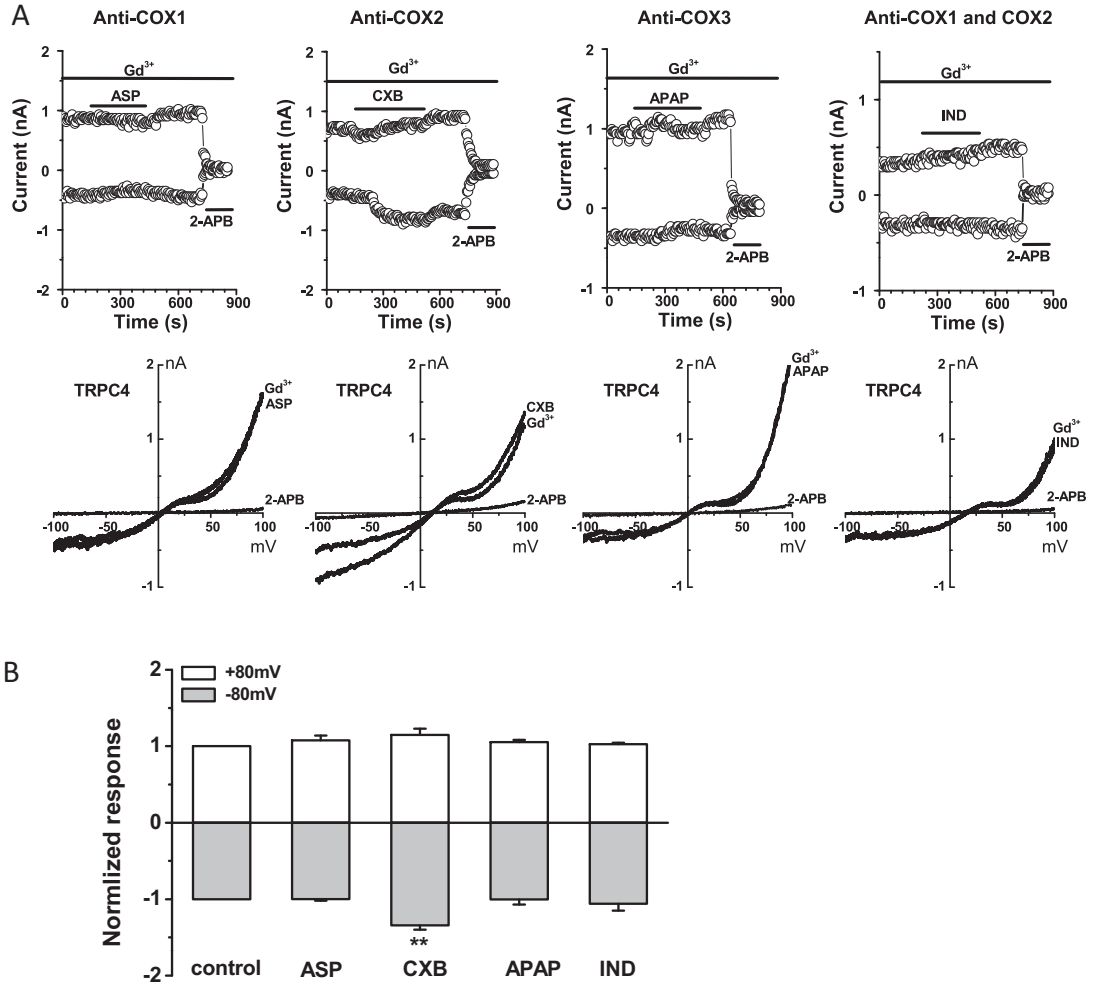


Fig. 3. Effect of non-fenamate COX inhibitors on TRPC4 channel. (A) Representative time course and IV curve for selective COX inhibitors on TRPC4 channels. Aspirin (ASP, 100 μM), celecoxib (CXB, 10 μM), acetaminophen (APAP, 100 μM), and indomethacin (IND, 10 μM) was used. (B) Mean ± S.E.M. data (n = 6–8, $^{**}P < 0.01$).

