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Amitriptyline has a dual effect on the conductive properties of the epithelial Na channel

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Abstract

This study was undertaken with the aim of testing the action of amitriptyline on the epithelial Na channel (ENaC), which belongs to the same family (Deg/ENaC) as ASICs (acid-sensing ion channels) and many other putative members in the brain. We assumed that, having a common protein structure, characterization of the amitriptyline–ENaC interaction could help to elucidate the analgesic mechanism of this tricyclic antidepressant. Na-channel characteristics were derived from the analysis of blocker-induced lorentzian noise produced by amiloride. The effect of amitriptyline, present in the mucosal bathing solution, on the transepithelial short-circuit current (I_{sc}) and conductance (G_t), and on the blocker-induced noise of apical Na channels, was studied on isolated ventral skin of the frog *Rana ridibunda*. Amitriptyline exerted a dual effect on the macroscopic short-circuit current and conductance of the epithelia, increasing these two parameters in the concentration range 0.1–50 μM, while at higher concentrations (100–1000 μM) it showed an inhibitory action. The decrease in the association rate (k_{01}) of amiloride to the apical Na channels from $15.6 \pm 4.2\ \mu M^{-1}\ s^{-1}$ in control Cl-Ringer to $7.4 \pm 1.7\ \mu M^{-1}\ s^{-1}$ at 200 μM amitriptyline in a concentration-dependent manner suggests a competitive binding of amitriptyline to the pyrazine ring binding site for amiloride.

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Introduction

Amitriptyline is a tricyclic antidepressant that also has an analgesic effect regardless of the presence of depression. It is used for the treatment of pain associated with various diseases such as diabetic neuropathy, chronic low back pain, chronic tension-type headache and postherpetic neuralgia (Vrethem et al 1997; Atkinson et al 1998; Cerbo et al 1998; Watson et al 1998). The analgesic mechanism of amitriptyline is unclear, but it is considered that its ability to block Na channels in dorsal root ganglia neurons (Song et al 2000), as well as different types of K channels (Wooltorton & Mathie 1995; Galeotti 2001), plays a role in reducing pain.

A new class of ion channels known as the Deg/ENaC (degenerins–epithelial Na channel) family was shown to be involved in sensory transduction (Canessa et al 1993; Lingueglia et al 1993; Waldmann et al 1997; Horisberger 1998; de la Rosa et al 2000). It was suggested that heteromultimers composed of subunits belonging to the acid-sensing ion channels (ASIC) group of channels (Bassilana et al 1997; Benson et al 2002), along with the capsaicin receptor VR1, may mediate the pain induced by acidosis that occurs in ischaemic, damaged or inflamed tissue (Caterina et al 2000; Price et al 2001).

This study was undertaken with the aim of testing the action of amitriptyline on native epithelial Na channels from frog skin. We assumed that, having a common protein structure, characterization of the amitriptyline–ENaC interaction could help to elucidate the analgesic mechanism of this antidepressant.

Materials and Methods

Preparation of frog skin

In our experiments we have followed the standards published by the International Association for the Study of Pain for the minimization of animal suffering.

Frogs (*Rana ridibunda*), weighing around 50 g, were kept at 17°C with free access to tap water. The abdominal skin of doubly pithed frogs was dissected and mounted in an Ussing-type Lucite chamber, as previously described (Flonta et al 1998). The chamber ensured negligible edge damage and allowed continuous perfusion with fresh solutions of both the mucosal and serosal sides of the epithelium, at a rate of 5 mL min^{-1}. The tissue area in contact with the bathing media was 1 cm^2.

The transepithelial potential was clamped to zero with a low-noise voltage lamp (Van Driessche & Lindemann 1978), and the short-circuit current (I_{sc}) was recorded on a standard X-T recorder. The transepithelial conductance (G_t) was calculated from the current changes caused by 256 ms voltage pulses of 5 mV amplitude, applied every 14 s.

