Comparison of pyrrolidinyl and piperidinyl benzamides for their anticonvulsant activity and inhibitory action on sodium channel

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1 A pair of benzamide analogues containing a pyrrolidinyl or piperidinyl group was examined for their anticonvulsant activity against the electroshock-induced seizures in mice and the ability to block the voltage-gated Na channel in N1E-115 cells, in comparison with the prototype compound, U-54494A, ($\pm$)-*cis*-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzamide, a potent anticonvulsant and a Na channel blocker.

2 The pyrrolidinyl benzamide (U-49524E) was found to be effective against the electroshock-induced seizures ($ED_{50} = 35 \text{ mg kg}^{-1}$, i.p.) whereas the benzamide with a piperidinyl moiety (U-49132E) was inactive ($ED_{50} > 100 \text{ mg kg}^{-1}$).

3 Using whole-cell patch clamp techniques we found that U-49132E was several times less potent, with an IC_{50} of $396 \mu\text{M}$ as compared to $118 \mu\text{M}$ for U-49524E at the holding potential of -80 mV , and was much slower in blocking Na channels with a half-time of $10.7 \pm 1.1 \text{ min}$ vs. $2.2 \pm 0.4 \text{ min}$ for its counterpart.

4 Qualitatively, their general modes of interaction with Na channels were similar to each other and to that of U-54494A in that they interacted with the resting and slowly-inactivated states of the channels and exhibited a use-dependent inhibition because of a slow recovery from the inactivated state in the presence of the drugs.

5 Comparison of their physicochemical properties, shows the less potent and slowly acting U-49132E is more hydrophobic and bulkier than U-49524E, but has the same pK_a . This suggests that the drugs approach the Na channel through a narrow and hydrophilic pathway.

6 Overall, this study underscores the importance of inhibiting the Na channel to the anticonvulsant activity of the benzamide compounds.

Keywords: Anticonvulsant; patch clamp; sodium channel; U-49132E; U-49524E; U-54494A

Introduction

Voltage-dependent Na channels are primarily responsible for the depolarizing phase of action potentials in neurones. Various compounds block the Na currents and are used as local anaesthetics or anticonvulsants. In particular, phenytoin and carbamazepine are known to interact with the slowly-inactivated Na channels. The slow recovery rate of the drug-bound channels from inactivation leads to a use-dependent block and is responsible for their anticonvulsant activity (Schwarz & Grigat, 1989; Rogawski & Porter, 1990). Recently, we have shown that U-54494A, ($\pm$)-*cis*-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzamide, is a potent anticonvulsant (VonVoigtlander *et al.*, 1987) and blocks the Na current in N1E-115 neuroblastoma cells (Zhu & Im, 1991). The drug has been shown to interact with the slowly-inactivated state of the Na channel and to retard the recovery process like phenytoin, in addition to its interaction with the resting state of the Na channel. In the course of our studies on the structure-activity relationship for benzamide compounds, we discovered a pair of U-54494A analogues which differ slightly in their structures, but vary widely in their anticonvulsant activities. They are U-49524E (*trans*- α,α,α -trifluoro-N-methyl-N-[2-pyrrolidinyl-cyclohexyl]-*p*-toluamide) and U-49132E (*trans*- α,α,α -trifluoro-N-methyl-N-[2-piperidino-cyclohexyl]-*p*-toluamide) (Figure 1). U-49524E containing a pyrrolidinyl group was a potent anticonvulsant whereas U-49132E containing a piperidinyl group was not. In

this study, we also found that U-49132E was less potent and much slower in blocking the Na channel than U-49524E. Their interactions with the Na channels were further characterized.

