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RESEARCH PAPER

Pharmacological comparison of novel synthetic fenamate analogues with econazole and 2-APB on the inhibition of TRPM2 channels

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BACKGROUND AND PURPOSE

Fenamate analogues, econazole and 2-aminoethoxydiphenyl borate (2-APB) are inhibitors of transient receptor potential melastatin 2 (TRPM2) channels and are used as research tools. However, these compounds have different chemical structures and therapeutic applications. Here we have investigated the pharmacological profile of TRPM2 channels by application of newly synthesized fenamate analogues and the existing channel blockers.

EXPERIMENTAL APPROACH

Human TRPM2 channels in tetracycline-regulated pcDNA4/TO vectors were transfected into HEK293 T-REx cells and the expression was induced by tetracycline. Whole cell currents were recorded by patch-clamp techniques. Ca^{2+} influx or release was monitored by fluorometry.

KEY RESULTS

Flufenamic acid (FFA), mefenamic acid (MFA) and niflumic acid (NFA) concentration-dependently inhibited TRPM2 current with potency order FFA > MFA = NFA. Modification of the 2-phenylamino ring by substitution of the trifluoromethyl group in FFA with $–CH_3$, –F, $–CF_3$, $–OCH_3$, $–OCH_2CH_3$, –COOH, and $–NO_2$ at various positions, reduced channel blocking potency. The conservative substitution of 3-CF_3 in FFA by $–CH_3$ (3-MFA), however, gave the most potent fenamate analogue with an IC_{50} of 76 μM, comparable to that of FFA, but unlike FFA, had no effect on Ca^{2+} release. 3-MFA and FFA inhibited the channel intracellularly. Econazole and 2-APB showed non-selectivity by altering cytosolic Ca^{2+} movement. Econazole also evoked a non-selective current.

CONCLUSION AND IMPLICATIONS

The fenamate analogue 3-MFA was more selective than other TRPM2 channel blockers. FFA, 2-APB and econazole should be used with caution as TRPM2 channel blockers, as these compounds can interfere with intracellular Ca^{2+} movement.

Abbreviations

2-APB, 2-aminoethoxydiphenyl borate; 3-MFA, 2-(3-methylphenyl)aminobenzoic acid; ACA, *N*-(*p*-amylcinnamoyl)anthranilic acid; IP_3, inositol trisphosphate; *IV*, current–voltage; FFA, flufenamic acid; MFA, mefenamic acid; NFA, niflumic acid; NMDG, N-methyl-D-glucamine; NSAIDs, non-steroidal anti-inflammatory drugs; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; Tet, tetracycline; TRP, transient receptor potential; TRPC, transient receptor potential canonical; TRPM2, transient receptor potential melastatin 2

Introduction

Ca^{2+} ions are second messengers controlling many cell signalling processes. The transient receptor potential (TRP) channel family (channel and receptor nomenclature follows Alexander *et al.*, 2011) is a class of Ca^{2+}-permeable cationic channels that are important for maintaining intracellular Ca^{2+} level. The transient receptor potential melastatin 2 (TRPM2) channel is one member of the TRPM channel subfamily (Ramsey *et al.*, 2006), which was first cloned in 1998 (Nagamine *et al.*, 1998). TRPM2 channels are highly expressed in the brain and ubiquitously distributed in the body (Hecquet *et al.*, 2008; Wehrhahn *et al.*, 2010). The pathophysiological role of TRPM2 channels is still unclear, but they have been implicated in the free radical-induced cell death of hippocampal neurons (Olah *et al.*, 2009), striatal cells (Fonfria *et al.*, 2005) and other cell types (Zhang *et al.*, 2003; Yang *et al.*, 2006; Ishii *et al.*, 2007); the stress-related inflammatory processes (Yamamoto *et al.*, 2008; Wehrhahn *et al.*, 2010); insulin secretion (Uchida *et al.*, 2010); immune response (Sano *et al.*, 2001; Knowles *et al.*, 2011); and oxidant-induced endothelial injury (Hara *et al.*, 2002; Hecquet and Malik, 2009).

Heterologous expression of TRPM2 protein gives rise to a voltage-independent, Ca^{2+}-permeable, non-selective cationic channel with a linear current–voltage (*IV*) relationship (Perraud *et al.*, 2001; McHugh *et al.*, 2003) and a characteristic activation by intracellular ADP-ribose. Flufenamic acid (FFA), econazole, 2-aminoethoxydiphenyl borate (2-APB) and Zn^{2+} as all act as blockers of TRPM2 channels (Hill *et al.*, 2004a,b; Yang *et al.*, 2011). FFA is one of the fenamate non-steroidal anti-inflammatory drugs (NSAIDs), which affects a variety of channels, inducing inhibition of Cl^- channels, voltage-dependent Na^+ or Ca^{2+} channels, and TRPM2, TRPM4, TRPM5, TRPC3 and TRPC5 channels (Lee *et al.*, 2003; Kraft and Harteneck, 2005; Ullrich *et al.*, 2005). FFA also activates TRPC6 (Inoue *et al.*, 2001; Jung *et al.*, 2002; Foster *et al.*, 2009) and TRPA1 channels (Hu *et al.*, 2010). The action of FFA on ion channels is not mediated by cyclooxygenase (COX), because selective COX inhibitors were found to have no direct effect on TRP cationic channels (Jiang *et al.*, 2012), suggesting there is direct conformational interaction between FFA and the channel protein. The fenamate NSAIDs are anthranilic acid derivatives with structural similarity to the PLA2 inhibitor *N*-(*p*-amylcinnamoyl)anthranilic acid (ACA). However, although ACA inhibited TRPM2 channels (Kraft *et al.*, 2006), other PLA2 inhibitors without the skeleton of anthranilic acid had no effect on these channels, suggesting that the parent structure of anthranilic acid was essential for the channel blockade. Moreover, other fenamates with different substituents on the 2-phenylamino ring, such as FFA, mefenamic acid (MFA) and diclofenac, exert different effects on elevation of intracellular Ca^{2+} (Poronnik *et al.*, 1992). Recently, we found that the substituents on the 2-phenylamino ring of the fenamate skeleton were important for regulating TRPC4 and TRPC5 channel activity, especially the position of the methyl groups in MFA. The replacement of 2-methyl with a methoxy group gave an analogue showing activation, rather than inhibition on TRPC4 and TRPC5 channels (Jiang *et al.*, 2012). Therefore, we proposed that the modification of 2-phenylamino ring would be important for understanding the structure–activity relationship of fenamate analogues on TRPM2 channels and might yield new leads for drug discovery.

In this study, we examined the effect of some new fenamate analogues on TRPM2 channels using inducible cells over-expressing TRPM2 protein. In order to understand the structure–activity relationship of the fenamates, we synthesized analogues with modifications of the 2-phenylamino ring and compared their potency on TRPM2 channels. To compare their pharmacological properties of the new compounds with those of known TRPM2 channel blockers, the effects of econazole and 2-APB were also investigated in our model system.