Solutions

All solutions were freshly prepared with highly purified water (Milli-Q Water Purification System, Millipore).

The Ringer solution, bathing both sides of skins, consisted of (in mM): 112.5 Na^+, 114 Cl^-, 2 K^+, 1 Ca^{2+}, 2.5 HCO_3^- and, to eliminate the contribution of Cl^- to the measured I_{sc} and G_t, Cl-free Ringer solutions, having SO_4^{2-} as the major anion, were used. Na_2SO_4 Ringer solution contained (in mM): 112.5 Na^+, 57 SO_4^{2-}, 2 K^+,1 Ca^{2+} and 2.5 HCO_3^-. The pH of this solution was 7.8–8.2. The concentrations in the mucosal compartment of amiloride hydrochloride (Sigma) and amitriptyline hydrochloride (Sigma), which were dissolved in distilled water and added to the mucosal Ringer solutions, amounted to final values of 2–40 μM, and 0.1–1000 μM, respectively.

Noise analysis

The fluctuation in current was recorded and analysed in the frequency domain, as previously described (Van Driessche & Zeiske 1980). The technique exploits the fact that the macroscopic Na^+ current (μA) consists of the random flickering of millions of independent channels (pA), which give rise to fluctuations (nA) around the mean current. These fluctuations can be resolved into frequency components using the Fourier transform.

The current signal was high-pass filtered using a 24 dB/octave Butterworth filter with a cut-off frequency of 0.3 Hz. Furthermore, to prevent aliasing, the signal was low-pass filtered with a 48 dB/octave Butterworth filter (cut-off frequency 850 Hz). Power density spectra (PDS) were calculated from Fourier transformed records of 4096 points collected over a 2-s period, yielding a fundamental frequency of 0.5 Hz. The averaged power densities at 2048 frequencies were obtained from 50 records. Frequencies above 760 Hz were discarded in further analysis. Spectra containing relaxation noise were fitted with the sum of a lorentzian component and a background noise term (A/f^{α}) according to equation 1:

$$S(f) = \frac{S_0}{1+(f/f_c)^2} + \frac{A}{f^{\alpha}} \quad (1)$$

The plateau value (S_0) and the corner frequency (f_c) of the lorentzian function, determined by non-linear regression of the PDS, were used for the calculation of single-channel current and channel density, according to a two-state model for channel opening and blocking. A represents the power density at 1 Hz and α the slope of the $1/f$ noise component.

The model predicts a linear dependence of f_c on the concentration of the fluctuation-inducing blocker ([B]):

$$2\pi f_c = k_{01}[B] + k_{10} \quad (2)$$

where k_{01} and k_{10} are the ON-(association) and the OFF-(dissociation) rate constants, respectively. It also allows calculation of the single-channel current (i_{Na}) and the number of open channels (N_0) in the presence of the blocker, from the plateau value (S_0) and the macroscopic Na^+ current (I_{Na}):

$$i_{Na} = \frac{S_0(2\pi f_c)^2}{4 I_{Na} k_{01}[B]} \quad (3)$$

$$N_0 = I_{Na}/i_{Na} \quad (4)$$

Statistical analysis

Data are reported as means±s.d., n representing in each case the number of experiments. The effects of Ringer type (Cl or SO_4) and amitriptyline concentration on various kinetic and electrophysiological parameters of the noise analysis were assessed via a two-way analysis of variance

