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Flufenamic acid as an ion channel modulator

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ABSTRACT

Flufenamic acid has been known since the 1960s to have anti-inflammatory properties attributable to the reduction of prostaglandin synthesis. Thirty years later, flufenamic acid appeared to be an ion channel modulator. Thus, while its use in medicine diminished, its use in ionic channel research expanded. Flufenamic acid commonly not only affects non-selective cation channels and chloride channels, but also modulates potassium, calcium and sodium channels with effective concentrations ranging from 10^{-6} M in TRPM4 channel inhibition to 10^{-3} M in two-pore outwardly rectifying potassium channel activation. Because flufenamic acid effects develop and reverse rapidly, it is a convenient and widely used tool. However, given the broad spectrum of its targets, experimental results have to be interpreted cautiously. Here we provide an overview of ion channels targeted by flufenamic acid to aid in interpreting its effects at the molecular, cellular, and system levels. If it is used with good practices, flufenamic acid remains a useful tool for ion channel research. Understanding the targets of FFA may help reevaluate its physiological impacts and revive interest in its therapeutic potential.

Contents

1. Introduction

Flufenamic acid (FFA), namely N-(alpha,alpha,alpha-trifluoro-m-tolyl) anthranilic acid (CI-440), is an aromatic amino acid consisting of anthranilic acid carrying an *N*-(trifluoromethyl)phenyl substituent (Fig. 1). Its anti-inflammatory and analgesic effects were recognized in the 1960s (Winder et al., 1963) and thus FFA is included in the family of non-steroidal anti-inflammatory drugs (NSAIDs) with mefenamic, meclofenamic (MFA) and niflumic acids (NA). Anti-inflammatory actions occur mainly through reduction of prostaglandin synthesis from arachidonic acid by inhibiting the cyclo-oxygenases (Fig. 1) (Flower et al., 1972).

Despite lower effectiveness than other NSAIDs (Flower, 1974), FFA was locally applied for analgesia against pain and inflammation

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Abbreviations: BK_{Ca}, big K^+ channel; BLINaC, brain liver intestine Na^+ channel; CaCC, Ca^{2+}-activated chloride current; CFTR, cystic fibrosis transmembrane conductance regulator; ClC, chloride channel; ClC-K, chloride channel kidney; Cx, connexin; EC_{50}, concentration for half maximal effect; FFA, flufenamic acid; GABA, γ-aminobutyric acid; HEK-293, human embryonic kidney cell line 293; HERG, human ether-a-gogo-related gene; $I_{Cl,swell}$, swelling-activated chloride current; IC_{50}, concentration for half maximal inhibition; K_{Ca}, Ca^{2+}-activated K^+ channel; K_v, voltage-gated K^+ channel; K_{2P}, two pores K^+ channel; MFA, mefenamic acid; nAchR, nicotinic acetylcholine receptor; NA, niflumic acid; NMDA, N-methyl-D-aspartate; NSC, non-selective cation channels; NSC_{Ca}, Ca^{2+}-activated non-selective cation channels; PanX, pannexin; TMEM16A, transmembrane protein 16A; TRP, transient receptor potential channels; TRPA, transient receptor potential ankyrin; TRPC, transient receptor potential canonical; TRPM, transient receptor potential melastatin; TRPV, transient receptor potential vanilloid.

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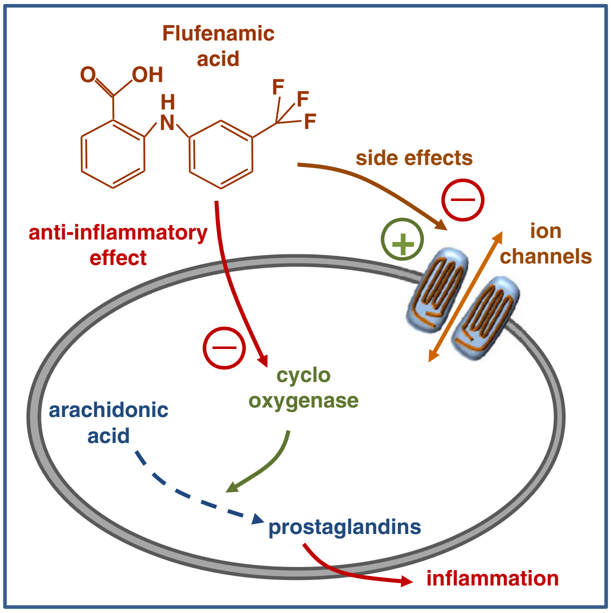

Fig. 1. Anti-inflammatory effect of flufenamic acid. Chemical structure of flufenamic acid and its main targets: cyclooxygenase for anti-inflammatory effect and ion channels for additional effects.

associated with musculoskeletal and joint disorders, peri-articular and soft tissue disorders. Oral administration was discontinued because of large intersubject variability in FFA absorption (Lentjes & van Ginneken, 1987). In addition, the dermal administration reduces first-pass metabolism (Roberts & Walters, 2008). FFA, similar to other NSAIDs, has side effects including gastrointestinal perturbations (which are reduced in dermal application) (Ravi et al., 1986) and renal damage. Due to these deleterious side effects, and because its benefits were weak compared to other NSAIDs, the use of FFA in medicine remained somewhat limited. Nevertheless, human trials in more than 10,000 patients in 1998 re-affirmed NSAIDs effectiveness for acute and chronic pain relief, and particularly emphasized FFA topical application in combination with salicylic acid (Moore et al., 1998).

Interest in FFA revived following the 1976 report of an effect on calcium and sodium uptake in lymphoid cells, which suggested that ion-handling proteins were affected (Famaey & Whitehouse, 1976). Indeed, during the 1990s FFA became recognized as a common regulator of ionic currents in native tissues. The story was elaborated in the 2000s as the molecular identities of the ion channels targeted by FFA were discovered, including Cl^-, Na^+, K^+ and, most notably, non-selective cation channels. Therefore, FFA made a comeback in basic research as a convenient pharmacological tool to study ion channels. However, its broad spectrum of targets may produce complex experimental results that are difficult, or impossible, to interpret unambiguously.

Here, we focus on ion channels modulated by FFA, including native currents and cloned channel proteins. We aim to provide an overview of the currents modulated by FFA to help differentiate its effects in physiological contexts where several ion channel types could be affected pharmacologically (see Table 1 and Fig. 2 for specific ion channel targets, permeability, FFA efficiency, and experimental conditions).

2. Flufenamic acid as an ion channel modulator

2.1. Chloride channels

Anion channels poorly differentiate between anions but because Cl^- is most abundant, the channels are referred to as chloride channels. These channels are implicated in a variety of physiological processes, depending on their regulatory properties, including sensitivity to voltage, cell volume, internal Ca^{2+}, cAMP, pH, and ligand binding (see Duran et al., 2010 for review). Chloride channels were the first family shown to be affected by FFA, which is considered to be a classical chloride channel blocker, along with disulfonic stilbenes (DIDS, SITS, DNDS), anthracene carboxylates (9-AC), arylaminobenzoates (DPC), indanylalkanoic acids (IAA-94), clofibric acid derivatives (CPP) and other fenamates such as NPPB and niflumic acid (see Suzuki et al., 2006 for review).

2.1.1. Cystic fibrosis transmembrane conductance (CFTR)

A cAMP-dependent chloride current, later recognized as the cystic fibrosis transmembrane conductance regulator (CFTR), was the first identified FFA target among ion channels (McCarty et al., 1993). CFTR is an ATP-binding cassette (ABC) protein containing 1480 amino acids divided into two domains, each composed of six transmembrane domains. CFTR forms a PKA and PKC-activated chloride channel mediating chloride transport in a variety of tissues, with a major role in airway epithelia. Altering its activity or expression leads to cystic fibrosis and secretory diarrhea. CFTR is intensively studied because of its role in pathology (Welsh et al., 1992; Duran et al., 2010 for review).

CFTR modulators were sought to correct chloride transport in cystic fibrosis (Becq & Mettey, 2004). FFA, which is membrane permeable, inhibits CFTR heterologously expressed in *Xenopus* oocytes (McCarty et al., 1993). FFA inhibition is stronger at positive voltages. In addition, since the effect is observed in inside-out patches, FFA inhibits CFTR by direct interaction with the channel, producing an open-channel block. However, high concentrations are necessary to inhibit the channel; indeed, the CFTR currents are reduced by only 20–30% by 200 μM FFA (McCarty et al., 1993).