Methods

Cell culture and transfection

Human TRPM2 protein (GenBank accession number BC112342) in pcDNA4/TO tetracycline-regulatory vector was transfected into HEK-293 T-REx cells (Invitrogen, Paisley, UK). The expression was induced by 1 $\mu g \cdot mL^{-1}$ tetracycline for 24–72 h before recording. The non-induced cells without addition of tetracycline were used as control. Cells were grown in DMEM-F12 medium (Invitrogen) containing 10% fetal calf serum (FCS), 100 $units \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin. Cells were maintained at 37°C under 95% air and 5% CO_2 and seeded on coverslips prior to experiments.

Electrophysiology

The procedure for whole-cell clamp is similar to that described earlier (Xu *et al.*, 2012). Experiments were performed at room temperature (25 °C). Briefly, electrical signal was amplified with an Axopatch 200B patch clamp amplifier and controlled with pClamp software 10. A 1 s ramp voltage protocol from −100 to +100 mV was applied at a frequency of 0.2 Hz from a holding potential of 0 mV. Signals were sampled at 3 kHz and filtered at 1 kHz. Glass microelectrodes with a resistance of 3–5 MΩ were used. The 200 nM Ca^{2+} buffered pipette solution (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) was used. ADP-ribose (0.5 mM) was added in the pipette solution. The same pipette solution was used for outside-out patches. The standard bath solution contained (mM) 130 NaCl, 5 KCl, 8 D-glucose, 10 HEPES, 1.2 $MgCl_2$ and 1.5 $CaCl_2$; pH was adjusted to 7.4 with NaOH.

Ca^{2+} measurement

Cells were pre-incubated 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 with a cooled Orca-R2 CCD camera (Hamamatsu, Hamamatsu City, Japan). The imaging system was controlled by software NIS-Elements 3.0 (Nikon, Tokyo, Japan). The ratio of Ca^{2+} dye fluorescence (F_{340}/F_{380}) was measured. For the experiment with single wavelength Ca^{2+} dye Fluo3-AM, the cuvette-based $[Ca^{2+}]_i$ assay system was used as described previously (Xu *et al.*, 2008). All experiments were performed at room temperature.

Materials

All general salts and reagents were from Sigma (Dorset, UK). FFA, MFA, niflumic acid (NFA), diclofenac , aspirin, indomethacin, 2-APB, tetracycline, ADP-ribose, econazole and N-methyl-D-glucamine (NMDG) were purchased from Sigma. Fura-PE3 AM was purchased from Invitrogen. Fura-PE3 AM (5 mM) and 2-APB (100 mM) were made up as stock solutions in 100% dimethyl sulphoxide (DMSO). Fenamate derivatives were synthesized in the Chemistry Department following the method reported by Mei *et al*. (2006) using the copper-catalysed coupling of either 2-chloro- or 2-bromobenzoic acid with the appropriate aniline derivative. For example, 2-chlorobenzoic acid (9.0 mmol), the appropriate aniline (9.5 mmol), K_2CO_3 (9.0 mmol), Cu (0.8 mmol), Cu_2O (0.4 mmol) and 5 mL of 2-ethoxyethanol were heated under a nitrogen atmosphere for 24 h. The cooled reaction mixture was poured into water; activated charcoal was added then the solution filtered. The crude product was precipitated upon acidification of the filtrate with 1 M HCl. The residue was purified by dissolution in 5% aqueous Na_2CO_3 solution, filtration and then re-precipitation by careful addition of 1 M HCl. In the case of compound 8 in Figure 3, 5[2-(4′-carboxyphenylamino)benzoic acid], the starting material was ethyl 4-aminobenzoate, but the ethyl ester group suffered *in situ* hydrolysis. All products gave satisfactory ^{1}H, ^{13}C-NMR and mass spectra; and their purity was estimated to be >95%.

Statistics

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

Results

TRPM2 channels activated by ADP-ribose and H_2O_2

The expression of human TRPM2 protein in HEK-293 T-REx cells was induced by tetracycline and confirmed by Western blotting as we previously described (Xu *et al*., 2008; 2012). The whole cell current was recorded by patch clamp after 24–48 h induction of gene expression, and the current carried by TRPM2 channels was activated by intracellular ADP-ribose with a linear *IV* curve (Figure 1A and B), in accordance with previous reports (Perraud *et al*., 2001; Sano *et al*., 2001; Hara *et al*., 2002; Wehage *et al*., 2002; McHugh *et al*., 2003). The activation achieved its maximum within 30 s after the formation of whole-cell patch configuration and was fully blocked by 2-APB (100 μM). Substitution of Na^+ with equimolar concentrations of $NMDG^+$ rapidly abolished the inward current and the outward current gradually decreased (Figure 1C). In the non-induced cells, a small current (<1 nA) was activated by ADP-ribose, which could be due to endogenous channel activity. 2-APB fully inhibited the endogenous current (Figure 1D and E). We also examined the effect of H_2O_2 on the cells with inducible TRPM2 channels. Bath application of H_2O_2 activated TRPM2 channels, but the current development was much slower and the maximum amplitude of the current was smaller than that after activation by ADP-ribose (Figure 1F). The *IV* curve induced by H_2O_2 showed an outward rectification and 2-APB at 100 μM did not fully block the current, suggesting that H_2O_2 may activate other 2-APB-insensitive channels. In addition, cytosolic Ca^{2+} concentrations were monitored using Ca^{2+} -sensitive dye. Influx of Ca^{2+} in cells with induced TRPM2 channels was robustly increased after perfusion with H_2O_2, but the non-induced cells showed a small increase (Figure 1G).

Comparison of the three experimental approaches indicated that whole-cell patch recording with intracellular ADP-ribose was the best methodology for examining TRPM2 channel pharmacology, as the large current (~10 nA) through TRPM2 channels evoked by ADP-ribose was clearly distinguished from the small endogenous current (0.64 ± 0.02 nA measured at –80 mV, $n = 12$) in the non-induced cells which also showed a linear *IV* relationship and 2-APB sensitivity. Therefore, the whole-cell patch was used in the subsequent experiments for pharmacological comparison.

Effect of NSAIDs on TRPM2 channels

We examined the effect of fenamates and non-fenamate NSAIDs on TRPM2 channels. FFA, NFA and MFA significantly inhibited the TRPM2 current; while diclofenac showed only a small inhibition. The IC_{50} values for FFA, MFA and NFA was 70 ± 2.5, 124 ± 11.9 and 149 ± 12.0 μM with a slope factor of 0.01776, 0.00872 and 0.00763 respectively. The non-fenamate NSAIDs, aspirin and indomethacin, had no significant effect (Figure 2). These data suggested that the blocking activity of fenamates could be a direct effect, rather than a class effect of NSAIDs on COX signalling pathways, as the non-fenamate COX inhibitors had no effect.