3.3. Non-fenamate COX inhibitors on TRPC4 channels

The effect of selective COX inhibitors on TRPC channels is unknown, therefore we examined the direct effect of non-fenamate COX1-3 inhibitors on TRPC4 (Fig. 3). Aspirin, celecoxib and acetaminophen are selective inhibitors for COX1, COX2 and COX3, and indomethacin inhibits both COX1 and COX2 [8]. The COX1 and COX3 inhibitors had no effect on TRPC4, however, the COX2 inhibitor celecoxib showed a small stimulating effect on TRPC4. Similar stimulating effect of celecoxib was observed on TRPC5 current (Supplementary Fig. 1). Indomethacin showed no effect on TRPC4, suggesting that the inhibition of COX1 and COX2 are not involved in the channel activation. Aspirin, celecoxib, acetaminophen and indomethacin are structurally different from the fenamate analogues, therefore the blocking effect of fenamates is supposed to be a direct interaction between the chemical structures and the channel protein, rather than the COX pathways.

3.4. TRPC4 and TRPC5 channels potentiated by FFA but not by NFA

As shown in Fig. 4, FFA initially inhibited the TRPC4 and TRPC5, and then gradually increased the currents of TRPC4 and TRPC5. This delayed channel potentiation occurred after the exposure to FFA for 3 min and achieved the steady-state at 8–10 min. This channel potentiating effect was observed in the TRPC4 or TRPC5 transfected cells, but not the non-induced cells, after perfusion with FFA and MFA at concentrations higher than 50 μM. However, NFA and DFS did not show potentiation on TRPC4 and TRPC5 channels. The FFA-induced *IV* curve showed a typical TRPC4 or TRPC5 current. These results suggested the delayed channel potentiation is due to the increased activity of TRPC4 or TRPC5 channels and specific for some structures of fenamate analogues.

3.5. Mitochondria Ca^{2+} release involved in the channel potentiation

In order to examine the underlying mechanism of the delayed channel potentiation on TRPC4 and TRPC5 channels, we compared the effects of FFA and NFA on mitochondrial Ca^{2+} release using primary cultured rat aortic smooth muscle cells (SMCs). The Ca^{2+} dye distribution in the mitochondria was evident after incubation with Fura-PE3 AM in Ca^{2+}-free solution for 30 min (Fig. 5). The pattern of mitochondrial Ca^{2+} dye loaded in the SMCs was similar to the previous report [33]. FFA robustly increased the intracellular Ca^{2+} concentration in both Ca^{2+}-free and 1.5 mM Ca^{2+} standard bath solutions, but MFA, NFA, DFS, ASP, and IND showed a small cytosolic Ca^{2+} increase compared to FFA, and CXB and APAP had no effect (Fig. 5C). The Ca^{2+} increase induced by FFA in the SMCs could be mainly contributed to by mitochondrial Ca^{2+} release, because pre-application of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) blocker thapsigargin (TG) did not inhibit the FFA-induced Ca^{2+} release (Fig. 5D).

Cytosolic Ca^{2+} increase evoked by FFA was also evident in the TRPC4 transfected HEK-293 cells even in the presence of TG, but