Probably due to its low efficiency, FFA has rarely been used to study CFTR in physiological preparations (Liu et al., 2006). However, because CFTR is expressed in apical membranes of epithelia, its inhibition has to be considered when using FFA at high concentrations in tissues such as airway epithelia, intestine, pancreas, kidney, sweat duct and testis, as well as cardiac cells that express CFTR (Duan, 2009 for review).

2.1.2. Ca^{2+}-activated chloride currents (CaCCs) and bestrophins

A Ca^{2+}-activated chloride current (CaCC) first described in *Xenopus* oocytes (Barish, 1983) has been similarly recorded in excitable and non-excitable cells (Huang et al., 2012a for review). For example, CaCC is present in Cl^- secretory epithelia and in tissues expressing cAMP-activated Cl^- current attributed to CFTR, where both channel types co-localize in the apical membrane (Cliff & Frizzell, 1990). Also, CaCC is present in cardiomyocytes; its cytosolic Ca^{2+} activation profile, outward rectification, and time-dependent inactivation contribute to cardiac action potential repolarization (Duan, 2009).

FFA inhibits the archetypal CaCC from *Xenopus* oocytes with an IC_{50} ranging from 28 to 35 μM (White & Aylwin, 1990; Oh et al., 2008). Inhibition by FFA has also been observed in other native CaCCs in rabbit portal vein, pig ventricular cardiomyocytes, as well as olfactory receptor neurons from moth *Spodoptera littoralis* (Greenwood & Large, 1995; Gwanyanya et al., 2010; Pezier et al., 2010). FFA appears to exert an open-channel block like in CFTR (Greenwood & Large, 1995). Despite its lack of specificity for CaCC, FFA remains a useful tool to study these currents because its IC_{50} is comparably low and, until now, no other CaCC-specific inhibitors have been identified.

The molecular identity of CaCCs remains unknown. Three major protein families have been proposed (Huang et al., 2012a). The first candidate comes from the Ca^{2+} activated chloride channel (CLCA) protein family, initially shown to produce chloride currents. However, its identity as an ion channel has been strongly debated and it is now considered as a secreted non-integral membrane protein (Winpenny et al., 2009). Moreover, the CaCC endogenous expression levels do not

Table 1
Information about ion channels and currents affected by FFA. Depending on the reports, a single FFA concentration was used ([FFA]) or concentration for half maximal effect (EC_{50}) or dissociation constant (K_D) was provided.

Channel name	Permeability	Current	Cell	Configuration	FFA effect	[FFA] in 10^{-6} M	EC_{50} in 10^{-6} M	K_D in 10^{-6} M	Other fenamates	Mechanisms	References
Chloride channels											
CFTR	Cl^-	cAMP-activated Cl^- current	*Xenopus* oocyte	Whole-cell Single channel	Inhibition			200 to 1000		Direct interaction in the open state	McCarty et al., 1993
ClC-Ka	Cl^-	Voltage-gated Cl^- current	*Xenopus* oocyte	Whole-cell	Inhibition			57 to 121	MFA>FFA	Direct interaction in the vestibule	Liantonio et al., 2006
ClC-Kb	Cl^-	Voltage-gated Cl^- current	*Xenopus* oocyte	Whole-cell	Activation	200			NA>FFA		Liantonio et al., 2006
ClC-1	Cl^-	Voltage-gated Cl^- current	*Xenopus* oocyte	Whole-cell	Inhibition			4.5	FFA>NA	Direct interaction	Liantonio et al., 2007
$GABA_A$-R	Cl^-	GABA-induced Cl^- current	*Xenopus* oocyte	Two electrodes voltage-clamp	Inhibition			16			Woodward et al., 1994
			HEK-293	Whole-cell	Inhibition			2			
PanX-1	Cl^-		HEK-293	Whole-cell	Inhibition		>1000		FFA=NA		Ma et al., 2009
Non-selective cation channels											
TRPC3	Na^+, K^+, Ca^{2+}	Redox-sensitive NSC current	HEK-293	Whole-cell	Inhibition	100					Inoue et al., 2001
TRPC4	Na^+, K^+, Ca^{2+}	Redox-sensitive NSC current	HEK-293	Whole-cell	Inhibition		55		FFA>NA>MFA	Direct interaction	Jiang et al., 2012
TRPC5	Na^+, K^+, Ca^{2+}		HEK-293	Whole-cell	Inhibition		37		FFA>MFA>NA	Direct interaction	Jiang et al., 2012
TRPC6	Na^+, K^+, Ca^{2+}	α-Adrenoreceptor -activated NSC current	HEK-293	Whole-cell	Inhibition		17		FFA>MFA>NA		Klose et al., 2011
TRPC6	Na^+, K^+, Ca^{2+}	α-Adrenoreceptor -activated NSC current	HEK-293	Whole-cell	Activation	100			FFA>>NA	Direct interaction	Inoue et al., 2001; Foster et al., 2009
TRPC7	Na^+, K^+, Ca^{2+}		HEK-293	Whole-cell	Inhibition	100					Inoue et al., 2001
TRPM2	Na^+, K^+, Ca^{2+}	Hydrogen peroxide-activated NSC	HEK-293	Whole-cell	Inhibition		155.1		FFA>NA=MFA		Klose et al., 2011
TRPM3	Na^+, K^+, Ca^{2+}	Hypoosmolarity -activated NSC	HEK-293	Whole-cell	Inhibition		33.1		MFA>FFA>NA		Klose et al., 2011
TRPM4	Na^+, K^+	NSC_{Ca}	HEK-293	Whole-cell	Inhibition		2.8				Ullrich et al., 2005
TRPM5	Na^+, K^+	NSC_{Ca} in taste cells	HEK-293	Whole-cell	Inhibition		24.5				Ullrich et al., 2005
TRPV1	Na^+, K^+, Ca^{2+}	Capsaicin-activated NSC current	*Xenopus* oocyte	Two electrodes voltage-clamp	Inhibition	100					Hu et al., 2010
TRPV3	Na^+, K^+, Ca^{2+}	Thermo-sensitive NSC current	*Xenopus* oocyte	Two electrodes voltage-clamp	Inhibition	100					Hu et al., 2010
TRPV4	Na^+, K^+, Ca^{2+}	Thermo-sensitive NSC current	HEK-293	Whole-cell	Inhibition		40.7		FFA>NA>MFA		Klose et al., 2011
TRPA1	Na^+, K^+, Ca^{2+}	Heat-activated NSC current	HEK-293	Whole-cell	Activation		57				Hu et al., 2010
α3−β2 nAchR	Na^+, K^+, Ca^{2+}	Neuronal-nicotinic Ach-receptor	*Xenopus* oocyte	Two electrodes voltage-clamp	Inhibition		90		FFA>NFA	Direct interaction	Zwart et al., 1995
α3−β4 nAchR	Na^+, K^+, Ca^{2+}	Neuronal-nicotinic Ach-receptor	*Xenopus* oocyte	Two electrodes voltage-clamp	Activation		30		FFA>NFA	Direct interaction	Zwart et al., 1995
Cx 43	Na^+, K^+, Ca^{2+}	Gap junction	Rat kidney fibroblast	Dye measurements	Inhibition		40		MFA>FFA		Harks et al., 2001
Cx 50	Na^+, K^+, Ca^{2+}	Gap junction	N2A neuroblastoma cells	Two electrodes voltage-clamp	Inhibition		47		NA>FFA=MFA	Reduction of open probability. Binding in a modulatory site within membrane	Srinivas & Spray, 2003
Potassium channels											
K_{Ca} 1.1	K^+	Ca^{2+}-activated K^+ current (BK_{Ca})	*Xenopus* oocyte	Two electrodes voltage-clamp	Activation		>300		FFA=NA		Gribkoff et al., 1996
K_V 11.1	K^+	Human ether à gogo related current (HERG)	*Xenopus* oocyte	Two electrodes voltage-clamp	Activation	100			FFA>NA		Malykhina et al., 2002

Table 1 (*continued*)