Fenamate analogues on TRPM2 channels

In order to explore the structure–activity relationship of varying the substituents on the 2-phenylamino ring of the fenamate skeleton, 10 analogues were synthesized replacing the 3-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 (Figure 3). Potency on the TRPM2 current was compared with the known channel blockers 2-APB and FFA. Substitution with –3-CH_3 (**1**) (abbreviated as 3-MFA), 3-F (**2**), 3-CH_3O (**3**) and –3-NO_2 (**4**) showed a significant difference in the inhibition of TRPM2 channel. The methyl substituent in the *meta* position (3-MFA) was critical for channel blocking effect. A $–CH_3$ group in the *ortho* (**5**) or *para* (**6**) position reduced potency. Other substituents at the *meta* position (**2**, **3**, and **4**) also showed less potency. Substituents with –4-CH_3CH_2O (**7**) and –4-COOH (**8**) showed weak inhibition. MFA and the analogue (**9**) have two methyl substituents in the ring; however, they showed a significant difference in their inhibitory activity (see Figures 2C and 3), which further suggested the importance of the methyl substituent at the *meta* position. Substitution of the 2-methyl with a methoxy group (**10**) or introduction of two Cl^- substituents in the *ortho* positions (as in diclofenac) showed reduced inhibition (see Figures 2D and 3). Moreover, the replacement of the benzoic

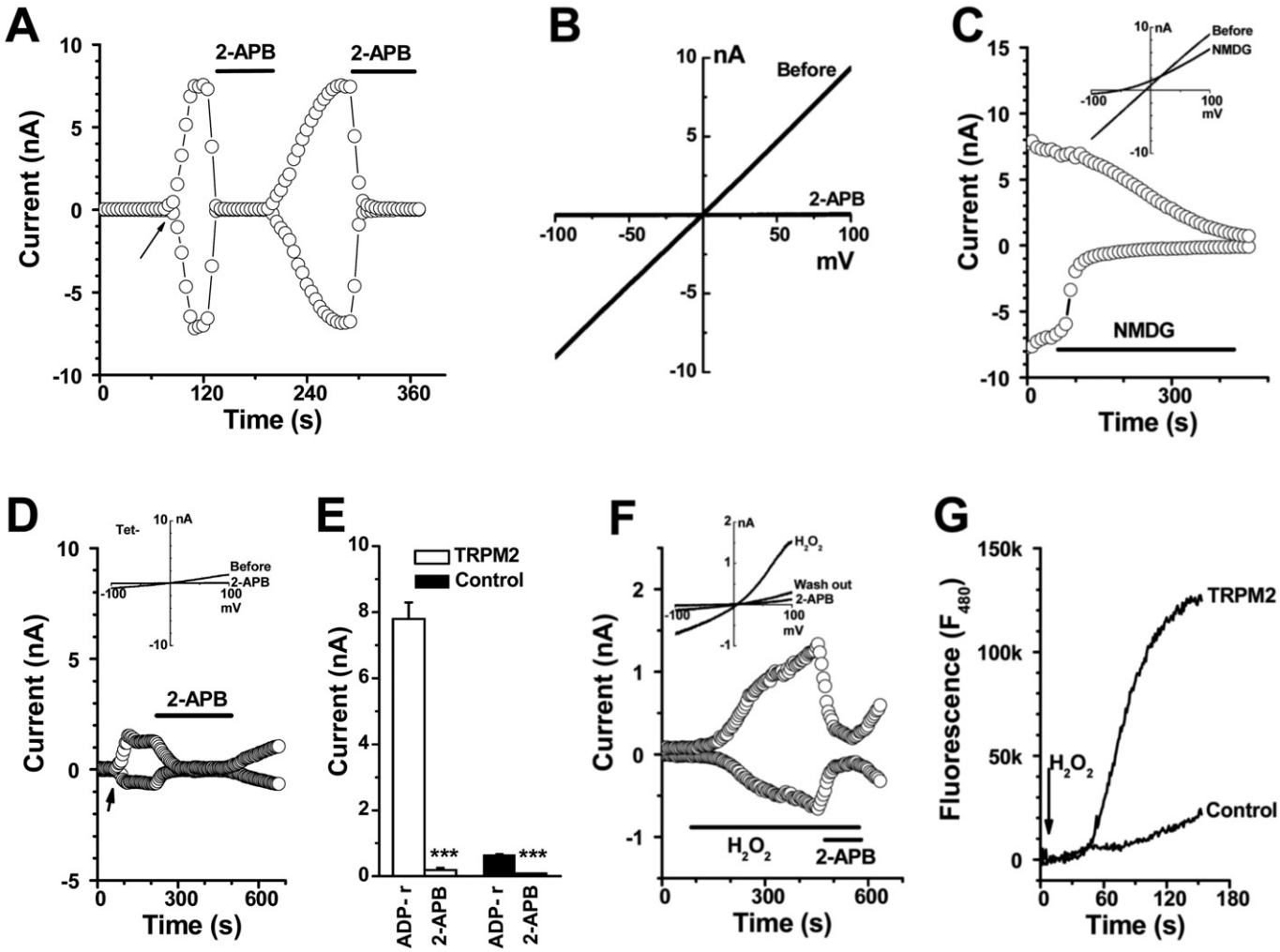

Figure 1

TRPM2 channels activated by ADP-ribose and H_2O_2. Whole-cell current in the HEK293 T-REx cells inducibly transfected with TRPM2 channels was recorded by patch clamp. (A) The time course for TRPM2 channel activation by 0.5 mM ADP-ribose (ADP-r) in pipette solution. The arrow shows the point of membrane breakthrough as whole-cell patch formation. 2-APB (100 μM) was used. (B) *IV* curve for (A). (C) Na^+ was substituted by equimolar concentrations of $NMDG^+$. The *IV* curves are shown in the inset. (D) Current recorded in the non-induced cells. (E) Summary data (means ± SEM) for the current at −80 mV in cells with induced TRPM2 channels (TRPM2) and non-induced cells (control) (n = 6). (F) TRPM2 channels activated by H_2O_2 (500 μM). (G) H_2O_2-evoked Ca^{2+} influx via TRPM2 channels. The cells without tetracycline induction (non-induced) were used as control.

acid ring in FFA with a nicotinic acid group (as in NFA) reduced potency, in comparison with FFA (Figure 2D). The dose–response curves of 3-MFA (**1**) and FFA were determined by single concentration application and fitted with the Boltzmann equation to yield IC_{50} values of 76 ± 2.8 and 70 ± 2.5 μM respectively. The inhibitory effect of 3-MFA on TRPM2 current was partially reversible but showed a voltage-independent block.

Intracellular effect of FFA and 3-MFA

The whole-cell patch recordings were performed using pipette solutions containing 200 μM FFA or 200 μM 3-MFA. The current induced by ADP-ribose was significantly prevented by the inclusion of FFA or 3-MFA in the pipette solution comparing with the control group with ADP-ribose only in the pipette solution (Figure 4A). The outside-out excised membrane patches also showed no effect for FFA and 3-MFA but 2-APB significantly inhibited TRPM2 channel activity (Figure 4B and C). In addition, the single channel activity was recorded in the outside-out patches that formed immediately by standard procedures after the whole-cell TRPM2 current evoked by ADP-ribose. The mean slope conductance was 66 pS, which is similar to the channel conductance of TRPM2 channels (60–64 pS) recorded by others (Perraud *et al.*, 2001; Starkus *et al.*, 2010). Perfusion with FFA (100 μM) and 3-MFA (100 μM) did not change single channel conductance and the events of channel opening. However, 2-APB (100 μM) abolished the TRPM2 single channel events (Figure 4D). These data suggest that the site of action for FFA and 3-MFA was intracellularly located.