Methods

Electroshock-induced seizures in mice

Charles River CF-1 (18–22 g) male mice were dosed intraperitoneally in the range of 6.25 to 200 mg kg^{-1} (drugs dissolved in 0.9% saline). Anticonvulsant activity, 1 h after the drug administration, was assessed by determination of electroshock-induced seizure thresholds as previously described (VonVoigtlander *et al.*, 1987). Generally, 10 mice were used to establish a threshold (mA) of the electroshock convulsions in the control which was typically 25 mA for a period of 0.2 s. The ED_{50} represents the dose of the drugs which enhance the threshold of the electroshock seizures to 100 mA for a 0.2 s stimulus.

Cell culture

Mouse neuroblastoma cells (N1E-115) were grown in Dulbecco's modified Eagle's medium (Sigma Chemical Co., USA) supplemented with 5% foetal calf serum, 20 mM HEPES, penicillin (100 u ml^{-1}) and streptomycin ($100 \mu\text{g ml}^{-1}$) at 37°C under humidified air containing 10% CO_2 (Quandt & Narahashi, 1984). The cells were differentiated by reducing

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▪ HC1

U-54494A

U-49524E

U-49132E

Figure 1 Structures of U-54494A, U-49524E and U-49132E.

foetal calf serum to 1% and adding 2% of dimethylsulphoxide in the medium for 6–9 days before use.

Voltage-clamp recording

The whole-cell configuration of the patch clamp technique (Hamill *et al.*, 1981) was used to record the Na channel current. Patch pipettes were made of borosilicate glass capillary tubes (Kimax-51, Kimble Products, U.S.A.) on a Flaming/Brown Micropipette Puller (P-80/PC, Sutter Instrument Co. U.S.A.). After fire polishing, the pipette tip had a resistance of 0.5 to 2 M Ω in solutions described below. Cells grown on the cover slip were transferred to a small chamber (1 ml volume) on the stage of an inverted light microscope and superfused by gravity with the external solution at a rate of 1.5 to 2 ml min⁻¹. The membrane current through the pipette was recorded by a current-voltage converter consisting of an Axopatch-1D amplifier and a CV-4 headstage (Axon Instrument Co., U.S.A.) and stored on diskettes by use of 'Pclamp 5.5' acquisition software (Axon Instrument Co.). Signal was filtered at 5 kHz. Linear leakage current was subtracted by a P/N procedure provided by the 'Pclamp 5.5' programme. The capacitive transient current and the series resistance were compensated for using the features of the Axopatch-1D amplifier. Each test pulse was applied at an interval of 20 s which is sufficiently long to avoid the use-dependent effect of the drug.

Solutions

Pipette solution contained (in mM): CsCl 100, CsF 50, Na-HEPES 10 and glucose 5, pH 7.0. The external solution contained (mM): NaCl 125, KCl 5.5, CaCl₂ 3, MgCl₂ 0.8, glucose 20 and Na-HEPES 20 (pH 7.4). Stocks of drugs were first made with water and later added to the external solution. All experiments were performed at room temperature. (22 $\pm$ 1°C).

Data analysis

In most cases, data were normalized to their appropriate maximal values under various conditions. When two drugs were tested on the same cell, a paired Student's *t* test was used. Otherwise, an unpaired *t* test was used to determine the significance of differences (Dudewicz & Mishra, 1988). *P* < 0.05 was considered significantly different. Data are presented as mean $\pm$ standard error in most cases.

Results

U-49132E and U-49524E, despite the minor structural difference, display widely different potency in anticonvulsant activity. Figure 2 shows that in mice treated intraperitoneally with the drugs, U-49524E enhanced the electroshock threshold from 25 to 100 mA with an ED₅₀ value of 35 mg kg⁻¹, but U-49132E was not effective up to the dose of 100 mg kg⁻¹. In similar experiments, U-54494A, the prototype compound in the benzamide series, was effective with an ED₅₀ value of 25 mg kg⁻¹.