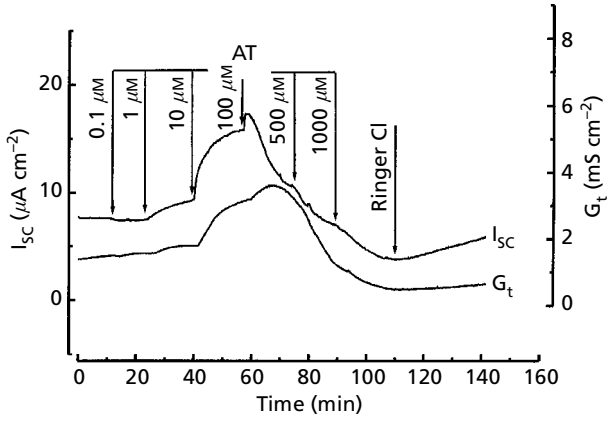

Figure 1 Effect of amitriptyline (AT), added to the mucosal compartment, on the short-circuit current (I_{sc}) and transepithelial conductance (G_t) of frog skin. Amitriptyline concentration was successively: 0.1, 1, 10, 100, 500 and 1000 μM. The bathing solution was Cl-Ringer. A dual effect of amitriptyline is visible. Up to 100 μM amitriptyline the short-circuit current and conductance increase, while at higher concentrations an inhibitory action on these two parameters is recorded.

(Rice 1995). The same method was employed to prove the influence of amiloride and amitriptyline concentration on the corner frequency extracted from the power density spectra.

Results

Figure 1 shows the dual response of the short-circuit current (I_{sc}) when adding increasing concentrations of amitriptyline (0.1–1000 μM) to the mucosal side of the epithelium. The current increases progressively up to 50 μM amitriptyline, but at higher concentrations an inhibitory effect appears. The effect is similar in Cl-Ringer or SO_4-Ringer, which excludes a specific effect of amitriptyline on the Cl^- conductance, indicating that the Na^+ flux is modulated by amitriptyline. Amitriptyline applied on the basolateral side of the epithelium has no effect on I_{sc}. In SO_4-Ringer, a transient overshoot in the stimulated I_{sc} is usually noted, which is larger at mid-range amitriptyline concentrations (20–200 μM).

To obtain further insight into the interaction of amitriptyline with the epithelial Na channel, we have calculated the kinetic parameters of the amiloride–Na channel interaction, in the absence or presence of various amitriptyline concentrations. Figures 2 and 3 show these protocols in the presence of a stimulating concentration of amitriptyline (20 μM) or an inhibiting concentration (200 μM), respectively. Figure 4 depicts the differential effect of

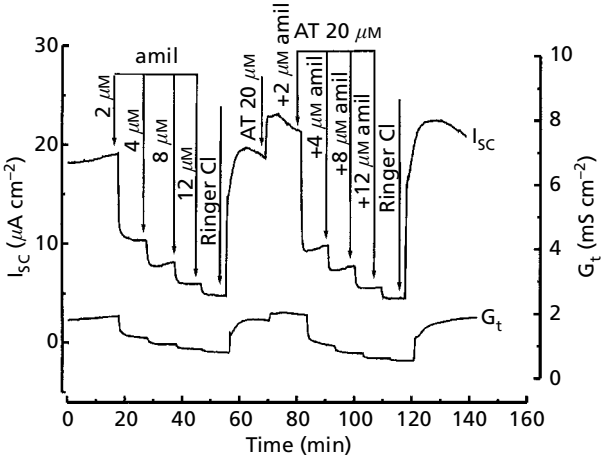

Figure 2 Effect of amitriptyline (AT) (20 μM in the mucosal compartment) on the short-circuit current (I_{sc}) and transepithelial conductance (G_t) of frog skin, in the presence of increasing concentrations of amiloride (amil) added on the mucosal side of the epithelium. The amiloride concentration was successively: 2, 4, 8 and 12 μM. The bathing solution was Cl-Ringer.