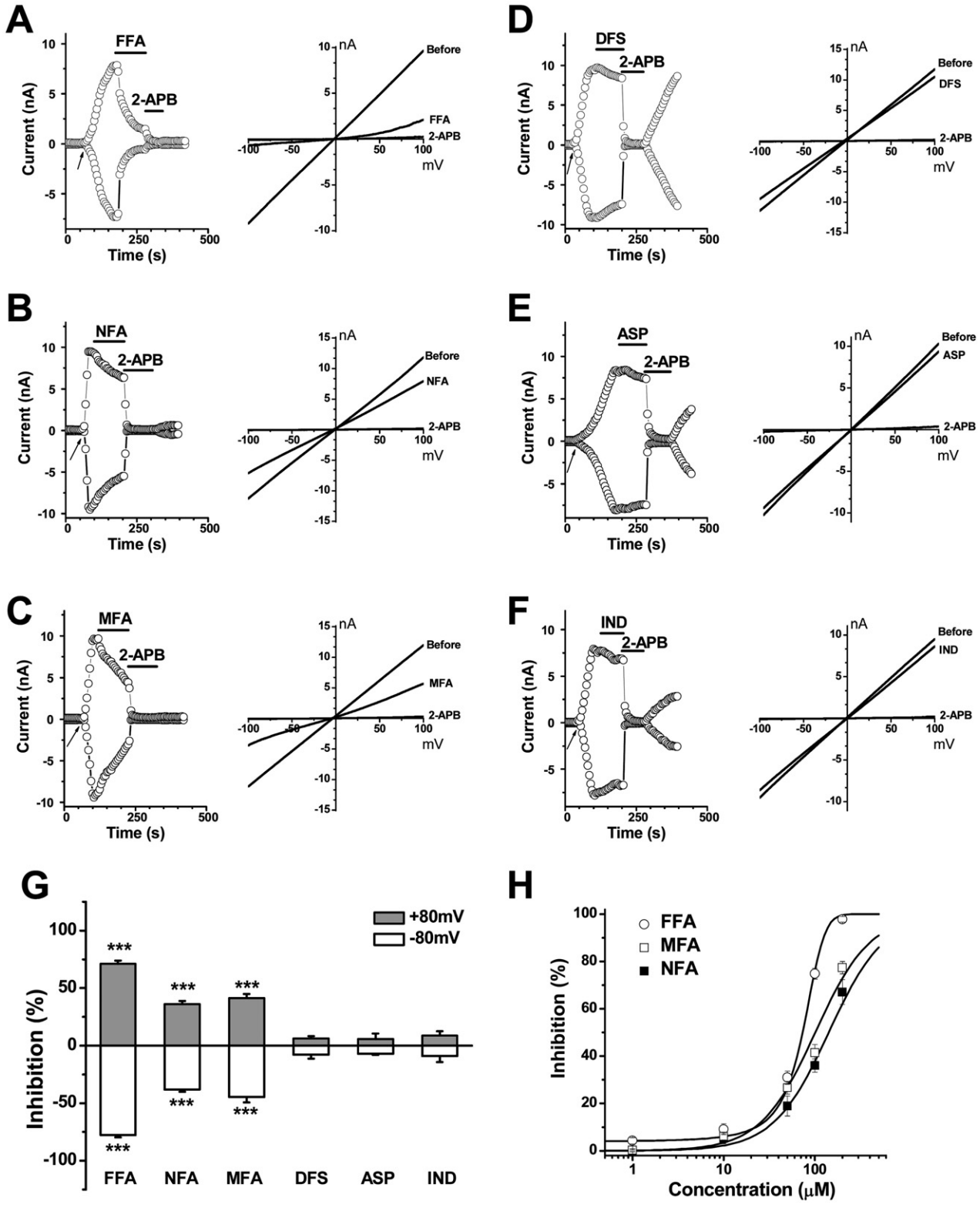

Figure 2

Effect of fenamates and non-fenamate NSAIDs on TRPM2 current. Representative time course and *IV* curves of TRPM2 channels were shown in (A–F). (A) FFA (100 μM). (B) NFA (100 μM). (C) MFA (100 μM). (D) diclofenac (DFS; 100 μM). (E) aspirin (ASP; 100 μM). (F) indomethacin (IND; 100 μM). (G) Summary data (means ± SEM) showing the percentage of inhibition of TRPM2 current. The amplitude was normalized to that blocked by 2-APB (100 μM) (n = 3–8). ***P < 0.001. (H) Concentration–response curves for FFA, MFA and NFA for the inhibition of TRPM2 current (n = 5–6 for each point).