Because the anticonvulsant activity of U-54494A has been ascribed to its block of the voltage-dependent Na channel currents (Zhu & Im, 1991), we compared in this study the interactions of its analogues with the Na channel in N1E 115 cells. Figure 3a shows typical recordings of the inward Na currents with and without drugs. U-49524E and U-49132E (at 50 μ M) reduced the peak Na current at all the depolarizing potentials. The drug effect was recorded after a steady-state effect of the drug was reached, typically 5 to 10 min for U-49524E and 20 min or more for U-49132E (see below). Figure 3b illustrates the current-voltage (*I*-*V*) relationships of the Na channels with and without 50 μ M U-49524E and U-49132E. The drugs showed no considerable effect on the shape of the *I*-*V* relationship and the apparent reversal potential. Figure 3c shows a superimposition of the control current trace to the drug-treated one obtained at the same depolarizing voltage as the control and multiplied by an appropriate factor. No noticeable deviations are observed

Figure 2 Dose-response of U-49524E (▽) and U-49132E (□) to the maximal electroshock seizures. The increase in the threshold of electroshock convulsion (MA₅₀) was determined 60 min after mice were injected intraperitoneally.

Figure 3 Effects of U-49524E and U-49132E on the current-voltage relationship of the Na channel. (a) Tracings of the whole-cell patch clamp recording of the Na currents generated upon a series of depolarizations from holding potential -80 mV to various potentials for 30 ms with and without drugs. (b) The peak amplitudes of the Na currents with and without drugs are plotted as a function of depolarizing potentials: (O) control; (●) 1 μ M tetrodotoxin; (▽) 50 μ M U-49524E; (□) 50 μ M U-49132E. (c) Superimposition of control traces and those in the presence of drugs (from a) generated upon depolarization to 0 mV. Traces with drugs have been multiplied.

between the two traces superimposed, indicating that the two compounds like U-54494A exert no effect on the kinetics of the channel activation or inactivation and have no apparent time-dependent block.

Figure 4 shows the time-course profiles for inhibition by U-49132E and U-49524E of the peak Na currents. The inhibitory action of U-49524E (50 μ M) rapidly reached a plateau with a half time of 2.2 ± 0.4 min ($n = 10$) (the same t_1 for U-54494A), but that of U-49132E at 50 μ M was much slower, its half time being 10.7 ± 1.1 min ($n = 12$, $P < 0.01$,

Figure 4 Time-course of resting block of Na channel by U-49524E (▽) and U-49132E (□). The peak Na current was measured after a depolarizing pulse from -80 mV to 0 mV at an interval of 10 s. The drugs were superfused and washed out at a rate of 1.5 to 2 ml min^{-1} .

unpaired t test). The values of the half time were not altered noticeably as the concentration of the drugs increased to 100 μ M (data not shown). All the current measurements in the presence of the drugs in this study, therefore, were performed as their inhibitory effects reached a plateau.

The general patterns of blocking the Na currents by two compounds were qualitatively similar. A quantitative difference, however, was observed in their potency to block the currents. Figure 5 shows dose-response profiles of the drugs at the holding potentials of -80 or -120 mV. The dose-response profiles were analyzed using the equation:

$$I_{\text{Na}} = 1/[1 + (C/IC_{50})^k]$$

where C is the concentration of drugs, IC_{50} is the half maximal inhibitory dose, and k is the slope of the curve. U-49524E was 3 to 5 times more potent than U-49132E in blocking the Na currents; the IC_{50} s were 118.2 and 396.1 μ M for U-49524E and U-49132E, respectively, at the holding potential of -80 mV, and 281 and 1435 μ M at -120 mV, respectively. The corresponding values for the parent compound, U-54494A, were 72.1 and 228.7 μ M at -80 and -120 mV, respectively. It should be mentioned that the inhibition of the Na current occurred at -120 mV is a true

Figure 5 Dose-response of the Na current to U-49524E (▽) and U-49132E (□). The peak Na current was measured after depolarization to 0 mV for 30 ms from holding potentials of either -120 (open symbols) or -80 mV (filled symbols) and normalized to the control values in the absence of drugs ($n = 3-7$). Curves were drawn with the function expressed in Results. Inset: holding potential (HP) dependent block of the Na channel by U-49524E (▽) and U-49132E (□) at 100 μ M.

