Nipecotic acid directly activates GABA$_A$-like ion channels

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1 The GABA-related compound nipecotic acid is commonly used to inhibit GABA uptake. This report shows that nipecotic acid can also directly activate GABA$_A$-like chloride channels.

2 When applied to outside-out patches of paraventricular neurones, nipecotic acid (1 mM) activated inward unitary currents (approximately 3 pA at a holding potential of -60 mV, $E_{Cl} + 44$ mV).

3 The EC_{50} for ion channel activation was approximately 300 μM, 3 fold greater than that found for GABA itself in this preparation.

4 The nipecotic acid activated channels had similar conductance and kinetic properties to those of GABA activated channels in the same patches, reversed near E_{Cl} and were inhibited by bicuculline (3 μM).

5 This study indicates that for experiments in which relatively high concentrations of nipecotic acid are used, possible direct GABA$_A$ receptor agonist properties should be considered.
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Abbreviations: *cmot*, corrected mean open time; E_{Cl}, chloride equilibrium potential; E_K, equilibrium potential for potassium ions; GABA, γ-aminobutyric acid; GAT, GABA transporter

Introduction

Nipecotic acid is a GABA-related compound which has been widely used as an inhibitor of both glial and neuronal GABA uptake (Krogsgaard-Larsen & Johnston, 1975; Johnston *et al.*, 1976; Brown & Galvan, 1977; Massey & Redburn, 1982; Brennan & Haywood, 1985; Pfeiffer *et al.*, 1996; Galindo *et al.*, 1999; Ciranna *et al.*, 2000). Its conventional mechanism of action is believed to be the competitive inhibition of the GABA transporter (GAT, Masson *et al.*, 1999). GABA is the primary inhibitory neurotransmitter in the mammalian CNS and so inhibition of its uptake is expected to lead to a facilitation of inhibitory neurotransmission (Sivilotti & Nistri, 1991). Exploiting this mechanism, the lipophilic nipecotic acid derivative N-4,4-di(3-methylthien-2-yl)but-3-enyl) nipecotic acid has recently entered clinical trials as an antiepileptic agent (Suzdak & Jansen, 1995; Beydoun, 1997; Adkins & Noble, 1998). Both *in vivo* and *in vitro* experiments have, as expected, revealed that application of nipecotic acid to various brain preparations (in the absence of exogenous GABA agonists); induces inward current (Draguhn & Heinemann, 1996), increases whole-cell conductance (Honmou *et al.*, 1995), inhibits neuronal firing (Reith & Sillar, 1999; Ciranna *et al.*, 2000) and catecholamine accumulation (Proll & Morgan, 1983). Although Draguhn & Heinemann (1996) suggested that nipecotic acid induced currents could be due to a direct action on GABA$_A$ receptors, all these effects are consistent with an increase in local concentrations of endogenously released GABA. It is, however, difficult in an *in vivo* or *in vitro* slice preparation, to distinguish between such 'GABA overflow' and any direct agonist-like action of nipecotic acid. The mediocellular paraventricular nucleus

(PVN) is an area of the hypothalamus rich in GABAergic innervation (Boudaba *et al.*, 1996; Hermes *et al.*, 1996; Bains & Ferguson, 1997; Tasker *et al.*, 1998; Zhang & Patel, 1998), and tonic activity of these GABA neurones is involved in regulation of the cardiovascular system (Martin & Haywood, 1993; Goren *et al.*, 1996). Our group has also observed that addition of nipecotic acid to slices of the PVN can evoke a small decrease in neuronal firing frequency and an increase in whole-cell conductance (unpublished observations). Again, however, these effects could be explained by either block of the GABA uptake protein, or a GABA mimetic action of nipecotic acid. The aim of this work was to investigate the action of nipecotic acid on cell free membrane patches, and determine whether nipecotic acid can directly gate GABA$_A$ ion channels.