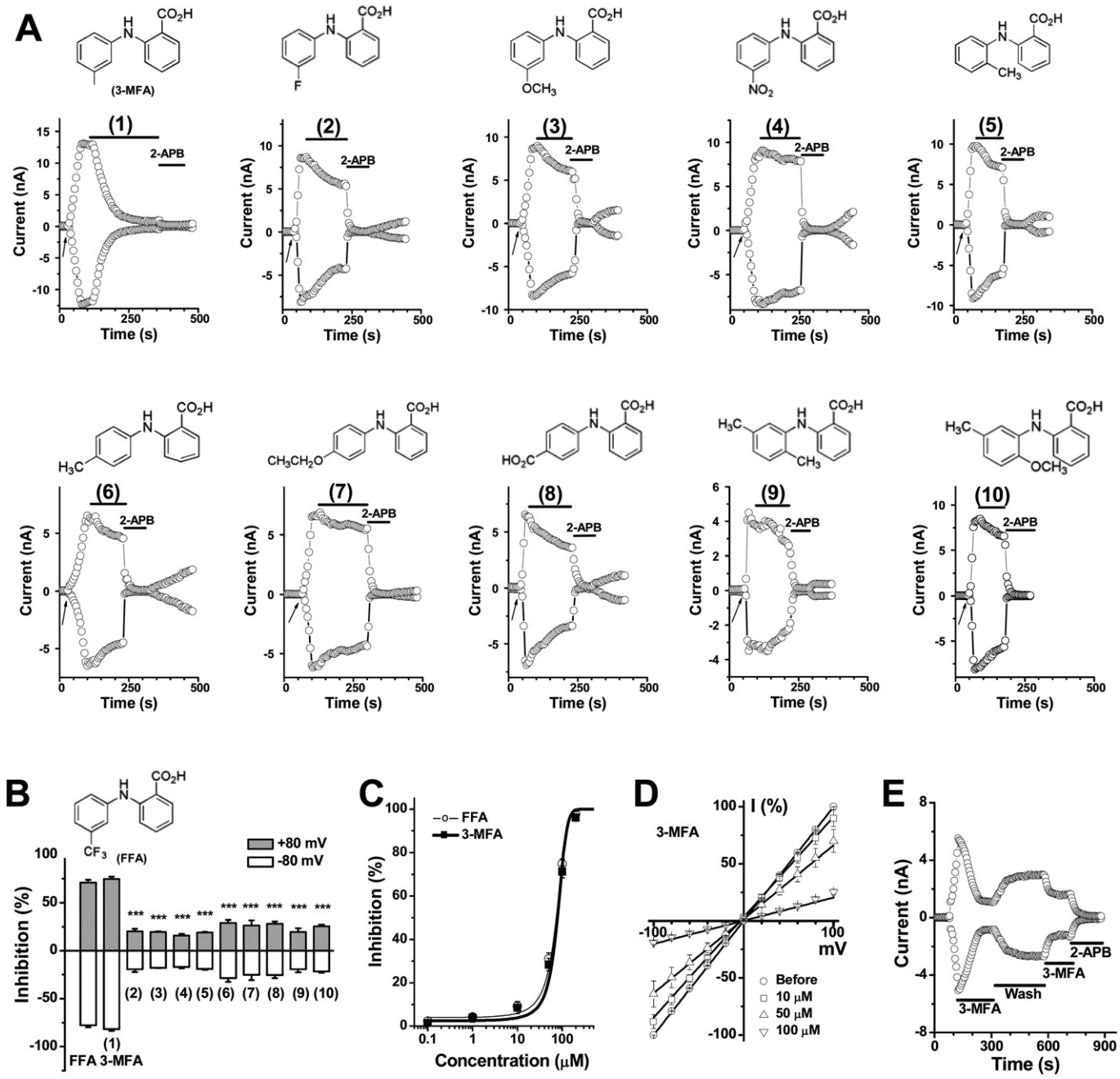

Figure 3

Synthetic fenamate analogues and the effect on TRPM2 current. (A) Time course showing the effect of fenamate analogues, compounds (**1**) to (**10**) at 100 μM. The structures are shown at the top of each panel. (B) Summary data (means ± SEM) for the effect on TRPM2 current. The current measured at ±80 mV was normalized to that blocked by 2-APB (100 μM). ***$P < 0.001$, significantly different from FFA group; ANOVA. $n = 3–6$ for each group. (C) Comparison of the concentration–response curves for 3-MFA (**1**) and FFA. (D) Current–voltage relationship and the inhibition of TRPM2 current by 3-MFA. (E) Inhibition of ADP-ribose-induced TRPM2 current by 3-MFA (100 μM) was partly reversed after wash-out and abolished by 2-APB (100 μM).

Comparison with fenamates, econazole and 2-APB

FFA, econazole and 2-APB are known to be TRPM2 channel blockers (Hill *et al.*, 2004a,b; Togashi *et al.*, 2008). Here we compared their pharmacological properties. FFA and 3-MFA (**1**) showed a similar potency for blockade of TRPM2 channels (Figure 3C), but FFA also caused a significant Ca^{2+} release (Figure 5A). This effect was partly exerted on the endoplasmic reticulum (ER) Ca^{2+} store, because the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) blocker thapsigargin reduced by nearly half (46%), the effect of FFA-induced Ca^{2+} release

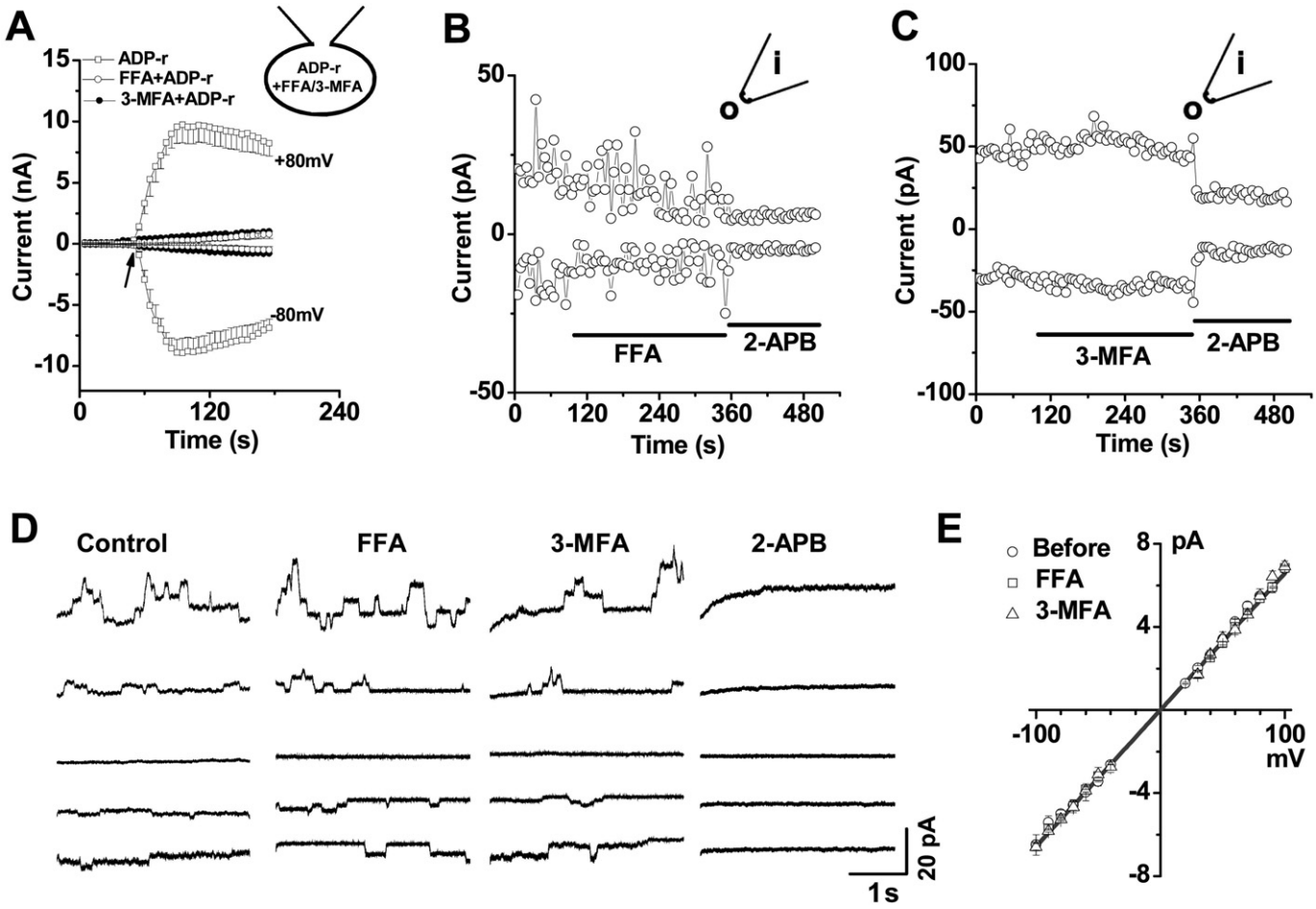

Figure 4

Effect of 3-MFA or FFA applied intracellularly. (A) Whole-cell patch was recorded in the TRPM2 cells using pipette solution containing 0.5 mM ADP-ribose (ADP-r) with or without FFA (200 μM) or 3-MFA (200 μM) (n = 5 for each group). (B) Example of outside-out patches showing the effect of FFA (100 μM) and 2-APB (100 μM). (C) Example of the effect of 3-MFA (100 μM) and 2-APB (100 μM). (D) Single channel activity of TRPM2 recorded by outside-out patches (n = 4). (E) Mean unitary current sizes for ADP-ribose-induced TRPM2 single channel events plotted against voltage. Straight lines were fitted, and the mean unitary slope conductance was 65.76 ± 0.23 pS (0.5 mM ADP-ribose). No effect of FFA (100 μM, 65.81 pS) and 3-MFA (100 μM, 65.61 pS) on the single channel conductance in the outside-out patches.