'resting block' since a further hyperpolarization (to -140 mV, data not shown) did not relieve the block. The Hill coefficients of the curve (k) for U-49524E were 0.969 at -120 mV and 1.22 at -80 mV, suggesting the possibility of more than one binding site for U-49524E at a holding potential more positive than -120 mV. The Hill coefficients for U-49132E are less than unity, 0.684 and 0.851 at -120 mV and -80 mV, respectively, suggesting a limited access of the drug to the channel.

The inset of Figure 5 illustrates that with a fixed concentration of drugs at $100 \mu\text{M}$, the percentage inhibition is a function of the holding potential. This indicates the block of the Na channel by those two agents is voltage-dependent; the more depolarized potential, the more inhibition.

The steady-state inactivation of the Na channel was examined (see Figure 6) by fitting the data to the function (Matsuki *et al.*, 1984):

$$h_{\infty}(\text{or } S_{\infty}) = 1/(1 + \exp[(E_m - E_i)/k])$$

where h_{∞} or S_{∞} is the fast and slow inactivation, respectively, E_m is the conditioning potential, E_i is the conditioning potential at which 50% of the Na channel is inactivated and k is the slope of the curve. The fast inactivation was produced with a short conditioning pulse (100 ms) and a 2 ms gap between the conditioning and test pulses while the slow inactivation was produced with a long conditioning pulse (2 s) and a long hyperpolarizing gap (50 ms) between the conditioning and test pulses to relieve the fast inactivation. The drugs did not alter the parameters for the fast inactivation (data not shown), but brought about hyperpolarizing shifts in the slow inactivation. The shift was -9.7 ± 1.0 mV ($n = 5$) and -6.4 ± 1.3 mV ($n = 5$) for U-49524E and U-49132E, respectively, at $100 \mu\text{M}$ from the control E_i of -39.4 ± 2.0 mV ($P < 0.01$ for both compounds, paired t test). U-49524E induced a larger shift than U-49132E ($P < 0.05$, paired), but brought about the same shift as U-54494A.

The recovery from the inactivated state of the Na channels was examined with a double exponential function (Chernoff & Strichartz, 1989):

$$I/I_{\max} = 1 - A_1 \exp(-t/\tau_1) - A_2 \exp(-t/\tau_2)$$

where A_1 , A_2 are proportional constants and τ_1 , τ_2 are the time constants of two phases of the recovery. Table 1 shows the results from the analysis of the recovery processes for the inactivated Na channels in the presence of 50 and $100 \mu\text{M}$ of U-49132E and U-49524E. The drugs at the two concentrations had a marginal effect on the fast phase of recovery, both the amplitude (A_1) and time constants (τ_1). The fraction associated with the fast phase (A_1) accounts for $84 \pm 7\%$ of the recovery processes in the control as well as in the presence of the drugs at $50 \mu\text{M}$, but for $80 \pm 6\%$ with the drugs at $100 \mu\text{M}$. τ_1 was 5.9 ± 0.3 ms for the control, 6.9 ± 0.3 ms for both U-49132E and U-49524E at $50 \mu\text{M}$, and 7.1 ± 0.5 and 8.4 ± 0.8 ms for the respective drugs at $100 \mu\text{M}$. As the drug concentration was raised to $100 \mu\text{M}$, the fraction associated with the slow recovery phase (A_2) marginally increased from 16 ± 3 to $20 \pm 4\%$. On the other hand, the time constant for the slow recovery (τ_2) changed as a function of

Figure 6 Effects of U-49524E (▽) and U-49132E (□) on the steady-state slow inactivation of the Na channel (○, control). A 2 s conditioning pulse to various potentials followed by a 50 ms rest at -100 mV was applied. The peak Na current was normalized to its maximal value (the first peak current in this case) in the absence or presence of drugs ($100 \mu\text{M}$).