Methods

Preparation of tissue

Slices of paraventricular nucleus were prepared using the methods of Barrett-Jolley *et al.* (2000). Briefly, rats were killed by an overdose of anaesthetic (Sagital) and the brain removed to iced low-Na$^+$ high-sucrose artificial cerebrospinal fluid (low-Na$^+$-ACSF). The general area of the hypothalamus was blocked and glued in the chamber of a Campden Instruments Vibroslice. The chamber was maintained at 0–4°C by frequent addition of iced low-Na$^+$-ACSF. One hundred and fifty to 200 μm slices were cut and removed to a multiwell culture dish containing normal ACSF. The culture dish was incubated at 35–37°C and bubbled with 95% O$_2$–5% CO$_2$, for at least 1 h prior to recording.

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Electrophysiology

Following incubation at 35–37°C, slices were transferred to a glass-bottomed recording chamber and perfused at 3 ml min^{-1} with a HEPES buffered flow solution at 26–28°C. Visualization of PVN neurones was made with a Nikon E600FN Eclipse upright microscope equipped with near infrared DIC optics. During pharmacological experiments, patch pipettes were lifted clear of the slice and placed near the mouth of a glass pipette carrying the flow solution. Patch-clamp electrodes were fabricated from GC150F electrode glass (Clark Electromedical) and pulled to a final (filled) resistance of 5–10 $\equiv\Omega$ on a Brown-Flaming MP P-80 horizontal puller (Sutter Instrument Co.).

Analysis

Patch-clamp recording was made with an Axon Instruments Axopatch 200A integrating patch-clamp amplifier, filtered at 2–3 kHz and digitized with a DigiData 1200 board (Axon Instruments).

Membrane current was measured in one of two ways; when channel activity was low (e.g., only one channel was observed) current was estimated as $Po \times i$, where i was the unitary current amplitude, and Po the average open probability of the channel per acquisition sweep (approximately 1 s per sweep). Alternatively, when membrane current was greater, it was measured simply as the peak average current.

'Maximum' current is difficult to assess in single channel experiments, and so data for dose response curves were normalized to a fixed reference concentration in each patch. pD_2 values were then obtained by fits to the mean normalized data with;

$$r = m \times \frac{c^h}{c^h + (10^{-pD_2})^h}$$

where r was the relative response, m the maximum response, c the concentration and h the Hill slope (Barrett-Jolley & McPherson, 1998). Errors for the pD_2 values quoted in the text are those returned by the non-linear least squares fitting algorithms of MicroCal Origin 6.0.

Kinetic analysis followed the procedures of Barrett-Jolley & Davies (1997). Briefly data was digitally filtered to 1 kHz, dwell times were log binned according to the methods of Sigworth & Sine (1987) and fitted with the standard maximum likelihood probability density function. Bursts were defined by the preceding closed time exceeding a critical time, t_c. This critical time was calculated for each patch by equalizing the proportions of intraburst closures misclassified as longer than t_c and of interburst closures misclassified as shorter than t_c.

Analysis was performed with the Axgox suite of programs, written by Noel Davies (University of Leicester, Davies, 1993) and with MicroCal Origin. Data is displayed as ±s.e. unless otherwise stated n refers to the number of patches.

Solutions

'Low−Na$^+$ ACSF' (mM): sucrose 250, KCl 2.5, NaHCO$_3$ 26, glucose 10, NaH$_2$PO$_4$ 1.25, CaCl$_2$ 1, MgCl$_2$ 1.2. pH 7.4 when bubbled with carbogen 95/5. 'Normal ACSF' (mM): NaCl 125, KCl 2.5, NaHCO$_3$ 26, glucose 10, NaH$_2$PO$_4$ 1.25, CaCl$_2$ 2, MgCl$_2$ 1.2, pH 7.4 when bubbled with carbogen 95/5. Bath solution (mM): NaSO$_4$ 60, NaCl 20, KCl 3, MgCl$_2$ 3.5, glucose 11, HEPES 10 (pH 7.4 with NaOH). Pipette solution (mM): CsCl 140, NaCl 10, EGTA 5, MgCl$_2$ 1, HEPES 5 (pH 7.2 with KOH).

Chemicals

All chemicals were obtained from Sigma. GABA and nipecotic acid were prepared as 1000× stocks in deionized water on the day of each experiment. Bicuculline was prepared as a 10,000× stock in DMSO. 1 : 1000 DMSO is inactive on these membrane patches or ion conductances.