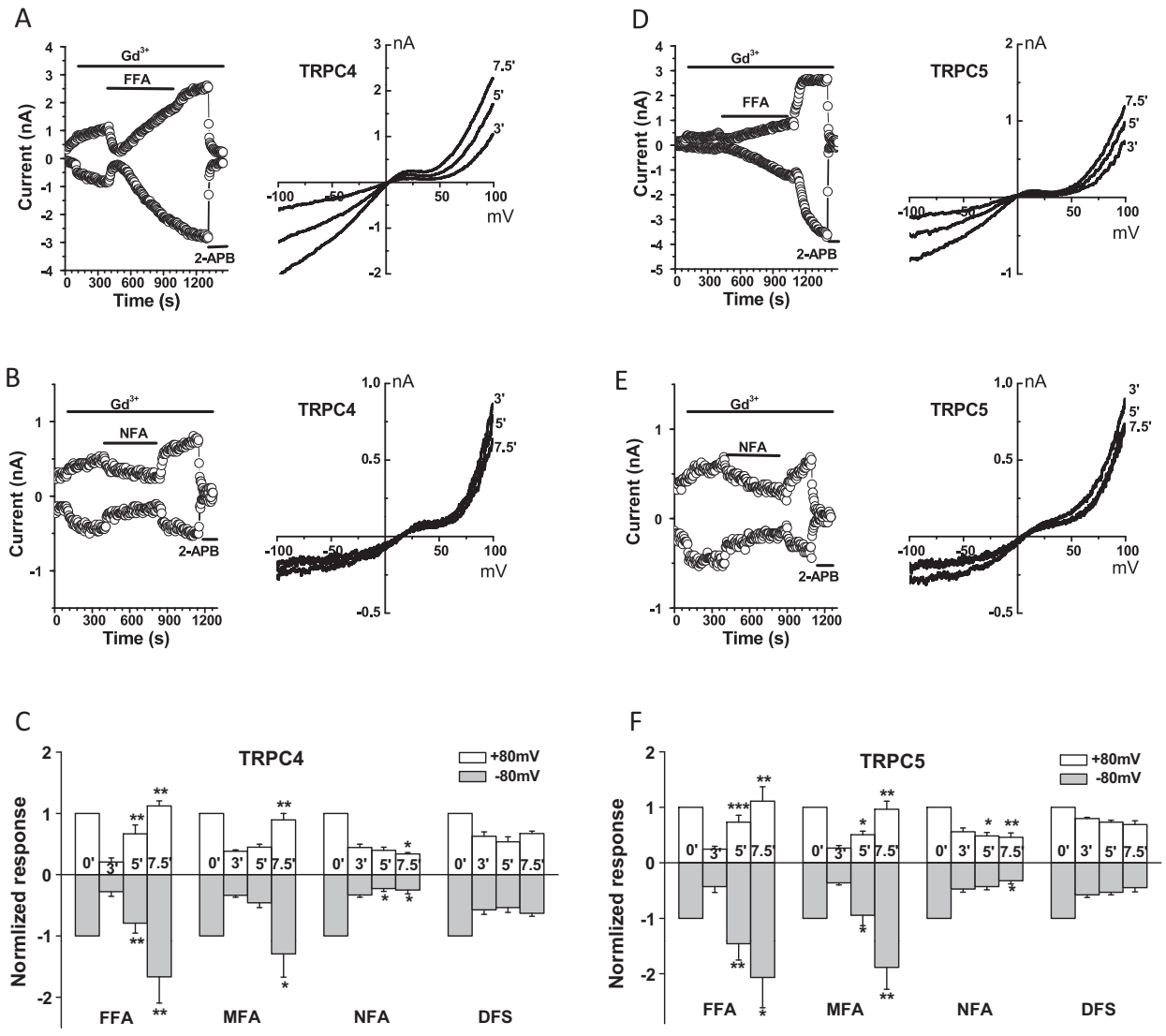


Fig. 4. Delayed potentiation on TRPC4 and TRPC5 currents by FFA. (A) FFA (100 μM) initially blocked the TRPC4 current and the gradually increased the amplitude of TRPC4 current in the presence of 100 μM Gd^{3+}. The *IV* curves were showed at 3, 5, and 7.5 min after initial blockade. (B) No potentiation by NFA (100 μM). The *IV* curves were shown in the right panel. (C) Mean ± S.E.M. data for the TRPC4 amplitude measured at ±80 mV after perfusion with FFA, MFA, NFA, and DFS for 3, 5 and 7.5 min. (D–F) As shown in A–C, but the cells expressing TRPC5 were used. $n = 6–8$ for each group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

the amplitude was reduced after the treatment with TG (Supplementary Fig. 2), suggesting that Ca^{2+} release from ER also contributes to the FFA-induced Ca^{2+} increase in this cell type. However, ER Ca^{2+} store depletion with TG was unable to abolish the channel potentiating effect of FFA (Fig. 5E and F), which further suggested the involvement of mitochondrial Ca^{2+} store in the mechanism.