Channel name	Permeability	Current	Cell	Configuration	FFA effect	[FFA] in 10^{-6} M	EC_{50} in 10^{-6} M	K_D in 10^{-6} M	Other fenamates	Mechanisms	References
Potassium channels											
K_V 7.1	K^+	Delayed -rectifier K^+ current	*Xenopus* oocyte	Two electrodes voltage-clamp	Activation	100				Slowing of channel deactivation	Busch et al., 1994
K_{Ca} 4.2	K^+	Two pores outward rectifier K^+ current	*Xenopus* oocyte	Two electrodes voltage-clamp	Activation		1100		MFA> FFA>NA	Binding in the pore region	Garg & Sanguinetti, in press
K_{2p} 2.1	K^+	Lipid-sensitive mechano-gated 2P domain K^+ channel	Cos-7	Perforated patch-clamp	Activation		100		FFA> NA=MFA		Takahira et al., 2005
K_{2p} 4.1	K^+	TWIK-related arachidonic acid -stimulated K^+ channel	Cos-7	Perforated patch-clamp	Activation		>500		FFA= NA>MFA		Takahira et al., 2005
K_{2p} 10.1	K^+	Inward rectifier K^+ channel	Cos-7	Perforated patch-clamp	Activation		>100		FFA> NA=MFA		Takahira et al., 2005
Sodium channels											
BLINaC	Na^+	Brain liver intestine Na^+ channel	*Xenopus* oocyte	Two electrodes voltage-clamp	Activation		>1000		FFA>NA	Increase of Na^+ selectivity	Wiemuth & Grunder, 2011

Current	Permeability	Cell	Configuration	FFA effect	EC_{50} in 10^{-6} M	K_D in 10^{-6} M	Other fenamates	Mechanisms	References
CaCCs	Cl^-	*Xenopus* oocyte	Two electrodes voltage-clamp	Inhibition	35.4	28	F=NA>MFA	Direct interaction in the open state	White & Aylwin, 1990 Oh et al., 2008
$ICl_{l,swell}$	Cl^-	Human gastric epithelial cells	Whole-cell	Inhibition	$50<IC_{50}<200$				Jin et al., 2003
NMDA-R current	Na^+, K^+, Ca^{2+}	Spinal cord neurons						Independent from NMDA	Lerma & Martin del Rio, 1992
Voltage-gated I_{Na}	Na^+	Rat hippocampal pyramidal neurons	Whole-cell	Inhibition	189			Modification of inactivation kinetic	Yau et al., 2010
Voltage-gated I_{Ca}	Ca^{2+}	Smooth muscle cells of rat carotid artery	Whole-cell	Inhibition	100				Shimamura et al., 2002

match the expression levels that characterize CLCA. The second candidate comes from the bestrophin family, so called because mutations of the prototypic member Best-1 causes Best disease, an inherited form of retinal macular dystrophy (Xiao et al., 2010). The bestrophin family is composed of four members found in the human genome. The expression of some of these four transmembrane domain proteins produces a Cl^- current activated by physiological levels of internal Ca^{2+}. In hippocampal astrocytes, 100 μM FFA inhibits a Ca^{2+}-activated anionic current by 75% (Park et al., 2009). This endogenous current is reduced by the expression of mBest-1-specific short hairpin RNA, which suggests that the Ca^{2+}-activated anion current corresponds to Best-1 and, thus, indirectly demonstrates Best-1 sensitivity to FFA. At present, to the best of our knowledge, there are no reports demonstrating the direct effects of FFA on bestrophins. The most recent candidate for the molecular identity of CaCCs is the transmembrane protein 16A (TMEM16A), which forms a CaCC channel subunit (Huang et al., 2012b). This eight transmembrane segment protein may form a functional channel as a homodimer. No existing data show TMEM16A modulation by FFA, even though the effects of FFA have been reported on TMEM16A-expressing cells such as pulmonary artery smooth muscle cells and human airway gland cells (Fischer et al., 2010; Yamamura et al., 2011).

2.1.3. Swelling-activated chloride currents (ICl_{swell}) and chloride channel-3

Chloride channels that activate under hypo-osmotic conditions can prevent cellular injuries associated with swelling. In conjunction with K^+ channels, they allow KCl leakage, leading to intracellular dilution, net water loss, and volume decrease. A swelling-activated Cl^- current named ICl_{swell} has been characterized in virtually every cell yet examined, including in the heart where ICl_{swell} may combat arrhythmias (Baumgarten & Clemo, 2003; Duran et al., 2010). Tissue-specific differences in biophysics and pharmacology suggest that different channel proteins give rise to ICl_{swell} in different cells. ICl_{swell} is supported by an outwardly rectifying Cl^- channel in rabbit and human myocytes (Duan et al., 1997a; Demion et al., 2006). Open probability is not voltage-dependent but the single-channel conductance increases from 10 to 80 pS as voltage ascends, resulting in a pronounced outward rectification (Duan et al., 1997a; Demion et al., 2006).

In human gastric epithelial cells, 100 μM FFA reduced ICl_{swell} by 82% (Jin et al., 2003). More recently, the same concentration has been shown to inhibit ICl_{swell} in microglia (Schlichter et al., 2011) and reduce regulatory volume decrease in bovine ciliary epithelium (Do et al., 2006).

The molecular identity of ICl_{swell} is a subject of debate. The confusion is probably due to several underlying channel proteins whose

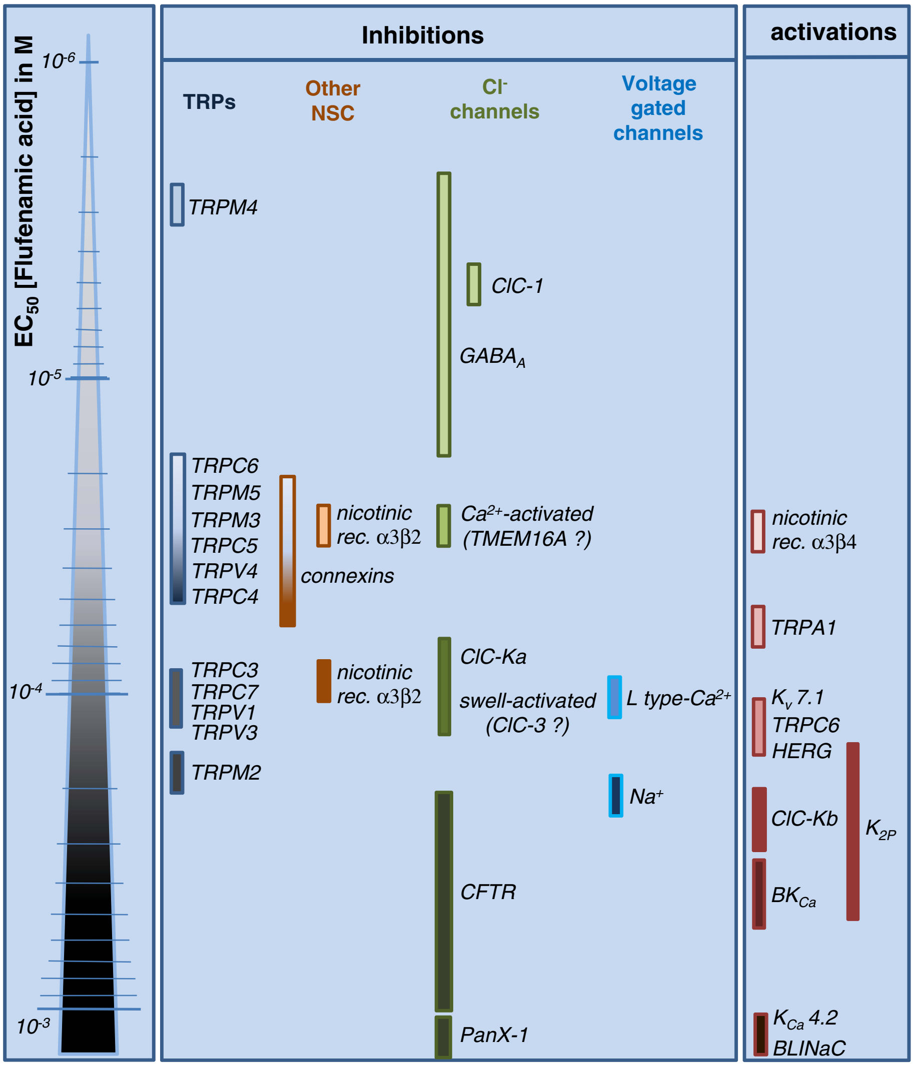

Fig. 2. Ion channels targeted by flufenamic acid. Flufenamic acid produces inhibition or activation of ion channels. Colored bars near ionic channel name correspond to the estimated EC_{50} for flufenamic effect. References are provided within the text.

expression differs with tissue type. One of the strongest candidates belongs to the chloride channel (ClC) family initially identified by the cloning of the voltage-gated Cl^- channel from the electric organ of the *torpedo* electric ray. Nine members comprise the ClC family in mammals (Duran et al., 2010). The constituent molecules, composed of 10 to 12 transmembrane domains, have two conducting pores. Among ClCs, ClC-3, cloned in 1997, is broadly distributed among tissues and its expression gives rise to an outwardly rectifying chloride channel activated by cell swelling (Duan et al., 1997b). Following the cloning of ClC-3, competing studies putatively demonstrated or, alternatively, invalidated the idea that ClC-3 mediated the endogenous swelling-activated chloride-current involved in cell volume regulation (Duan et al., 2001; Weylandt et al., 2001; Duran et al., 2010). It is unfortunate for our purposes that none of these studies directly evaluated the effects of FFA on the ClC-3 cloned protein. Nonetheless, FFA and anti-ClC-3 antibodies attenuated ICl_{swell} in human gastric epithelial cells and disrupted the attendant regulatory volume decrease, which suggests that ClC-3 is sensitive to FFA (Jin et al., 2003).