Results

Outside-out patches were drawn from neurones in the parvocellular/mediocellular region of the PVN and moved approximately 1 mm away from the slice. The patches were then moved to near the opening of a perfusion tip and continuously bathed in fresh flow solution to ensure that they were not affected by GABA released from the slice (in some cases the slice was removed from the tissue chamber altogether).

Application of 1 mM nipecotic acid to patches held at −60 mV evoked GABA-like inward currents (−0.57 ± 0.14 pA $n = 11$, Figure 1A). The peak average current evoked by 1 mM nipecotic acid was similar to that evoked by a near maximal dose (300 μM) of GABA ($I_{nipecotic\ acid}$ / I_{GABA} ratio 1.8 ± 0.3, $n = 4$; non-responsive patches excluded). I found no patches that responded to GABA, but not to nipecotic acid.

The nipecotic acid activated channels appeared similar to those activated by GABA in terms of their single channel amplitude (Hp-60 mV: nipecotic acid: −3.0 ± 0.68(SD) pA $n = 5$ vs GABA: −2.5 ± 0.75(SD) pA $n = 6$, Figure 2). In some patches, other, much less frequent conductance levels were observed, but these have been excluded from the analysis. In terms of their kinetics, 1 mM nipecotic acid evoked events were somewhat longer than those of 300 μM GABA (Hp-60 mV: nipecotic acid corrected mean open time, *cmot*: 6.0 ± 0.6 ms $n = 4$ vs GABA *cmot*: 3.0 ± 0.2 ms $n = 3$, Figure 3), but burst lengths were similar (nipecotic acid: 32.7 ± 1.8 ms $n = 4$ vs GABA: 25.6 ± 2.5 ms $n = 3$), with the number of openings per burst slightly increased (nipecotic acid: 3.7 ± 0.3 $n = 4$ vs GABA: 5.5 ± 0.7 $n = 3$).