To examine the role of mitochondrial Ca^{2+} release in the FFA-induced channel potentiation, we pre-treated the stable TRPC4 cells with cyclosporine A (CsA). CsA is an inhibitor of mitochondrial permeability transition pore, which also inhibits mitochondrial Ca^{2+} movement across the membrane [34,35]. Preincubation with CsA (10 μM) significantly decreased the FFA-induced TRPC4 channel potentiation (Fig. 5G), suggesting the involvement of mitochondrial Ca^{2+} release in the FFA-induced TRPC4 channel potentiation.

4. Discussion

In this study, we have found that the fenanates FFA, MFA, NFA and DFS showed inhibition on TRPC4 and TRPC5 channels with a potency sequence of FFA > MFA > NFA > DFS. Replacement of the trifluoromethyl group in FFA with a range of substituents leads to a significant change in the channel blocking activity for those derivatives. Introduction of 2-methoxy-5-methyl substituents to the phenylamino ring (**10**) gave an analogue with a stimulating effect on the channels. COX-1 and COX-3 inhibitors did not show any direct effect on TRPC4 or TRPC5 channels, but the highly selective COX-2 inhibitor celecoxib slightly increased the activity of TRPC4 and TRPC5. Interestingly, there was differential effect of fenamate analogues on TRPC4 and TRPC5 channels, i.e., FFA and MFA showed a potentiating effect after initial inhibition, but NFA and DFS did not potentiate the channels. The TRPC4 and TRPC5 channel potentiation by FFA is likely explained by Ca^{2+} release from mitochondria, because the inhibition of mitochondrial permeability transition pore by CsA partially prevented the FFA-induced channel potentiation. In addition, NFA did not show any channel potentiation, which could be explained by its low potency in mitochondrial Ca^{2+} release compared with FFA [31].

Fenamates are effective anti-inflammatory agents through COX-1 and COX-2 inhibition, and also inhibit a variety of ion channel activities in many cell types. Early studies have shown that