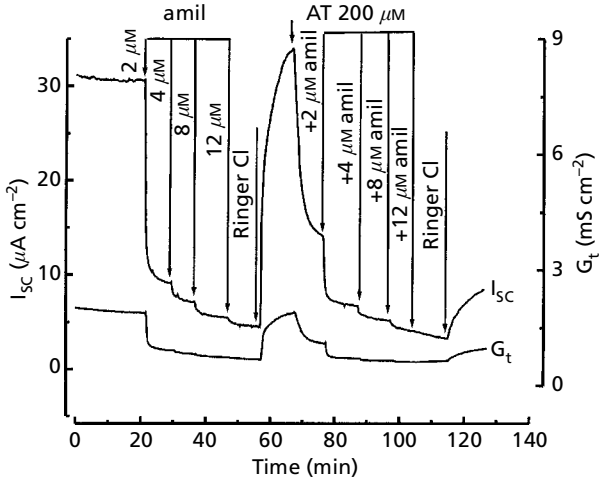

Figure 3 Effect of amitriptyline (AT) (200 μM in the mucosal compartment) on the short-circuit current (I_{sc}) and transepithelial conductance (G_t) of frog skin, in the presence of increasing concentrations of amiloride (amil). Amiloride concentration was successively: 2, 4, 8 and 12 μM. The bathing solution was Cl-Ringer.

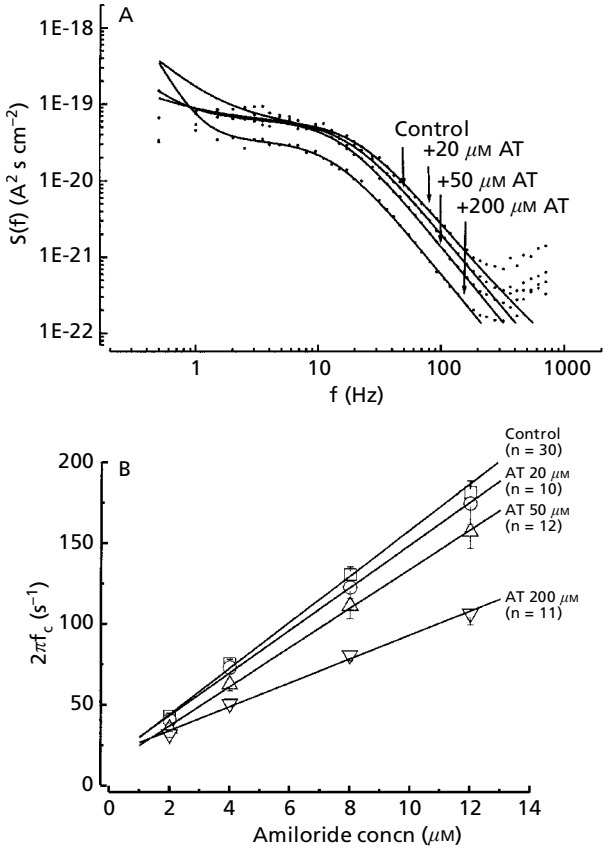

Figure 4 The steps which allow the calculation of the association (ON) and dissociation (OFF) rate constants of amiloride to the Na channel. A. Power density spectra in the presence of 8 μM amiloride, during control or after addition of amitriptyline (AT) in the mucosal bath, in the given concentrations. B. Effect of different concentrations of amitriptyline (AT) on the relationship between $2\pi f_c$ (f_c = corner frequency) and the amiloride concentration at the apical side. Values of f_c were obtained from non-linear regression of the power density data with the sum of a low frequency and a Lorentzian function. The slope and the Y-intercept of the linear regression indicate the ON and OFF rates, respectively, of the interaction between amiloride and the channel. Error bars represent s.e.m.

Table 1 Electrophysiological and kinetic characteristics of the interaction between amiloride and ENaC under control conditions and after mucosal addition of 20, 50 and 200 μM amitriptyline, as revealed by blocker-induced noise analysis.