Application of nipecotic acid to patches held at differing membrane potentials allows estimation of the reversal potential and conductance (Figure 4). The channels reverse near E$_{Cl}$ (+44 mV) and show slight inward rectification. At −60 mV chord conductance was 24 ± 1.0 pS ($n = 3$) for GABA and 29 ± 1.3 pS ($n = 4$) for nipecotic acid. GABA itself is known to activate both chloride and potassium channels by acting at GABA$_A$ and GABA$_B$ (G-protein coupled) receptors respectively. The possibility that nipecotic acid activated K$^+$ channels rather than Cl$^-$ channels can be excluded because, under these recording conditions E$_K$ is near −50 mV and so K$^+$ channel currents would be outward at 0 mV.
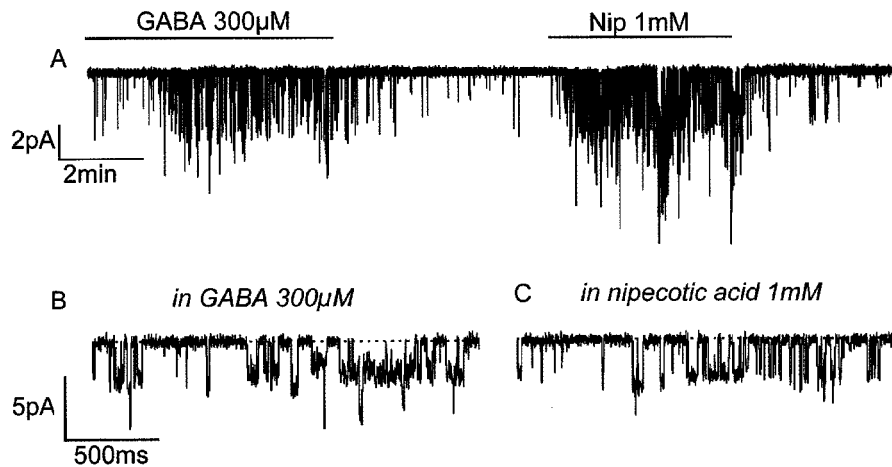
Figure 1 Activation of ion channels by nipecotic acid. (A) A compressed record. Application of GABA (300 μM) or nipecotic acid (Nip 1 mM), where indicated by the horizontal bars, activates brief inward channel events (inward events are represented by downward deflections; note that the amplitudes appear variable due to the compression routine used for display). (B) Time expanded record of GABA activated channels. Both (B) and (C) are taken from (A) at appropriate points. The scale bar for B applies to both B and C.
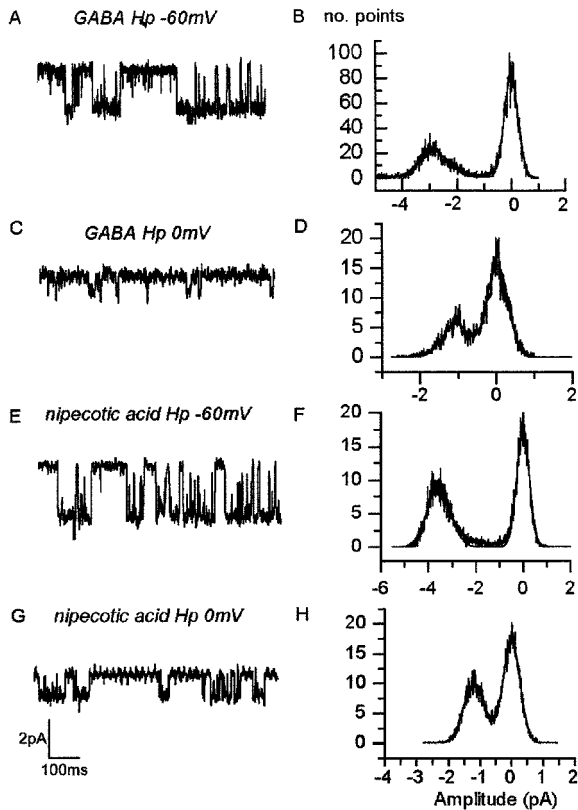
Figure 2 All points amplitude histograms for GABA and Nipecotic acid activated single channels. All points amplitude histograms for 300 μM GABA (A, B, C and D) and 1 mM Nipecotic acid (E, F, G and H) activated channels at −60 mV (A, B, E and F) and 0 mV (C, D, G and H). The histograms shown have each been fit with a two peak Gaussian curve (solid lines, Microcal Origin), fit parameters were as follows; B., *peak* 0 pA *width* 0.51 pA and *peak* −2.84 pA *width* 1.1 pA. D., *peak* −0.02 pA *width* 0.55 pA and *peak* −1.07 pA *width* 0.69 pA. F., *peak* −0.01 pA *width* 0.5 pA and *peak* −3.54 pA *width* 0.98 pA and H., *peak* −0.01 pA *width* 0.54 pA and *peak* −1.17 pA *width* 0.65 pA. In each case '*width*'; is equivalent to the standard deviation for the fitted Gaussian; mean values are given in the text (the data illustrated comes from different patches).

Although nipecotic acid is frequently used as an uptake blocker at concentrations of 1 mM, it actually inhibits GABA uptake with a IC_{50} of approximately 10 μM (Ruiz *et al.*, 1994). To investigate the concentration dependence of nipecotic acid on channel activity, concentration effect curves were constructed to both GABA and nipecotic acid (Figure 4B). Nipecotic acid (pD_2 3.5, Figure 4B) was somewhat less potent than GABA (pD_2 4.0, Figure 4B).

The evidence detailed above implies that nipecotic acid activates a chloride current. To examine if this current is carried by GABA_A channels, some experiments were repeated in the presence of the selective GABA_A receptor antagonist, bicuculline (Figure 4C). Three μM bicuculline inhibited the action of nipecotic acid by 80±4%, $n=4$, suggesting that nipecotic acid may act as an agonist at a GABA_A-receptor GABA binding site.

Discussion

The GABA uptake blocker nipecotic acid is a widely used research tool in both *in vitro* and *in vivo* experiments, however the present work, using single channel recording from isolated membrane patches, suggests that nipecotic acid can also act as a GABA agonist. This action would be difficult to detect in certain *in vivo* or *in vitro* (brain slice) experiments.