2.1.4. Renal transepithelial Cl^- transport and chloride channel kidney

Within the ClC family, the expression of ClC-K channels is restricted to the basolateral membrane of kidney cells (from the thin ascending limb to the collecting duct), where they play a major role in urine concentration. ClC-K is also expressed in the inner ear where these channels participate in endolymph production (Fahlke & Fischer, 2010). Two human ClC-K isoforms (ClC-Ka and ClC-Kb) correspond to ClC-K1 and ClC-K2 orthologs in rat. Unlike other ClCs, ClC-K channels require the presence of an additional β-subunit called barttin (Estevez et al., 2001), which produces a chloride current with moderate outward rectification (Estevez et al., 2001; Waldegger et al., 2002). Mutations in ClC-Kb that reduce channel activity cause type III Bartter's syndrome, a renal disease characterized by severe salt wasting (Simon et al., 1997; Seyberth & Schlingmann, 2011). Mutations in barttin cause Bartter's syndrome type IV, which is characterized by renal failure and sensorineural deafness (Birkenhager et al., 2001).

Experiments sought to identify ClC-K ligands to discover pharmacological interventions for Bartter's diseases. FFA inhibits ClC-Ka in *Xenopus* oocytes with a binding constant ranging from 57 μM at −140 mV to 121 μM at +60 mV (Liantonio et al., 2006). The authors predicted 1:1 binding based on the dose response curve. Therefore, K_D might be equivalent to the EC_{50} for ClC-Ka. This FFA inhibition is abolished by the N68D mutation, a residue putatively located on the extracellular vestibule (Liantonio et al., 2006). A non-coplanar conformation in the aromatic group of FFA is necessary for the inhibitory binding site (Liantonio et al., 2008); FFA derivatives with coplanar aromatic groups are too rigid to enter the narrow part of the extracellular vestibule. Nonetheless, coplanar ligands bind to an activating site and activate ClC-Ka (Gradogna & Pusch, 2010).

ClC-Kb does not exhibit the same FFA sensitivity: 200 μM FFA increases ClC-Kb current by two-fold while, at the same dose, it reduces ClC-Ka current by half (Liantonio et al., 2006). At present there are no clear explanations for these discrepancies.

To our knowledge, the effects of FFA on renal and inner ear transepithelial salt transport systems remain unknown even though other ClC-K blockers were recently shown to increase water dieresis in rat (Liantonio et al., 2012).

2.1.5. Skeletal muscle voltage-gated chloride current and chloride channel-1

In a study designed to evaluate skeletal muscle chloride current sensitivity to niflumic acid, the authors also observed that 100 μM FFA abolished about all the endogenous chloride current from native rat muscle fibers (Liantonio et al., 2007). Because skeletal muscle chloride conductance is mainly attributable to the ClC-1 chloride channel protein (Steinmeyer et al., 1991), they subsequently tested the effect of FFA on ClC-1 expressed in *Xenopus* oocytes. The blocking potency of FFA, with a K_D value of 4.5 μM, was enhanced compared to niflumic acid (Liantonio et al., 2007). This unique report of FFA-sensitive ClC-1 awaits further confirmation since it is based on only five recordings. However, if confirmed, the inhibition of ClC-1 by FFA might have physiological importance because it occurs at low concentrations and dysfunction of this channel causes congenital myotonia from both autosomal dominant (Thomsen type) and autosomal recessive (Becker type) inherited patterns (Tang & Chen, 2011 for review).

2.1.6. Synaptic inhibition and γ-aminobutyric acid A-receptor

The γ-aminobutyric acid (GABA) receptor mediates fast inhibitory neurotransmission in the central nervous system by opening anion channels. GABA channels share a five-subunit structure with other ligand-gated ion channels, in which each subunit is composed of four transmembrane domains. Of the three types of GABA receptors, $GABA_A$ and $GABA_C$ form Cl^- channels ($GABA_B$ receptors are G-protein coupled and linked to K^+ channels). The single-channel conductance of $GABA_A$ and $GABA_C$ ranges from 10 to 30 pS. Its current–voltage relationship is linear at the single channel level, yet exhibits weak outward rectification at the macroscopic level (Bormann et al., 1987; Macdonald et al., 1989). $GABA_A$ receptor subunit mutations that reduce GABA-activated currents are associated with epilepsy (Baulac et al., 2001; Wallace et al., 2001; Macdonald et al., 2010).

FFA, like other NSAIDs, modulates $GABA_A$ receptors. Nevertheless, whereas most NSAIDs exert a potentiating effect, FFA reduces the GABA-induced current with an IC_{50} of 16 μM in a model of $GABA_A$ receptors expressed in *Xenopus* oocytes (Woodward et al., 1994) and 2 μM in a model of $GABA_A$ receptors expressed in HEK-293 cells (Rae et al., 2012). FFA effects on $GABA_A$ receptors may depend on the subunit composition in mammalian brain, because FFA exerts a potentiating effect on several $GABA_A$ subunits, while inhibiting others (Smith et al., 2004). Interestingly, FFA is specific for the $GABA_A$ isoform because it does not exert any effect on $GABA_C$ (Jones & Palmer, 2011).

The impact of $GABA_A$ modulation by FFA on neurophysiology is incompletely understood. It has been shown that FFA suppresses epileptiform activity (Schiller, 2004; Fernandez et al., 2010). However, at least in the hippocampus, this effect is more likely due to NMDA receptor modulation than effects on $GABA_A$ receptors.

2.1.7. Pannexins

The recently identified mammalian pannexins (PanX) are molecules that bear amino acid sequence homologies with innexins, the gap junction-forming invertebrate proteins. PanX was considered to have a structure similar to connexins (see Section 2.2.3) and thus suspected to form non-selective transmembrane pores. However, the three known isoforms form typical anion channels and do not form gap junctions but most likely function as hemi-channels when expressed in HEK-293 cells (Ma et al., 2012). PanX1 is inhibited by FFA at very high concentrations; the IC_{50} is estimated to exceed 1 mM (Ma et al., 2009).

2.2. Non-selective cation channels

Following its description as a Cl^- channel blocker, FFA was shown to modulate non-selective cation channels (NSC). NSC channels are a heterogeneous family whose members do not strongly differentiate between permeable cations. Initially characterized at the current level in native cells, now a large number of cloned genes are known to code for NSC channels. They are usually classified as ligand-gated NSC channels (e.g., nicotinic acetylcholine receptors, glutamate receptors, P2X purinergic receptors), cyclic nucleotide-gated channels (cGMP-gated or cAMP-gated channels), connexins and, the large group of transient receptor potential (TRP) channels. Despite their heterogeneity in structure, FFA modulates members in all subfamilies of NSCs except cyclic nucleotide-gated channels.

2.2.1. Transient receptor potential channels

TRP channels, first characterized in tissue from the *Drosophila* eye (Minke, 1977; Montell & Rubin, 1989), are classified for mammals into six sub-families: TRPC (canonical, seven members), TRPV (vanilloid, six members), TRPM (melastatin, eight members), TRPP (polycystin, three members), TRPML (mucolipin, three members) and TRPA (ankyrin, one member) (Gees et al., 2010). Most TRPs are permeable to Ca^{2+} as well as monovalent cations. However, some are strictly Ca^{2+} selective (TRPV5, TRPV6), whereas others are Ca^{2+} impermeable (TRPM4, TRPM5). Major physiological functions of TRP channels include Ca^{2+} signaling, sensory detection in peripheral neurons, as well as burst-generating functions in central neurons (Gees et al., 2010). TRP proteins are composed of subunits containing six transmembrane domains that assemble as tetramers. A large variety of TRP modulators have been described, including intracellular or extracellular messengers (e.g., ATP, $Ca^{2+,}$ phosphatidylinositol 4,5-bisphosphate), as well as biophysical modulators such as voltage and temperature. FFA inhibits a wide spectrum of TRP channels, including: C3, C7, M2, M3, M4, M5, M7, M8, V1, V3, and V4; but FFA activates at least two TRP channels (C6 and A1), as described below.