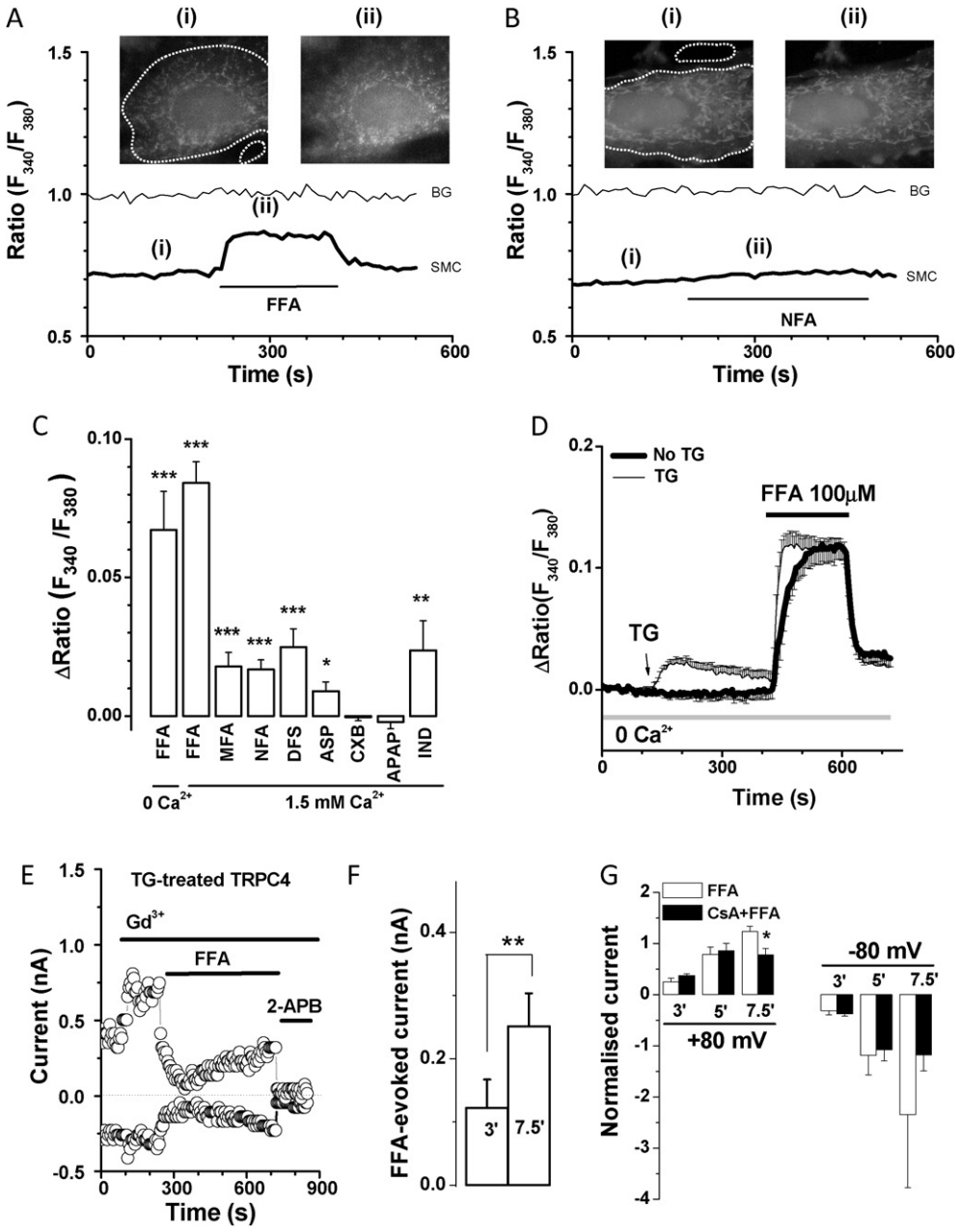


Fig. 5. Involvement of mitochondrial Ca^{2+} release on the TRPC4 channel potentiation. (A) Rat aortic smooth muscle cells were loaded with Fura-PE3 AM. The cytosolic Ca^{2+} increase induced by FFA at 100 μM. (i) and (ii) are the images before and after FFA treatment. The thin trace is the background (BG) signal and the thick line is smooth muscle cell (SMC). (B) Effect of NFA (100 μM). (C) Mean ± S.E.M. data for the intracellular Ca^{2+} increase in the SMCs induced by 100 μM of FFA, MFA, NFA, DFS, ASP, APAP, and IND and CXB (10 μM), n = 17–26 cells for each group. (D) SMCs were incubated with 1 μM TG (n = 7) or without TG (n = 10). (E) TRPC4 cells were pre-treated with TG (1 μM) for 10 min. The whole cell current was recorded in the presence of TG. FFA (100 μM), Gd^{3+} (100 μM) and 2-APB (100 μM) were used. (F) Mean data for FFA-induced current after perfusion with FFA for 3 and 7.5 min. The amplitude of TRPC4 current was measured at −80 mV (n = 6). (G) Pretreatment with CsA (10 μM) for 5 min, the amplitude of TRPC4 current was measured at ±80 mV after perfusion with FFA for 3, 5 and 7.5 min. n = 6–8 for each group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

fenamates inhibited Ca^{2+}-activated chloride channels [36–38], Ca^{2+}-activated potassium channels [39], and the Ca^{2+}-activated non-selective cationic channel [40]. Recently, it has been demonstrated that FFA activated TRPC6 [28], TRPA1 [29], and an OAG-sensitive cationic current in A7r5 cells [26], but inhibited TRPM2, TRPM3, TRPM4 and TRPM5 channels [22,24,41–44]. Here we give the new evidence that fenamates also inhibit TRPC4 and TRPC5. The potency of inhibition depends on the modification on the skeleton of the 2-phenylaminobenzoate. Apart from the initial inhibition, there is a delayed channel potentiation evoked by some fenamate analogues, such as FFA and MFA. The delayed channel potentiation could be explained by the mitochondrial Ca^{2+} release evoked by FFA, because both TRPC4 and TRPC5 channels have been demonstrated as Ca^{2+}-sensitive channels [45–48], and thus the increased cytosolic Ca^{2+} level caused by Ca^{2+} release from mitochondria in turn facilitates TRPC4/TRPC5 channel activity [45,47]. Our data showed that the FFA-induced Ca^{2+} increase in the cytosol was fast, robust and reversible in SMCs, while the effect for NFA was small. This difference has also been described in mouse submandibular salivary cells [31]. Our data suggested that the FFA-induced Ca^{2+} increase in the smooth muscle cells was mainly contributed by mitochondrial Ca^{2+} release, because the Ca^{2+} release signal was not influenced by the depletion of ER Ca^{2+} store. Tu and co-workers also showed that FFA-induced mitochondrial