This work clearly shows the activation of GABA_A-like channel currents by nipecotic acid. Nipecotic acid has previously been found to activate inward currents through GAT proteins in catfish horizontal retinal cells (Cammack & Schwartz, 1993). Theoretical predictions of the microscopic currents expected for GAT transporter proteins themselves are 10^{-17} to 10^{-19} pA (Masson *et al.*, 1999), nevertheless, Cammack & Schwartz (1996) identified 1–1.4 pA inward unitary currents associated with GAT-1 proteins in transfected HEK293 cells (Hp 50 pA). These currents remained in the absence of both GABA and chloride and are thought to represent a leak state of GAT-1. The currents activated by
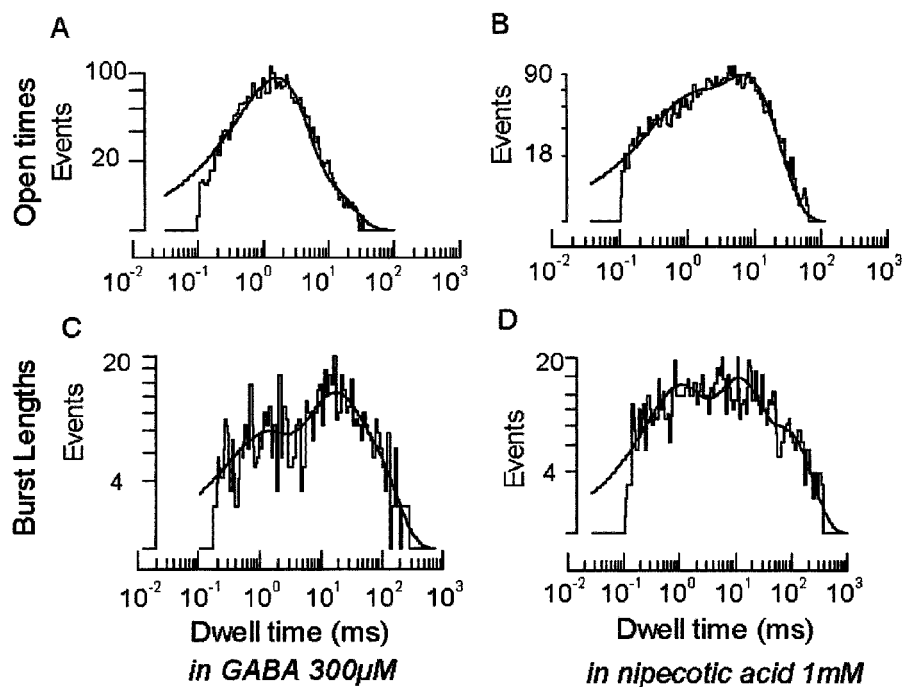
Figure 3 Kinetics of nipecotic acid activated ion channels. Kinetic analysis of nipecotic acid (1 mM) and GABA (300 μM) activated channels. (A) and (B) show open dwell times – fitted with two exponentials (GABA open: τ_1 1.5 ms, τ_2 6.1 ms; nipecotic acid open: τ_1 0.8 ms, τ_2 6.9; mean values are given in the text). (C) and (D) show burst lengths – fitted with three exponentials (GABA burst lengths: τ_1 0.9 ms, τ_2 13.7 ms, τ_3 48.2 ms; nipecotic acid burst lengths: τ_1 0.9 ms, τ_2 10.98 ms, τ_3 75.96 ms; mean values are given in the text).
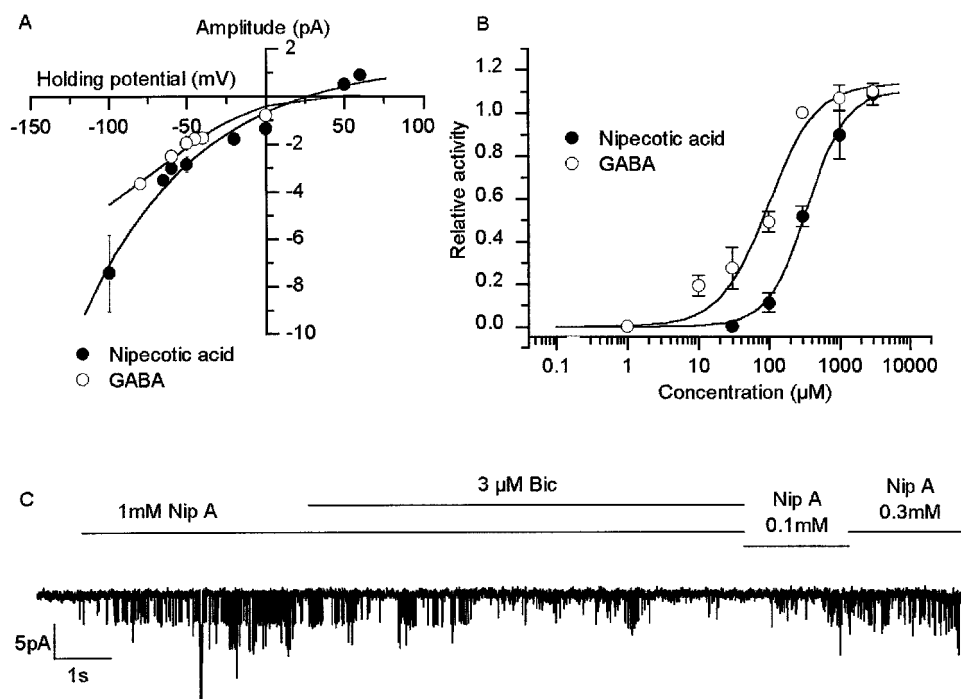
Figure 4 Nipecotic acid activates GABA chloride channels. (A) Current-voltage curves for GABA and nipecotic acid from a number of experiments similar to those of Figure 3. (B) Concentration effect curves for GABA (open circles) and nipecotic acid (filled circles) activation of channels. The smooth lines are Hill equations fitted to the mean data with MicroCal Origin (GABA pD_2 3.46 $\pm$ 0.05, h 1.60 $\pm$ 0.2 and nipecotic acid pD_2 4.04 $\pm$ 0.11, h 1.44 $\pm$ 0.46, $n=11$, see Methods). (C) Bicuculline ('Bic'), and nipecotic acid ('Nip A') were added at the concentrations stated, where indicated by the horizontal bars. Bicuculline reversibly inhibits nipecotic acid activated channels.