Conditions		k_{01} ($\mu mol^{-1}\ s^{-1}$)	k_{10} (s^{-1})	i_{Na} (pA)	$N_0 \times 10^{-6}$ (cm^{-2})
Amiloride-treated					
NaCl Ringer	Control	15.55±4.24	19.79±14.49	0.42±0.13	17.65±11.86
	Amitriptyline 20 μM	14.74±2.56	17.51±4.54	0.41±0.03	8.83±3.11
	Amitriptyline 50 μM	12.33±2.22	11.07±6.34	0.26±0.05	22.72±10.72
	Amitriptyline 200 μM	7.40±1.72	19.94±7.62	0.21±0.16	21.59±12.17
Na_2SO_4 Ringer	Control	12.74±2.16	15.78±5.81	0.40±0.17	18.48±5.42
	Amitriptyline 20 μM	11.3±4.44	16.70±11.53	0.47±0.02	15.80±13.67
	Amitriptyline 50 μM	10.43±1.64	17.05±6.50	0.36±0.02	16.93±12.47
	Amitriptyline 200 μM	7.06±1.31	20.94±5.32	0.33±0.08	14.43±5.84

k_{01}, association (ON) constant; k_{10}, dissociation (OFF) constant; i_{Na}, single-channel current and N_0, open channel density, obtained by linear regression of the values at four amiloride concentrations (2, 4, 8 and 12 μM). Values are given as mean±s.d.

amitriptyline on the association (k_{01}) and dissociation (k_{10}) rates of amiloride–ENaC interaction. The k_{01}, which is proportional to the slope of the linear fit, decreased at progressive amitriptyline concentration, while k_{10}, which corresponds to the Y-intercept, did not change. A two-way analysis of variance proved that the effect of both amitriptyline ($F = 7.77$, $P = 0.0072$) and amiloride ($F = 63.99$, $P < 0.0001$) were statistically significant.

The kinetic parameters of the interaction between amiloride and the Na channel under control conditions and after addition of various amitriptyline concentrations are presented in Table 1. A two-way analysis of variance revealed no significant influence of the Ringer type (Cl or SO_4) on the kinetic rates, single-channel current or channel density, while the amitriptyline concentration exerted a significant effect (decrease) only on the opening rate k_{01} ($F = 20.27$, $P = 0.017$).

Since the apical membrane of epithelial cells is rate limiting for transepithelial Na transport, the increase in I_{sc} noticed at amitriptyline concentrations up to 50 μM could be due to an increase in single-channel current, total Na-channel density or open probability. Table 1 summarizes the influence of amitriptyline on single-channel current and open channel density. Single-channel current decreased with increasing amitriptyline concentration in both Cl-Ringer and SO_4-Ringer solution, from 0.42 to 0.21 pA, and from 0.40 to 0.33 pA, respectively, but this change was not statistically significant ($F = 6.20$, $P = 0.084$). The open channel density, obtained by linear regression of the values at different amiloride concentrations, did not vary significantly with amitriptyline concentration ($F = 1.01$, $P = 0.498$).

Discussion

Amitriptyline has a dual effect on the macroscopic short-circuit current and conductance of the epithelia, increasing these two parameters in e concentration range 0.1–50 μM, and exerting an inhibitory action at higher concentrations (100–1000 μM). The decrease in the association rate (k_{01}) of amiloride to the apical Na channels from 15.6±4.2 $\mu M^{-1}\ s^{-1}$ to 7.4±1.7 $\mu M^{-1}\ s^{-1}$ in an amitriptyline-dependent manner suggests a competitive and stronger binding of amitriptyline to the amiloride binding site. The same decrease is noticed in SO_4-Ringer bathing solution (from 12.7±2.2 $\mu M^{-1}\ s^{-1}$ to 7.1±1.3 $\mu M^{-1}\ s^{-1}$), indicating that Cl^- does not influence this phenomenon. Our results suggest that amitriptyline interferes in some way with the Na channel, reducing the specific blocking efficacy of amiloride. The amiloride molecule is composed of two modules, a pyrazine ring and a guanidinium moiety. It was suggested that the guanidinium moiety binds to the entrance of the selectivity filter, penetrating 20% of the transmembrane electrical field (Palmer 1990), whereas the pyrazine ring binds to an extracellular domain of the channel (Li et al 1987; Ismailov et al 1997). Amitriptyline, a tertiary tricyclic amine, could interfere with the binding of the pyrazine ring of amiloride to ENaC, hindering the blocking effect of amiloride and keeping the channel open for a longer time. The bound amitriptyline could also be the cause of the decreased single-channel current (i_{Na}). The subsequent inhibitory effect on the Na^+ flux at higher amitriptyline concentrations can be explained by the known phenomenon of ENaC self-inhibition at increased intracellular Na^+ concentrations (Ishikawa et al 1998; Awayda 1999).