(Figure 5B). The other part of FFA-induced Ca^{2+} release could be due to mitochondrial Ca^{2+} release which has been described by us (Jiang *et al.*, 2012) and other groups (McDougall *et al.*, 1988; Poronnik *et al.*, 1992; Tu *et al.*, 2009). Although MFA had a small effect on Ca^{2+}-release, but its analogue 3-MFA (**1**) had no effect on this variable (Figure 5C and D). FFA-induced Ca^{2+} release was unrelated to the expression of TRPM2 channels, because the amplitude of the Ca^{2+} release signal in the induced cells (after tetracycline) was similar to that in the non-induced cells. These data suggested that 3-MFA was more selective than FFA and MFA as it did not affect Ca^{2+} release from intracellular stores.

Application of econazole (10 μM) inhibited TRPM2 current evoked by intracellular ADP-ribose. The onset of blockade was rapid and partly reversed by wash-out. However, econazole at 10–100 μM showed an inhibition at first and followed by a gradual increase of the whole cell current. 2-APB (100 μM) and FFA (100 μM) were unable to block the current evoked by econazole (Figure 6A–D, Supplementary Figure S1). Substitution of Na^+ with equimolar $NMDG^+$ showed a small reduction of the inward current through TRPM2 channels, but the inward current in the cells without econazole treatment was nearly abolished. In addition, the econazole-induced current was irreversible and resistant to the Cl^- channel blocker tamoxifen (10 μM) (Supplementary Figure S1C). The non-induced cells also showed the current induced by econazole (data not shown), suggesting that this econazole-induced current could be a non-selective current, which may result from the non-specific effects of antifungal drugs on membrane permeability. On the other hand, econazole (100 μM) induced significant Ca^{2+} oscillations in the T-REx cells. These oscillations were reversed on washing and inhibited by 100 μM 2-APB (Figure 6E and F). Pretreatment with the SERCA blocker thapsigargin (1 μM) prevented the Ca^{2+} oscillations (Figure 6G), suggesting that the econazole-induced Ca^{2+} oscillations were related to Ca^{2+} release from the ER.

2-APB at 100 μM nearly abolished the TRPM2 current, which was consistent with recent reports (Togashi *et al.*,

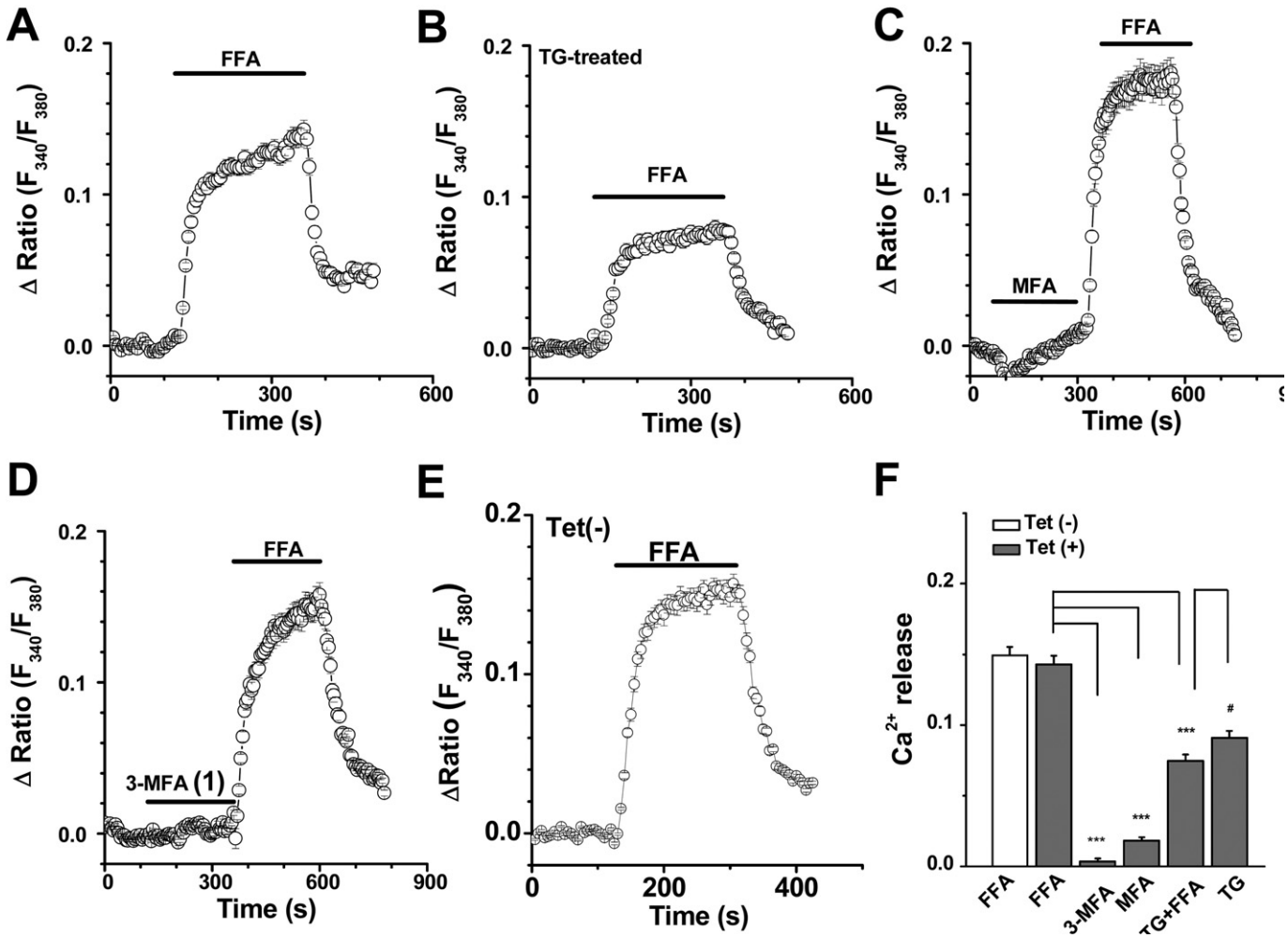

Figure 5

Ca^{2+} release induced by FFA. Cytosolic Ca^{2+} concentrations were monitored in the T-REx cells perfused with Ca^{2+}-free bath solution. (A) FFA (100 μM) induced Ca^{2+} release in the Ca^{2+}-free bath solution. (B) The FFA (100 μM) -induced Ca^{2+} release decreased in cells treated with 1 μM thapsigargin (TG). (C) Perfusion with MFA (100 μM) followed by FFA (100 μM). (D) No effect of 3-MFA (1) (100 μM) on Ca^{2+} release, but FFA (100 μM) evoked Ca^{2+} release. (E) FFA induced Ca^{2+} release in the non-induced cells [Tet(–)]. (F) Summary data (means ± SEM) for the amplitude of Ca^{2+} release signal. FFA, 3-MFA and MFA at 100 μM and TG at 1 μM were applied (n = 20–26 cells). ***P < 0.001, significantly different from FFA Tet(+) group. $^{\#}P$ < 0.05 significantly different from TG+FFA group; ANOVA.