Ca^{2+} release occurred in the presence of TG [30]. Therefore the TRPC4 and TRPC5 channel potentiation by FFA could be explained as the increased cytosolic Ca^{2+} due to mitochondrial Ca^{2+} release. In addition, the contribution of mitochondrial Ca^{2+} release to the channel potentiation was further demonstrated by the application of CsA that can partially prevent the mitochondrial Ca^{2+} movement in this study.

The delayed potentiation on TRPC4 and TRPC5 channel could be a mechanism for the pharmacology of fenamate NSAIDs, especially in situations with high dosage or long-term medication. The peak plasma concentration of FFA or MFA achieved 6-20 $\mu g\ ml^{-1}$ (equal to 21–71 μM) in volunteers [49,50], suggesting that the effect on mitochondrial Ca^{2+} release and channel potentiation could happen in patients receiving fenamate drug treatment. The cardiovascular risks of some NSAIDs have been recognized and some NSAIDs have been withdrawn from the market by FDA due to the cardiovascular side effects [51]. The mechanisms for these side effects are unclear, but the interference with intracellular Ca^{2+} homeostasis or TRPC4/5 channel potentiation could be a new explanation for hypertension related to some fenamate analogues and celecoxib, which needs to be investigated further.

The effect of non-fenamate selective COX inhibitors was also examined in this study. COX-1 is involved in platelet aggregation, gastric mucosa protection and renal electrolyte homeostasis. Aspirin is a selective COX-1 inhibitor and irreversibly acetylates platelet COX-1 and inhibits the formation of the potent platelet agonist thromboxane A_2 (TXA_2) [52]. Perfusion with aspirin did not change the activity of TRPC4 and TRPC5 channels, suggesting that COX-1 is not directly involved in their modulation. In order to dissect COX-2 involvement, we tested the selective inhibitor celecoxib that significantly increased the channel activity of TRPC4 and TRPC5. The activation mechanism for celecoxib is unknown, but unlikely due to the COX2 inhibition, because other COX2 inhibitors have no direct effect.

Modification of the phenylamino ring in FFA is useful for finding the structure–activity relationship of those analogues. The substituents at R^3 position (analogues **1**–**4**) significantly changed the channel blocking potency, suggesting the importance of R^3 in the structural modification. The comparison between the methyl substituents at R^2, R^3 and R^4 (**5**, **1** and **6**) showed that the modification at *meta* and *para* positions could be more effective than the *ortho* position. Substituents at *para* position gave a compound (**7**) with similar potency to FFA, but the introduction of a polar substituent (**8**) could be ineffective. Our data also showed that the combination of groups at R^2 and R^5 could be a determinant as a channel opener or blocker, since the substituent $-OCH_3$ at R^2 (in **10**) yielded a channel stimulator, while the substituent $-CH_3$ at R^2 (in **9**) yielded a channel antagonist. We suggest that this could be due to the conformational changes in the diphenylamine core as the bulkier methoxy group is introduced into an *ortho* position. Introduction of a single $-CH_3$ or $-OCH_3$ group at R^3 (**1** or **5** respectively) however, without the second *meta* methyl residue, yielded channel inhibitors less effective than FFA.

In summary, our results show that fenamate analogues have differential effects on TRPC4 and TRPC5 channels with inhibition acutely and potentiation with long exposure. The analogues may have different properties for mitochondrial Ca^{2+} release and caution should be exercised in the explanation of drug class effect of these agents. The development of new fenamate analogues may have potential for identification of selective TRPC activators and inhibitors, which could yield new tools for investigating the roles of these channels in pathophysiological conditions.

Conflicts of interest

No potential conflicts of interest were disclosed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2012.01.014.

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