the drug concentrations. τ_2 increased from 225 ± 28 ms to 914 ± 96 ms with U-49524E and to 582 ± 63 ms with U-49132E at the concentration of $50 \mu\text{M}$, and further increased to 5744 ± 620 and to 722 ± 110 with the respective drug at a concentration of $100 \mu\text{M}$ (Figure 7). This indicates that the dissociation of the drug from the inactivated channels is very slow, particularly with the U-49524E. The marked dependence of the slow recovery rate for 49524E on its concentration suggests that the drug stabilizes the channel in the slow inactivated state and leads to an increase in the apparent time constant for recovery. With U-49132E, on the other hand, its apparent recovery rate was much less dependent on the concentration, implying that U-49132E has a limited ability to stabilize the channel in the inactivated state.

The recovery rate of Na channels from the slow inactivation in the presence of the drugs appears also to be responsible for the drug-induced use-dependent block. To examine the use-dependency arising solely from the slow inactivation, we have eliminated the accumulation of block from the resting and the fast inactivation block of Na channels by using a long gap, 300 ms at -100 mV, between pulses. Under these conditions, the control show no accumulation of block during the 9 consecutive pulses. This is consistent with our analysis that the slow inactivated state of the channel in the absence of the drugs recover with a time constant of 225 ± 28 ms, which is too short to survive the 300 ms gap. We have compared the degree of the use-dependent block at the 9th pulse at the two concentrations of the drug. At $50 \mu\text{M}$, the use-dependent block was $10 \pm 3\%$ for U-49132E and $16 \pm 4\%$ for U-49524E. At a concentration of $100 \mu\text{M}$, the extent of block by U-49132E was not measurably altered

Table 1 Kinetic parameters for the recovery of Na channels from inactivation states

Parameters	Control	U-49132E		U-49524E	
		50 μM	100 μM	50 μM	100 μM
A_1	0.85 ± 0.07	0.89 ± 0.05	0.76 ± 0.05	0.85 ± 0.07	0.76 ± 0.06
A_2	0.17 ± 0.03	0.19 ± 0.03	0.18 ± 0.04	0.18 ± 0.03	0.19 ± 0.03
τ_1 (ms)	5.9 ± 0.3	6.9 ± 0.3	7.1 ± 0.5	6.9 ± 0.3	8.4 ± 0.8
τ_2 (ms)	225.0 ± 28	582.0 ± 63	722.0 ± 110	914.0 ± 96	5744.0 ± 620

Na channels in N1E 115 cells were inactivated by use of the pulse protocol in Figure 7. The time courses for the recovery of Na channels from inactivated states in the presence of U-49132E and U-49524E at concentrations of 50 and $100 \mu\text{M}$ were analyzed with a double exponential equation: $I/I_{\max} = 1 - A_1 \exp(-t/\tau_1) - A_2 \exp(-t/\tau_2)$ where A_1 , A_2 are proportional constants and τ_1 , τ_2 are the time constants of two phases of the recovery. The data represent the mean of three to nine separate experiments with a standard error.

Figure 7 Effects of U-49524E (∇) and U-49132E ($\square$) on the recovery from the inactivated channel ($\circ$, control). Cells were first depolarized from -120 mV to 0 mV for 100 ms, then were allowed various durations at -120 mV to recover before a test pulse. The peak current was normalized to a maximal value (which was not treated with any conditioning pulse) in the absence or presence of $100 \mu\text{M}$ drugs.