2008; Naziroglu *et al.*, 2011). 2-APB not only blocked the TRPM2 current in the induced cells but also inhibited the current in the non-induced cells. The effect was rapid in onset and the current recovered fully after wash-out (see Figure 1). This result was contrary to our previous report (Xu *et al.*, 2005). After re-examining the effect of 2-APB, we believe the difference could be due to cell injury and membrane leak after long-lasting activation in our previous study. The massive Ca^{2+} influx through TRPM2 channels resulted in plasma membrane blebbing and cell shape change (Supplementary Figure S2). The non-induced TRPM2 cells had no such membrane blebbing phenomena after the membrane breakthrough with the patch pipette containing 0.5 mM ADP-ribose, suggesting that the plasma membrane blebbing was dependent on the activity of TRPM2 channels.

Discussion

In this study, we have compared the effect of some fenamate analogues, econazole and 2-APB on TRPM2 channels. Modification of the 2-phenylamino ring by substitution of the trifluoromethyl group in FFA with various substituents led to significant changes in channel blocking activity. The introduction of a *meta* $-CH_3$ group into the phenylamino ring (3-MFA, **1**) yielded a more selective inhibitor of TRPM2 channels that did not affect Ca^{2+} release from intracellular stores, but with a potency similar to FFA. This compound could therefore offer a new and useful tool for the selective study of this TRPM2 channel.

FFA and its analogues inhibit several types of ion channels. Early studies have shown that fenamates inhibited Ca^{2+}-activated Cl^- channels (Korn *et al.*, 1991; Hogg *et al.*,

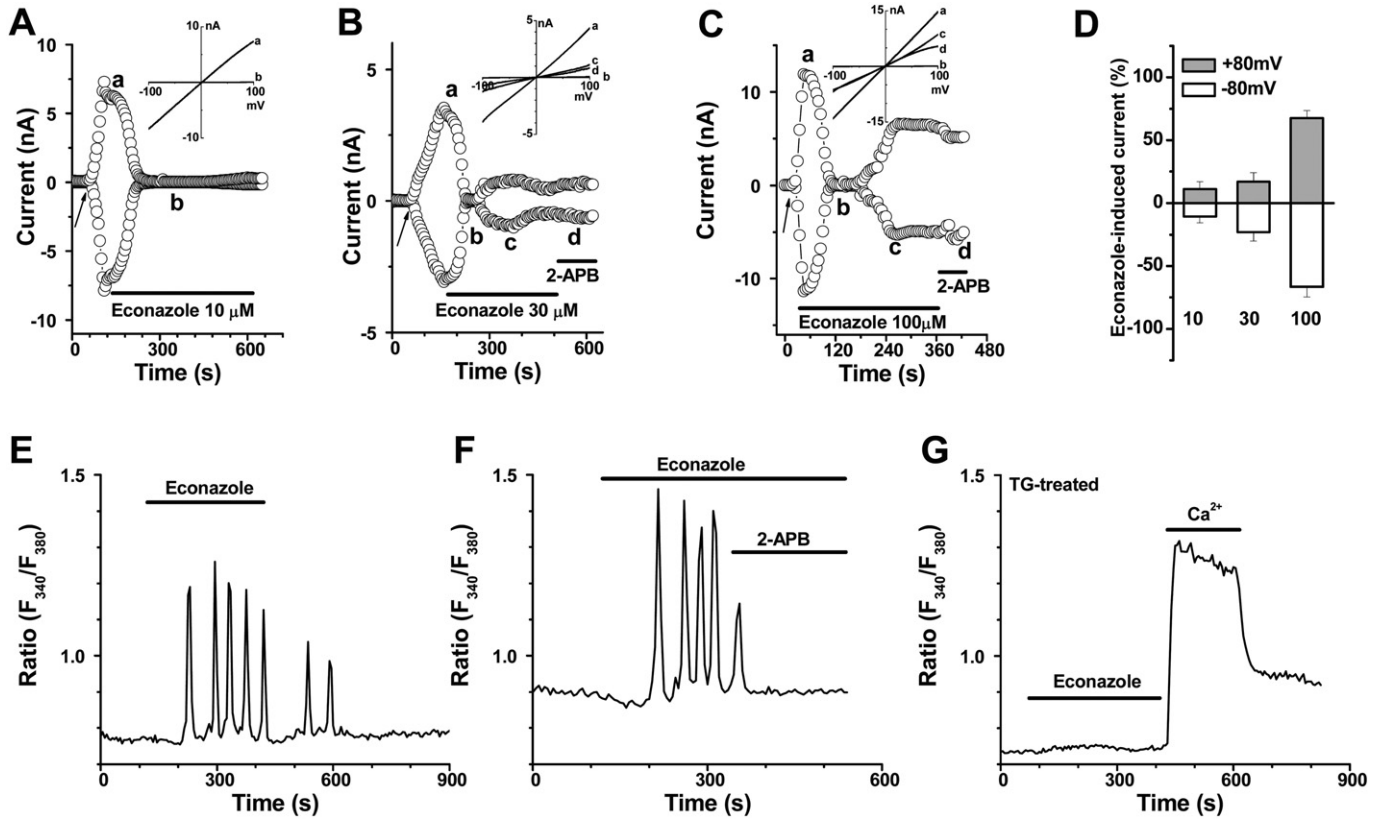

Figure 6

Effect of econazole on TRPM2 currents. (A–C) Examples of the time course for TRPM2 current inhibited by econazole (10, 30, 100 μM) and followed by an increase of the whole cell current. The *IV* curves are shown in the inset and the traces labelled as a, b, c, and d are indicated in the corresponding time course. (D) Summary data (means ± SEM) for current evoked by econazole. (E) Cytosolic Ca^{2+} oscillations induced by econazole (100 μM) in HEK-293 T-REx cells. (F) Ca^{2+} oscillations inhibited by 2-APB (100 μM) in the T-REx cells. (G) Ca^{2+} levels in cells pretreated with thapsigargin (TG; 1 μM) for 30 min and then perfused with econazole (100 μM) and then with a bath solution containing 1.5 mM Ca^{2+}.