2.2.1.1. Transient receptor potential canonicals. An α-adrenoreceptor-activated and Ca^{2+}-permeable NSC channel is activated by FFA in rabbit portal vein smooth muscle (Yamada et al., 1996). TRPC6 is responsible for this current, and, when the protein is expressed in HEK-293 cells, its amplitude doubles in the presence of 100 μM FFA (Inoue et al., 2001). Interestingly, the FFA activating effect is not reproduced by niflumic acid, which suggests that TRPC6-activation is not a general property of the fenamate family (Foster et al., 2009). In addition, cyclo-oxygenase inhibitors do not affect this activating effect, which favors a direct interaction of FFA with the channel (Foster et al., 2009). Surprisingly, a recent paper reported an inhibitory effect of FFA ($IC_{50} = 17$ μM) on TRPC6 heterologously expressed in HEK-293 cells (Klose et al., 2011). The effect of FFA has also been evaluated on the closely related channels TRPC3 and TRPC7 that share, with TRPC6, activation by diacylglycerol, thus forming a subgroup of TRPCs. 100 μM FFA inhibits TRPC3 and TRPC7 by 60 and 90%, respectively (Inoue et al., 2001). This inhibitory action of FFA was reproduced in TRPC3-like native currents from rabbit ear arterial myocytes (Albert et al., 2006). The fact that FFA exerts opposite effects on TRPC6 vs. TRPC3/7 channels indicates that FFA and diacylglycerol may act through different mechanisms on channel activity.

In the other TRPC subgroup (TRPC1/4/5), mouse TRPC5 current is reduced by 92% by 100 μM FFA (Lee et al., 2003). A more recent study reports the inhibition by FFA of human TRPC4 and TRPC5 heterologously expressed in HEK-293 cells with IC_{50} of 55 and 37 μM respectively (Jiang et al., 2012).

2.2.1.2. Transient receptor potential melastatins. TRPM4 and TRPM5 are unique among TRPs because they do not conduct Ca^{2+} but instead are activated by internal Ca^{2+} (Guinamard et al., 2011 for review). TRPM4/5 support one of the major NSC currents often called the Ca^{2+}-activated non-selective cation current (NSC_{Ca}) and sometimes Ca^{2+}-activated non-specific cation current (I_{CAN}). NSC_{Ca} has been recorded in a wide variety of tissues, and is inhibited by FFA in, for example, pancreatic acinar cells (IC_{50} <10 μM), rat liver cells (Simon et al., 2002), cardiomyocytes (Gogelein et al., 1990; Guinamard et al., 2002), and neurons (Partridge & Valenzuela, 2000; Pace et al., 2007). It is now well established that TRPM5 is responsible for NSC_{Ca} in taste receptor cells (Liman, 2007a,b). In contrast, insulin secretion, immune response, constriction of cerebral arteries, neural burst discharge in breathing-related neurons, and cardiac dysfunctions are associated with TRPM4 function (or dysfunction) (Guinamard et al., 2011). TRPM4 occupies a special position, particularly in the present review, because of its high sensitivity to FFA. Indeed, TRPM4 is inhibited with an IC_{50} of 2.8 μM when expressed in HEK-293 cells. Interestingly, in native tissue, our group measured a similar IC_{50} of 5.5 μM for the inhibition of an endogenous TRPM4 current in rat cardiomyocytes (Guinamard et al., 2006b). The closest relative, TRPM5, is inhibited with 10 fold higher doses, the IC_{50} for TRPM5 being 24.5 μM (Ullrich et al., 2005). Low concentrations of FFA (~10 μM) may be appropriate to evaluate the physiological role of TRPM4 in situ, which would be expected to have little to no effect on other ion currents whose FFA sensitivity is much lower. Consistent with this idea, 10 μM FFA was used to differentiate breathing-related neurons that depend putatively on TRPM4 for I_{CAN}-mediated neural bursts in the respiratory oscillator pre-Bötzinger complex in mice (Del Negro et al., 2005). TRPM4 modulation may represent a major common explanation for the physiological effects of FFA given its ubiquitous expression profile and high sensitivity to FFA. This is particularly important because plasma concentrations of 4–12 μM, measured in conditions of FFA clinical use, are sufficient to strongly inhibit TRPM4 (Aly et al., 2000).

FFA also inhibits TRPM2, the most abundant TRP in the brain, which is implicated in cell death resulting from oxidative stress (Hill et al., 2004). FFA inhibits 90% of the TRPM2 current in HEK-293 cells at a dose of 50 μM (Hill et al., 2004) or 200 μM (Togashi et al., 2008). Interestingly, the inhibitory effects of FFA increase in response to extracellular acidification. This phenomenon can be explained by the fact that FFA assumes its uncharged form at acidic pH, which favors membrane crossing to the cytosolic face of TRPM2. It can also be also explained by a modification of the channel itself, which favors FFA interaction (Hill et al., 2004). A more recent study in the same preparation reports an IC_{50} of 155 μM for TRPM2 inhibition and an IC_{50} of 33 μM for TRPM3 inhibition (Klose et al., 2011). The inhibitory effect of FFA has been further established using peroxide-stimulated endogenous TRPM2 currents from CR1-G1 insulinoma cells and CHO cells (Hill et al., 2004; Naziroglu et al., 2007) or endogenous currents from hippocampal neurons (Olah et al., 2009) and dorsal root ganglion from rat (Naziroglu et al., 2011).

Three recent publications report a 50% reduction of TRPM7-like currents by 10^{-4} M FFA in rat brain microglia, the human breast cancer cell line MCF-7, and in mouse renal tubule (Jiang et al., 2003; Guilbert et al., 2009; Guinamard et al., 2012). Nevertheless, the direct inhibition of TRPM7 by FFA remains to be clearly demonstrated. In addition, a tiny inhibition of 16 to 30% by 10^{-4} M FFA has been also reported for TRPM8 heterologously expressed in *Xenopus* oocyte (Hu et al., 2010).

2.2.1.3. Transient receptor potential vanilloids. Sensitivity to vanilloid characterizes TRPV1, which became the founding member of the thermo-sensitive TRP channels (Xia et al., 2011). Subsequently, this channel was shown to be modulated by capsaicin (Cortright et al., 2001) and has been implicated in somatic pain sensing. As a consequence, TRPV1 became an attractive target for pharmaceutical research in order to identify new analgesic drugs. Human TRPV1 is not only mainly expressed in dorsal root ganglia (and trigeminal root ganglia) but also in the central nervous system, kidney and liver (Cortright et al., 2001). TRPV1 is not only expressed in the plasma membrane but also in intracellular organelles such as the endoplasmic reticulum membrane (Wisnoskey et al., 2003). Therefore, TRPV1 is a target for molecules that are membrane permeable such as FFA, as previously shown (McCarty et al., 1993).

Unfortunately, only one study reports the FFA sensitivity of TRPV1; 10^{-4} M FFA reduces the TRPV1 current by 57–75% when heterologously expressed in *Xenopus* oocytes (Hu et al., 2010). TRPV3, in the same TRPV family, is inhibited to the same extent (57–67%) by 10^{-4} M FFA, as measured in *Xenopus* oocytes (Hu et al., 2010).

The mechanosensitive TRPV4 channel is inhibited by FFA with an IC_{50} of 41 μM when stably expressed in HEK-293 cells (Klose et al., 2011), which must be considered when investigating the effects of FFA in cell swelling.

2.2.1.4. Transient receptor potential ankyrin. Among the most recently cloned TRP channels, TRPA1 is expressed in sensory neurons and is implicated in inflammatory pain as well as nociception (Gees et al., 2010). Given the anti-inflammatory properties of fenamates, TRPA1 seemed to be an obvious target to study in detail. A variety of fenamates including niflumic, mefenamic and flufenamic acids were shown to activate TRPA1 current following expression in HEK-293 cells, with an EC_{50} of 57 μM for FFA (Hu et al., 2010). This activation effect has also been observed for the TRPA1 endogenous current from WI-38 fibroblasts (Hu et al., 2010). Nevertheless, warming (from 23 to 39 °C) prevents TRPA1 activation by FFA (300 μM) (Wang et al., 2012).

2.2.2. Ligand-gated non-selective cation channels

FFA effects have been described for three types of ligand-gated non-selective cation channels activated by acetylcholine, glutamate or ATP. However, the physiological significance of these FFA effects remains incompletely understood.