These results add to the evidence of a multiplicity of amitriptyline effects on different ion channels and receptors: it blocks tetrodotoxin-sensitive and -resistant Na^+ currents in dorsal root ganglia neurons (Song et al 2000) and heart muscle (Barber et al 1991; Nau et al 2000), various K^+ (Lee et al 1997; Casis & Sanchez-Chapula 1998; Teschemacher et al 1999; Dreixler et al 2000; Jo et al 2000) and Ca^{2+} channels and transporters (Lavoie et al 1990), cholinergic receptors (Schofield et al 1981; McKinney et al 1988; Park et al 1998), various glutamate (Sills & Loo 1989; Cai & McCaslin 1992; Tohda et al 1995), α_2-adrenergic (Collis & Shepherd 1980; Leighton

1982), GABA (Malatynska et al 1999) and histaminergic (Kachur et al 1988) receptors. Furthermore, it inhibits the reuptake of serotonin and noradrenaline (norepinephrine) (Hogberg et al 1988; Auerbach et al 1995) and interacts with opioid (Wahlstrom et al 1994; Gray et al 1998) and adenosine receptors (Sakuta 1994; Yoshida et al 1996). Amitriptyline affects Na^+-channel activation in rat dorsal root ganglion neurons, a result that is opposite to that found in bovine chromaffin cells, where amitriptyline had no effect on the activation process of the Na channel (Pancrazio et al 1998).

Our results suggest that the analgesic effect of amitriptyline uses a mechanism other than cold analgesia. Despite the fact that it has been found that cold temperatures markedly increase the currents through epithelial Na^+ channels and related Deg/ENaC family members (Askwith et al 2001), we do not believe that cold reception is transduced by ENaC, because work in this laboratory recently demonstrated an amiloride-insensitive cold-induced current in a small group of dorsal root ganglia neurons (7%) (Reid & Flonta 2001a) and also a cold-evoked inhibition of background K^+ channels in 25% of dorsal root ganglia neurons (Reid & Flonta 2001b).

All these various effects of amitriptyline can be useful when trying to explain its antidepressive and analgesic effects, and all its unwanted side effects. However, it should be noted that the concentration of amitriptyline used in some of these experimental studies is higher than the usual therapeutic plasma concentration, which is around 1 μM (Baldessarini 1996). This suggests the existence of specific high-affinity receptors responsible for the therapeutic effects of the drug.

Conclusions

Amitriptyline exerts a dual effect on the macroscopic short-circuit current and conductance of frog epithelia, increasing these two parameters in the concentration range 0.1–50 μM, and inhibiting them at higher concentrations (100–1000 μM). Amitriptyline decreases the association rate (k_{01}) of amiloride to the apical Na channels from 15.6 ± 4.2 μM^{-1} s^{-1} in control Cl-Ringer to 7.4 ± 1.7 μM^{-1} s^{-1} at 200 μM amitriptyline, while it has no significant influence on the dissociation rate (k_{10}). These results suggest a competitive binding of amitriptyline to the recently identified high-affinity binding site for the pyrazine ring of amiloride (the sequence WYRFHY on the α subunits), that stabilizes the open state of the channel.

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