1994; Shaw *et al.*, 1995), Ca^{2+}-activated K^+ channels (Greenwood and Large, 1995), 1-oleoyl-2-acetyl-*sn*-glycerol (OAG)-sensitive cationic current (Jung *et al.*, 2002), Ca^{2+}-activated non-selective cationic channel (Yamashita and Isa, 2003) and a cationic channel in cardiac myocytes (Macianskiene *et al.*, 2010). For the TRP channel family, FFA activated TRPC6 (Inoue *et al.*, 2001) and TRPA1 (Hu *et al.*, 2010), but inhibited TRPM2, TRPM3, TRPM4 and TRPM5 channels (Hill *et al.*, 2004a; Ullrich *et al.*, 2005; Harteneck *et al.*, 2007; Wilkinson *et al.*, 2008; Klose *et al.*, 2011; Naziroglu *et al.*, 2011), suggesting that fenamate analogues are useful tools in the study of TRP cationic channels. However, there are several reports that FFA induced mitochondrial Ca^{2+} release (Poronnik *et al.*, 1992; Hu *et al.*, 2010), which could indirectly alter the activity of TRP channels, especially the Ca^{2+}-sensitive forms including TRPM2 (Tang *et al.*, 2001; McHugh *et al.*, 2003; Zeng *et al.*, 2004). In order to find a relatively selective channel blocker, we modified the structure of FFA and found that 3-MFA (**1**) was the most promising compound among the analogues we synthesized. This compound had no effect on Ca^{2+} release and showed a slight inhibition of TRPC4 and TRPC5 channels (Jiang *et al.*, 2012) but was potent as FFA in blocking TRPM2 channels, suggesting that 3-MFA (**1**) was a more selective compound for analysing TRPM2 channel function. However, further study is still required to characterize its specificity, because we have not tested the effect of 3-MFA on other TRPM channels or native cationic channels. The IC_{50} value for FFA was lower in this study than that measured by the $FLIPR^{tetra}$ system (Klose *et al.*, 2011), which could be due to different methodology and the channel activator used. We noticed that the TRPM2 current showed significant rundown after full current development, so we used a single concentration to determine the IC_{50}, rather than a series of cumulative concentrations. In addition, the percentage of inhibition was measured at 100 s after perfusion with each tested reagent and the current rundown was corrected by linear fitting.

Econazole is an antifungal imidazole and early reports showed that it inhibited I_{CRAC} channels (Franzius *et al.*, 1994; Gamberucci *et al.*, 1998). Inhibition of TRPM2 channels was reported by Hill *et al.* (2004b), and the binding site on TRPM2 channels was extracellularly located. Our data also showed potent inhibition of TRPM2 channels by econazole, with 10 μM producing nearly full block of TRPM2 current. However, longer perfusion with econazole evoked a non-specific current. The amplitude of the non-specific current induced by econazole was concentration-dependent and achieved nano-amp values for the whole cell current, when

high concentrations of econazole (100 μM) were applied. 2-APB and FFA did not inhibit the econazole-evoked current. Substitution of Na^+ with NMDG slightly reduced the inward current, and Cl^- channel blocker tamoxifen did not affect the current. These findings suggest that the econazole-induced current was not mediated by endogenous TRPC channels or gap junctional channels or a Cl^- channel, but was a non-specific current that could be due to the membrane hyperpermeability caused by antifungal drugs (Georgopapadakou *et al.*, 1987; Matsui *et al.*, 2008). In addition, we found econazole at 100 μM caused significant cytosolic Ca^{2+} oscillations. These oscillations were probably due to inositol trisphosphate-mediated Ca^{2+} release from ER Ca^{2+} stores (Hajnoczky and Thomas, 1997), because depletion of the ER Ca^{2+} stores by the SERCA blocker thapsigargin or the inhibition of inositol trisphosphate receptors by high concentration of 2-APB can abolished the activity. Such cytosolic Ca^{2+} increase was also reported by other groups with lower concentrations of econazole in human osteosarcoma cells (Chang *et al.*, 2005), corneal epithelial cells (Chien *et al.*, 2008) and lymphocytes (Mason *et al.*, 1993), suggesting that econazole should be used with caution as a TRPM2 channel inhibitor.

The pH and temperature dependence of TRPM2 channels have been described (Hill *et al.*, 2004a; Togashi *et al.*, 2008; Yang *et al.*, 2011). In our study, we did not measure blocking activities of 3-MFA or FFA at different pH or temperature. All the experiments were performed at room temperature (23–25°C) with normal bath and pipette solutions. Unlike 2-APB, which showed a rapid and reversible inhibition of TRPM2 channels, 3-MFA and FFA only showed a small recovery after wash-out, which was comparable to that after FFA (Hill *et al.*, 2004a). The block of TRPM2 by 3-MFA was voltage-independent and its site of action was intracellularly located, in contrast to clotrimazole and 2-APB which are known to act on an extracellular site on the channel (Hill *et al.*, 2004b). The TRPM2 channels are ubiquitously expressed Ca^{2+}-permeable channels. A relatively large TRPM2-like endogenous current has been reported in the rat insulinoma cell line CRI-G1, which was blocked by FFA (Hill *et al.*, 2004a). We have not tested the effect of 3-MFA on such cells, but we have examined the effect of 3-MFA, FFA and 2-APB on the endogenous current in the non-induced HEK293 cells. 3-MFA only slightly changed the endogenous current, whereas both FFA and 2-APB showed clear inhibition and their inhibition was fully reversible after wash-out (data not shown), suggesting that the endogenous current may have different properties from the expressed TRPM2 channels or could be a non-specific current, sensitive to 2-APB and FFA, which needs to be further studied.

In conclusion, our results show that some fenamates, econazole and 2-APB are all useful blockers of TRPM2 channels. However, FFA, econazole and 2-APB have non-specific effects on intracellular Ca^{2+} movement and on other channels, so these unwanted effects should be considered when the blockers are used for pharmacological studies of TRPM2 channels. The fenamate analogue, 3-MFA (**1**), was as potent as FFA but had no effect on Ca^{2+} release from intracellular stores and may thus be used as a more selective TRPM2 channel blocker. However, we have not yet tested the effect of 3-MFA on other ion channels, which are targeted by FFA; therefore, the specificity and utility of 3-MFA as a TRPM2 blocker need to be further investigated. The information on structure-activity correlations would be useful for further improvement of the design of new fenamate-based channel blockers.

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Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Econazole-induced current and the effect of $NMDG^+$, tamoxifen and FFA. (A) TRPM2 current was induced by 0.5 mM ADP-ribose in the pipette solution. Perfusion with econazole (30 μM) inhibited the TRPM2 current and followed by the activation of another current. Perfusion with solutions containing $NMDG^+$ (140 mM), an equimolar substitute for Na^+, slightly reduced the inward current. (B) TRPM2 current inhibited by Na^+ substitution with $NMDG^+$ in the cells without econazole treatment. (C) Effect of FFA (100 μM) on econazole-induced current. (D) Effect of tamoxifen (10 μM).
Figure S2 Plasma membrane blebbing in cells with induced TRPM2 channels, during the patch recording. (A) Image showing the cells after the formation of whole-cell configuration for 3 min. (B) The same cells as in (A) photographed at 10 min after TRPM2 channels had been fully activated. Membrane blebbing is indicated by arrow.

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