An inhibitory, non-competitive effect of FFA has been described for the N-methyl-D-aspartate (NMDA) glutamate receptors in spinal cord neurons (Lerma & Martin del Rio, 1992). NMDA receptors form non-selective cation channels that flux Ca^{2+}, which can subsequently activate an NSC_{Ca}. Because NSC_{Ca} are inhibited by FFA, as described above, the effects of FFA on NMDA-induced responses must be interpreted with caution. NMDA receptors are implicated in epilepsy and their inhibition by 100 μM FFA has been shown to suppress epileptiform activity in the hippocampus (Fernandez et al., 2010). Nevertheless, this effect of FFA may involve the inhibition of NSC_{Ca} subsequently activated by NMDA receptor-mediated Ca^{2+} current (Schiller, 2004). Interestingly, FFA does not affect other types of glutamate receptors (Lerma & Martin del Rio, 1992).

Neuronal nicotinic acetylcholine receptors (nAChRs) form pentameric non-selective cation channels. FFA exerts differential effects on nAChRs in *Xenopus* oocytes, depending on the β subunit that is expressed. FFA inhibits the α3β2 nAChR current with an IC_{50} of 90 μM, whereas FFA activates the α3β4 nAChR current with an EC_{50} of 30 μM (Zwart et al., 1995). Once again, interpreting FFA effects is problematic because nAChRs are Ca^{2+} permeable, and their activation can elevate intracellular Ca^{2+} and subsequently evoke FFA-sensitive NSC_{Ca}, as shown in mesencephalic dopamine neurons (Zwart et al., 1995).

ATP induced Ca^{2+}-entry is reduced by FFA with a low EC_{50} of 655 nM in the 1321N1 astrocytoma cell line stably transfected with the purinergic receptor P2X7R, which also forms a non-selective cation channel (Suadicani et al., 2006). The authors attributed this reduction to the inhibition of the P2X7R. However this interpretation is now controversial since it was observed that 100 μM FFA had no effect on P2X7R currents in HEK-293 transfected cells (Ma et al., 2009).

2.2.3. Gap junction channels

FFA inhibits gap junctions, channels that electrically connect adjacent cells. Gap junctions are composed of two hemichannels that associate in series and can span the plasma membrane of neighboring cells. Hemichannels are composed of six connexin subunits, wherein each connexin is composed of four transmembrane segments. There are 21 connexin (Cx) isoforms in humans; nomenclature depends on molecular weight, from Cx26 to Cx62 (Maeda & Tsukihara, 2011 for review). The single-channel conductance of homomeric connexin channels spans 20–300 pS. These channels are permeable to most cations, sometimes anions, and several intracellular signaling molecules. The principal characteristic that influences permeability is size, which has to be under 1 kDa. A wide variety of tissues express connexins, which can synchronize intracellular Ca^{2+} signaling and membrane potential trajectory among cells. Gap junction modifications perturb the development of cerebral, cardiac, and auditory functions (Kar et al., 2012). Consequently, connexins represent important targets for pharmacological research (Bodendiek & Raman, 2010).

A variety of fenamates inhibit gap junctions in rat kidney fibroblasts, a result reproduced in SKHep1 cells overexpressing Cx43 (Harks et al., 2001). In this model, FFA inhibits intercellular communication with an IC_{50} of 40 μM. This inhibitory effect was later described for Cx46 and Cx50 expressed in *Xenopus* oocytes (Eskandari et al., 2002). The effect was further investigated at the current level after overexpressing a variety of connexins in N2A neuroblastoma cells; Cx23, 32, 40, 43, 46, and 50 are inhibited by FFA with an IC_{50} ranging from 20 to 60 μM (Srinivas & Spray, 2003). Interestingly, FFA does not appear to affect single-channel conductance. The molecule does not bind connexin within the conduction pore but rather in a modulatory site, presumably within the membrane, inducing channel closure (Srinivas & Spray, 2003).

2.3. Potassium channels

K^+ channels form the largest ion channel family with close to one hundred genes that encode such channels that have an extensive array of physiological functions. There are only a few noteworthy effects of FFA on these channels. K^+ channels are subdivided according to biophysics as voltage-gated K^+ channels (K_v), Ca^{2+}-activated K^+ channels (K_{Ca}), inward rectifier K^+ channels (K_{ir}), and two-pore K^+ channels (K_{2P}). In contrast to its effect on most other channels, FFA exerts an activating effect on K^+ channels in nearly all cases.

FFA affects a large conductance Ca^{2+}-activated K^+ channels, known as the Ca^{2+}-activated big K^+ channels (BK_{Ca}), as shown in coronary smooth muscle membrane vesicles incorporated in lipid bilayer for electrophysiological recordings (Ottolia & Toro, 1994), rabbit portal vein smooth muscle cells (Greenwood & Large, 1995), and cultured Vero kidney cells (Kochetkov et al., 2000), among others. The K_{Ca} 1.1 gene (or Slo1) encodes BK_{Ca} current. Expression of mouse or human K_{Ca} 1.1 in *Xenopus* oocytes results in a K^+ current activated by FFA with an EC_{50} that exceeds 0.3 mM (Gribkoff et al., 1996). FFA may be more efficient in native K_{Ca} channels, because the activation of BK currents in coronary and portal vein smooth muscle cells was on the order of 50 μM (Ottolia & Toro, 1994; Greenwood & Large, 1995). Moreover, in human trabecular meshwork 10^{-5} M FFA stimulated BK_{Ca} current by 400% (Stumpff et al., 2001).

FFA has also been shown to activate the channel encoded by the human ether-a-gogo related gene (HERG), also called K_v 11.1. This gene encodes for the pore forming subunit of the rapid component of the delayed rectifier K^+ channel participating in action potential repolarization in cardiac myocytes. When heterologously expressed in *Xenopus* oocytes, K_v 11.1 produces a current enhanced by 20% in the presence of 10^{-4} M FFA (Malykhina et al., 2002). Interestingly, 10^{-4} M FFA also enhances the slow component of the delayed rectifier K^+ current encoded by K_v 7.1 by slowing its deactivation (Busch et al., 1994).

Recently FFA was shown to stimulate the two-pore outwardly rectifying K^+ channel K_{Ca} 4.2 (or Slo 2.1) expressed heterologously in *Xenopus* oocytes, although at a high dose (EC_{50} of 1.1–1.4 mM) (Dai et al., 2010; Garg & Sanguinetti, in press). Interestingly, the mutant A278R, which substitutes a residue in the transmembrane domain six segment flanking the pore, is 19-times more sensitive to FFA, indicating that FFA binding might occur in this region (Garg & Sanguinetti, in press). K_{Ca} 4.2 encodes a K^+ channel gated by voltage as well as internal Na^+ and Cl^-, which is also inhibited by ATP. The physiological functions of Slo 2.1 are not yet established, but its relative "slack" (or Slo 2.2) may be involved in neural burst generation and termination in particular in central pattern generating neural circuits (Wallen et al., 2007; Krey et al., 2010). FFA also activates the lipid-sensitive mechano-gated two-pore channels encoded by K_{2P} 4.1, K_{2P} 10.1 and K_{2P} 2.1 with EC_{50} in the range of 1 mM (Takahira et al., 2005).

2.4. Sodium channels

Action potentials in all excitable cells depend on voltage-activated Na^+ channels. After an initial depolarization reaches the threshold of activation, Na^+ channels open and produce the rapid upstroke of the action potential. Repolarization is achieved, in part, by time-dependent channel inactivation. The Na^+ channel protein is composed of one α subunit (four major repeat units, each of which is composed of six transmembrane domains) and two β subunits (each is comprised of one transmembrane segment) encoded by genes SCNXA (or Na_v) and SCNXB (Catterall, 2010). A recent paper describes the inhibition of the voltage-activated Na^+ channel in hippocampal pyramidal neurons by FFA with an IC_{50} of approximately 0.2 mM (Yau et al., 2010). FFA affects inactivation by shifting the steady-state inactivation curve to more hyperpolarized membrane potentials.

FFA activates another Na^+ conductance in ventricular cardiomyocytes with an EC_{50} that exceeds 0.2 mM (Macianskiene et al., 2010). The underlying channel remains unknown but may correspond to the brain liver intestine Na^+ channel (BLINaC) that is activated by high levels of FFA ($EC_{50} > 1$ mM) when heterologously expressed in *Xenopus* oocytes (Wiemuth & Grunder, 2011). BLINaC belongs to the degenerin/epithelial Na^+ channel superfamily. It is predominantly expressed in non-neuronal tissues, in particular epithelia, and weak expression has been observed in the heart (Sakai et al., 1999). Its physiological function was unknown until the recent demonstration that the BLINaC channel is expressed in cholangiocytes and is activated by bile acids, suggesting its role in bile duct sensing of bile acid concentrations (Wiemuth et al., 2012).