nipecotic acid in this study were, however, similar in size and kinetics to those activated by GABA itself, reversed near to E_{Cl} and were sensitive to (although not totally abolished by) bicuculline.

The concentration of nipecotic acid which I have shown here to act as an agonist is well above the IC_{50} for block of the GABA uptake pathways (IC_{50}: approximately 10 μM: Ruiz et al., 1994; Mantz et al., 1994; Falch et al., 1999. Here, pD_2 3.54 equivilent to $EC_{50} \sim 300$ μM), but in the order of the concentrations frequently used (Brown & Galvan, 1977; Massey & Redburn, 1982; Fern et al., 1995; Pfeiffer et al., 1996 (1 – 5 mM); Arnarsson & Eysteinsson, 1997; Galinado et al., 1999).

The lipophilic derivative of nipecotic acid, (R)-N-(4,4-di-(3-methylthien-2-yl)but-3-enyl)nipecotic acid, which recently entered clinical trails as an anti-epileptic agent, is a more potent blocker of GABA uptake than nipecotic acid itself

(IC$_{50}$ $<$1 μM: Pavia et al., 1992), however, it remains to be seen whether this compound retains any GABA agonist - like potency. Further experiments are also required to determine whether the agonist action of nipecotic acid is general to all GABA$_A$ receptors, or specific to one particular subtype.

In conclusion, the GABA uptake inhibitor nipecotic acid appears to also to act as a GABA$_A$ receptor agonist at concentrations of 1 mM and below. Caution should be used when using this compound as it may be difficult to distinguish between its GAT inhibiting and GABA mimetic actions.

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