2.5. Calcium channels

Voltage-gated Ca^{2+} channels activate in response to depolarization and participate in Ca^{2+} transients that induce muscle cell contraction as well as a variety of excitable responses in neurons including, notably, chemical synaptic transmission. Ca^{2+} channels are composed of a central α subunit (organized according to four repeat units of six transmembrane segments each, similar to Na^+ channels) encoded by the Ca_v genes and four additional regulatory subunits (α2, β, γ, δ) (Catterall, 2010). The channels are divided in L, P/Q, N, R and T subtypes. FFA inhibits smooth muscle tone in carotid arteries by directly inhibiting L-type Ca^{2+} channels with an IC_{50} of ~0.1 mM (Shimamura et al., 2002). No experiments have been reported to identify the subunit targeted by FFA.

3. Mechanisms involved in current modulation by flufenamic acid

The activating or inhibiting effects of FFA are well described. However, the underlying mechanisms remain largely unknown. Because FFA targets numerous ion channels with different structures, biophysics, and regulatory properties, the underlying mechanisms might be different from one to the other.

As illustrated in Fig. 3 and in most studies reported in this review, modulation of ion currents by FFA is not likely to occur via gene expression since the effect develops within minutes. While indirect effects on ion channels through modulation of intracellular pathways may occur, the major accepted mechanism is a direct interaction between FFA and channel proteins. That is particularly evident when FFA is used in excised patch-clamp configurations, as example for CFTR (McCarty et al., 1993), TRPM4 and TRPM5 (Ullrich et al., 2005; Guinamard et al., 2006b) or Cx50 (Srinivas & Spray, 2003). The FFA effect can be abolished by channel mutation such as in ClC-Ka (Liantonio et al., 2006), which also suggests a direct interaction between FFA and channel proteins. This direct effect assumes a binding site within the channel itself. Such a site was suspected for Cl$^-$ channels (CFTR and ClC-K) within the narrow part of the protein vestibule since NA is not able to reach the site in ClC-Ka (Liantonio et al., 2006) and FFA showed an apparent binding site at 40–50% of the electrical distance from the cytoplasmic face in CFTR (McCarty et al., 1993). This binding site may be different in non-selective cation channels, at least in Cx50, where it may be a modulatory site comprised within the membrane but not in the pore (Srinivas & Spray, 2003). Although the binding site was not described, FFA interacts directly with TRPC4, C5, and C6 (Jiang et al., 2003; Foster et al., 2009).

The insights above regarding FFA binding sites cannot be extended to other channels because of large variations in channel structure despite their (sometimes) common sensitivity to FFA.

4. Impact of ion channel modulation by flufenamic acid on physiological processes

The effect of FFA has been observed in a wide variety of physiological processes; too many to cover thoroughly in one review. Here, we focus on a few representative examples to illustrate the large spectrum of targets.

FFA affects neurons, smooth muscle cells, and cardiomyocytes. FFA reduces firing rates in neurons, and in particular reduces the rhythmic burst-generating capabilities of inspiratory neurons from the respiratory pre-Bötzinger complex, studied in thin medullary slices from neonatal rodents at concentrations from 10 to 500 μM (Pena et al., 2004; Del Negro et al., 2005). This effect occurs through inhibition of a Ca^{2+}-activated non-selective cation current (I$_{CAN}$, see above) that was later attributed to the TRPM4 or TRPM5 proteins, both expressed in this tissue (Crowder et al., 2007; Del Negro et al., 2010). The effective dose of FFA was later determined to be ~100 μM (Pace et al., 2007). FFA (100 μM) has been also shown to suppress epileptiform activity in rat CA1 pyramidal neurons of the hippocampus through diminution of glutamatergic excitatory synaptic transmission (Fernandez et al., 2010) and by blocking I$_{CAN}$ (Schiller, 2004). Therefore, FFA was proposed as a potentially effective agent for the treatment of epilepsy. FFA (30 μM) reduces the peptide-induced intra-cardiac neuron firing rate (Merriam et al., 2012), which may involve the TRPC channel inhibition. A reduction of firing rate by FFA (20 μM) was also reported in GABAergic neurons, possibly through TRP current inhibition (Lee et al., 2011b). Finally, FFA (3 μM) reduced dopamine-induced oscillations in pyloric pacemaker neurons of the spiny lobster (Kadiri et al., 2011).

FFA modulates gastrointestinal tract motility by reducing pacemaker potentials of intestinal cells of Cajal in mice (Han et al., 2012; Lee et al., 2012). This effect has also been observed at 50 μM in human intestinal cells of Cajal and attributed to the inhibition of the TRPM7 channel (Kim et al., 2009).

In neuroendocrinology, FFA (100 μM) inhibits pacemaker activity in rat pituitary lactotrophs through non-selective cation channel

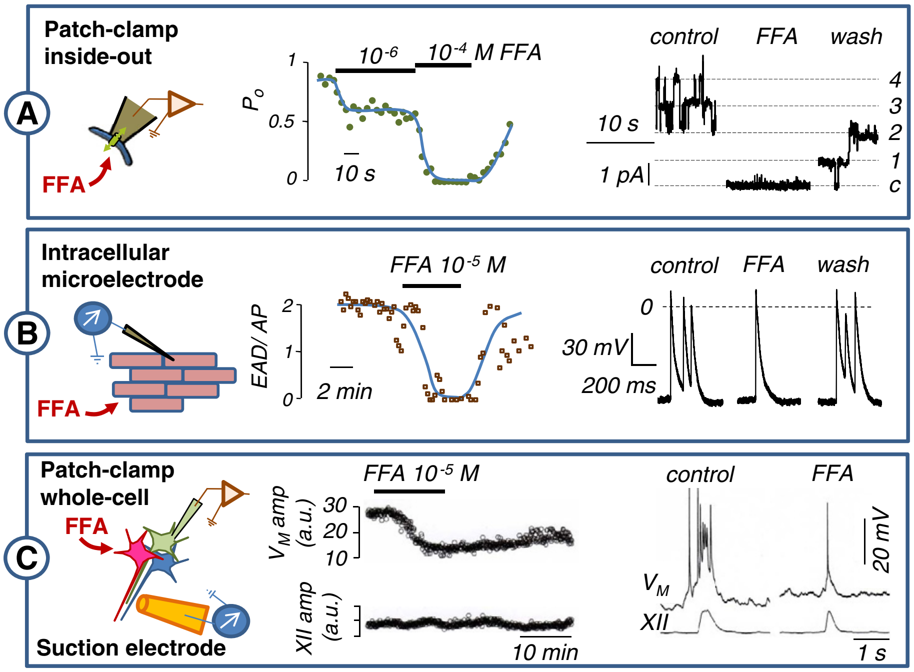

Fig. 3. Effects of flufenamic acid on several preparations. **A**: Inside-out patch-clamp recording of TRPM4 current on rat ventricular isolated myocyte (Vm = +40 mV). FFA produced a dose-dependent and reversible channel inhibition (see Guinamard et al., 2006-b for protocol). **B**: Action potential recorded by an intracellular microelectrode on isolated mouse ventricle submitted to a hypoxia and reoxygenation protocol (see Simard et al., 2012 for protocol). FFA superfusion reversibly reduced the number of early after depolarization by action potential (EAD/AP). **C**: Respiratory bursts recorded in rhythmogenic neurons of the pre-Bötzinger complex (preBötC) as well as hypoglossal nerve root (XII) from neonatal mouse brainstem-slice preparations. Whole-cell patch-clamp recordings in preBötC neurons show that 100 μM FFA attenuates respiratory bursts at the whole-cell level by attenuating I$_{CAN}$, but has a relatively mild affect motor output from the XII nerve root output (see Picardo et al., in press for protocol).

modulation, leading to decrease in prolactin secretion (Kucka et al., 2012).

Our group recently reported a cardioprotective effect of 10 μM FFA in a model of hypoxia reoxygenation-induced arrhythmia in mouse (Simard et al., 2012). The mechanism is related to the fact that FFA abolishes TRPM4-mediated early after depolarizations observed following reoxygenation. This FFA effect mimics the specific TRPM4 antagonist 9-phenanthrol, suggesting that FFA effect occurs through TRPM4 inhibition. Therefore, FFA may be regarded as a cardiac antiarrhythmic agent. FFA (25 μM) may also modulate Ca^{2+} signaling by inhibiting Cx43 in rat ventricular myocytes (Li et al., 2012). A similar result was observed in the murine fibroblast cell line L929, where FFA (100 μM) inhibits ATP release and Ca^{2+} transients that polarize the actin/myosin complex via inhibition of connexins (Marimuthu et al., 2012). FFA (50 μM) also modulates vascular endothelial growth factor secretion in human retinal pigment epithelial cells by inhibiting Cx43 (Pocrnich et al., 2012).

The impact of FFA is not restricted to excitable cells. Partial reduction of Cl^- secretion in human airway gland cells occurs in response to 100 μM FFA, an effect that might be attributed to inhibition of the chloride channel TMEM16A (Fischer et al., 2010). FFA also regulates cell volume in hypotonic as well as hypertonic conditions. Regulatory volume decreases in hypotonic conditions are reduced by FFA, due to a FFA-inhibited swelling-activated Cl^- channel (Jin et al., 2003; Do et al., 2006). A regulatory volume increase under hypertonic conditions that protects against apoptosis is reduced by FFA with an EC_{50} of 300 μM, which occurs through FFA-mediated inhibition of cation current (Wehner et al., 2003). Alpha-subunit of the epithelial Na^+ channel (ENaC) was shown to participate in this hypertonicity-induced current in the human hepatocellular liver carcinoma cell line HepG2 (Bondarava et al., 2009) whereas the current was recently shown to be supported by the TRPM2 channel in the HeLa cells (Numata et al., 2012).

5. Using flufenamic acid in research

In the following section we evaluate the advantages and caveats of using FFA in research. The caveats pertain to several FFA targets in the same preparation and FFA exerting opposite effects on a target channel in a dose-dependent fashion. We discuss the use of FFA in comparison to other NSAIDs and finally identify assets of FFA.

5.1. Multiple targets in the same preparation

As described above, FFA modulates a wide spectrum of ion channels. The same cell can express several FFA-sensitive channels. For example, gonadotropin-releasing hormone neuroendocrine neurons express non-selective cation channels and BK channels (Wang & Kuehl-Kovarik, 2010). Mammalian cardiomyocytes not only express ion channels inhibited by FFA including TRPC3, TRPC6, TRPM4, TRPM7, and $I_{Cl,swell}$, but also channels that are activated by FFA such as HERG (Malykhina et al., 2002; Demion et al., 2006; Inoue et al., 2006; Guinamard et al., 2006a, 2006b). Similarly, guinea pig cardiac neurons express TRPC3, TRPC4, TRPC5 and TRPC6 (Merriam et al., 2012).

The presence of multiple FFA-sensitive channels must be considered when analyzing the effect of the drug at the whole-cell or system levels. Because FFA has different affinities for different ion channels, interpreting and analyzing its effects depend on the sensitivity of each possibly affected channel type and the drug concentration used. For example, FFA modifies fictive swim patterns of the lamprey spinal cord, which is attributable to modulation of both Ca^{2+} channels and NMDA receptors (Wang et al., 2006). Similarly, FFA targets different channels in *Aplysia* bag cell neurons, modulating K^+ channels, voltage-gated Ca^{2+} channels and Ca^{2+}-dependent cation conductances (Gardam et al., 2008).

In addition to ion channels, FFA also affects other targets that indirectly impact ion channels and excitable cell behavior. For example, FFA activates the cAMP-activated protein kinase, (Chi et al., 2011), and yet inhibits the mouse GABA transporter GAT4 (Liantonio et al., 2007) and glycine transporters (Steinmeyer et al., 1991). Finally, FFA can also alter mitochondrial Ca^{2+} homeostasis, impacting Ca^{2+}-dependent channels (Macdonald et al., 2010). Since our review focuses on the direct effects of FFA on ion channels, we will not describe the effects above in detail, but we emphasize that there are other biochemical and integral membrane proteins that may be affected by FFA. Therefore, these other targets must be taken into account when analyzing the effects of FFA in the context of physiological experiments.

A recent publication reevaluating the chemical structure of FFA demonstrated that this molecule possesses at least nine polymorphs (Lopez-Mejias et al., 2012), which may influence the bioavailability of the drug and thus provide new opportunities for investigating the channel types targeted by FFA, depending on these polymorphs.

5.2. Opposite effects on the same channel

Another FFA-related caveat comes from its ability to exert opposite effects on the same channel, depending on concentration. FFA inhibits TRPC6 with an IC_{50} of 17.1 μM (Klose et al., 2011) but 100 μM FFA activates the same channel (Inoue et al., 2001). TRPM8 is inhibited at 100 μM FFA but slightly activated at higher concentrations (Hu et al., 2010). A worse situation was reported for BK_{Ca} modulation since FFA activates the channel below 10 μM, inhibits the channel between 10 and 50 μM, and then activates the channel above 50 μM (Kochetkov et al., 2000).

5.3. Flufenamic acid or other fenamates

Other NSAIDs, including fenamates, are also known to modulate a variety of ion channels (Gwanyanya et al., 2012). Most ion channels modulated by FFA are also affected by other fenamates. A few studies provide a comparative analysis of the effects of several fenamates on the same ion channel, ranking fenamates according to their potencies to block or activate channels. Because the rank order of efficacy among fenamates differs from one channel to the other, we will not review all of them. However, in the majority of reports, FFA appears to be more effective than niflumic acid (NA) and mefenamic acid (MFA), two of the most commonly tested fenamates. This sequence was observed for TRPM2, TRPV4 and TRPC6 inhibition (Klose et al., 2011; Chen et al., 2012), TRPC4 and TRPC5 inhibition (Jiang et al., 2012), TRPA1 activation (Hu et al., 2010), BK_{Ca} activation (Ottolia & Toro, 1994), Cx43 inhibition (Harks et al., 2001), as well as K_{2P} 2.1 and K_{2P} 10.1 channel activation (Takahira et al., 2005). he sequence of fenamate sensitivity might be somewhat different for chloride channels, since MFA is more effective than FFA in ClC-K and $GABA_A$ receptor modulation (Woodward et al., 1994; Liantonio et al., 2006), whereas NA is more effective than FFA on ICl_{Ca} (Greenwood & Large, 1995; Oh et al., 2008). For the Slo2.1 potassium channel, the sequence is MFA > FFA > NA (Garg & Sanguinetti, in press).

Most of the FFA-targeted ion channels are sensitive to other fenamates, but this does not necessitate non-specificity of the FFA binding site within channel proteins. Indeed, FFA and NA do not use the same binding site on the ClC-Ka channel (Zifarelli et al., 2010).

5.4. Assets of flufenamic acid

Despite its promiscuity, FFA remains a convenient toll for physiological studies. FFA can be used in a wide variety of experimental models ranging from molecular preparations such as inside-out single-channel recordings, to cellular preparations such as whole-cell recordings on isolated cells as well as isolated tissue slices in vitro and in situ. Instead of reviewing all these preparations, which have been already presented in the above sections and Table 1, we illustrate several examples of FFA applications using different experimental models (Fig. 3).

FFA is lipophilic and thus membrane permeable (McCarty et al., 1993; Hill et al., 2004). Accordingly, FFA can access intracellular or extracellular targets whatever is its side of application, as illustrated for TRPM4 inhibition (Fig. 3). FFA access can be achieved by drug application in the bath during inside-out patch recordings, when the inside of the channel faces the bath (Guinamard et al., 2006b) or in the whole cell-configuration when external side is exposed (Pena et al., 2004; Pace et al., 2007).

The effects of FFA develop and reverse rapidly. Examples in Fig. 3 show that, even when applied on a multicellular isolated tissue preparation (mouse right ventricle, Fig. 3B; (Simard et al., 2012)) or a rhythmically active respiratory rhythmogenic network (Fig. 3C; (Picardo et al., in press)), the effect of FFA develops within a few minutes and washes out with a commensurate time course. When applied to isolated cells, the effects of FFA occur (and reverse) in the range of few seconds.

6. Conclusion

FFA appears to be a broad spectrum ion channel modulator, with preference for non-selective cation channels and chloride channels. However, it remains a convenient tool if used with precaution, keeping in mind the caveats recapped above. That is particularly true for studies investigating the role of channels with higher sensitivity for FFA such as TRPM4. In combination with other more specific tools, FFA can provide a useful tool to identify ion channels and probe their physiological role(s) in a range of reduced preparations in vitro or in situ.

Extensive knowledge of ion channels targeted by FFA may revive interest in the use of this molecule for therapeutic purposes, as was suggested for NSAIDs, especially fenamates, in the treatment of neurological disorders (Khansari & Coyne, 2012). The recently developed FFA hydrophobic derivative nanoprodrugs show an increase in the drug efficiency (Lee et al., 2011a). Accordingly, lower doses might be efficient in medical use and, thus, a better targeting of different physiological actors might be achieved.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Written assurance

This manuscript has not been published and is not under consideration for publication elsewhere.

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