Figure 8 Use-dependent block of the Na channel by U-49524E (∇) and U-49132E ($\square$). Cell was treated with trains (varied from 0 to 9) of conditioning pulses from -100 to 0 mV before the test pulse. The interval between each conditioning pulse was 300 ms. The peak Na current was normalized to the first one in the absence ($\circ$) or presence of drugs ($100 \mu\text{M}$).

but that by U-49524E increased to $25 \pm 4\%$ (Figure 8). These results indicate the importance of the slow recovery rates (τ_2) in the extent of the use-dependent block: the marginal dependence of τ_2 for U-49132E on its concentration may account for the unnoticeable change in the use-dependent block as its concentration was raised from 50 to $100 \mu\text{M}$ whereas the large increase in the τ_2 for U-49524E (from 914 ± 96 to 5744 ± 620 ms) with the same change in its concentration appears to be responsible to the enhanced use-dependent block.

Discussion

In this study, we have shown that the two benzamide analogues containing either pyrrolidinyl (U-49524E) or piperidinyl (U-49132E) substituent at C2 of the cyclohexyl ring displayed remarkable differences in the anticonvulsant activity against electric shock in mice and their interactions with the voltage-dependent Na channel in neuroblastoma (N1E-115) cells. It appears that U-49524E has a very similar profile to its prototype compound U-54494A in both anticonvulsant activity and Na channel blocking actions whereas U-49132E lacks anticonvulsant activity and has a very weak Na channel blocking action. The determining factors for anticonvulsant activity of a drug *in vivo* are its intrinsic activity and pharmacokinetic parameters. Considering similar physicochemical properties between the two compounds (see below), the lack of anticonvulsant activity *in vivo* by U-49132E could be attributed to a combination of its low potency in the resting block of the Na channel, slow onset of action, and the poor use-dependent block. It appears, therefore, that blocking the Na channel (probably a rapid onset of block) is an important mechanism for the anticonvulsant activity of U-54494A and its active analogues.

The modes of the interaction of two benzamides with the Na channel, nevertheless, were qualitatively similar to each other. The drugs interact with the resting and slowly-inactivated states of the Na channel without affecting activation and inactivation kinetics and the shape of the *I-V* curve. The two agents induce a slow recovery from the inactivated state and lead to use-dependent block which is thought to be crucial for anticonvulsants like phenytoin and carbamazepine (Matsuki *et al.*, 1984; Willow *et al.*, 1985).

It is of interest to explore why the piperidinyl group in place of the pyrrolidinyl in the benzamide series markedly delayed the onset of inhibition and reduced the inhibitory potency on the Na channel. It has been suggested that potency in blocking the Na channel may be dependent upon several factors such as molecular weight, hydrophobicity, pK_a , or spacial configuration (Yeh & Ten Eick, 1987; Courtney, 1990). We compared the physicochemical properties of the two compounds. The pK_a values of U-49132E and U-49524E are not expected to be different from that of U-54494A which was 8.4 . For instance, the pK_a of piperidine, 11.28 , is the same as that of pyrrolidine, 11.27 , and the sigma value for the CF_3 group, 0.46 , is also identical to the sum of *para* and *meta* chloro groups (Perrin *et al.*, 1981). Their hydrophobicity as estimated by Hansch-Leo Fragment Approach in octanol/water system are somewhat different, log *p* being 4.23 for U-49524E and 4.79 for U-49132E. Given the same pK_a for the both compounds, U-49132E, the more hydrophobic one, would penetrate hydrophobic phase more readily than U-49524E. In fact, however, U-49524E, the more hydrophilic drug, shows the faster rate of action on the Na channels. This suggests that the interaction between the drug and channel is not all hydrophobic but includes a critical hydrophilic action. Another explanation for the different drug effects would be the size effect as suggested by Courtney (1988) for cardiac Na channel blockers. The spacial configuration of a piperidinyl group is considerably more nonplanar and bigger than the pyrrolidinyl group, about 30% larger in a spacial computational model. It appears that drugs reach the receptors through somewhat sterically restricted pathways and that the passage may be more limited for the more hydrophobic and bulkier U-49132E. It is conceivable that the active species of the drugs is the protonated form.

In conclusion, our results support the importance of the ability of benzamide analogues to block the Na channel for their anticonvulsant activity and are in agreement with the steric restriction proposal for Na channel blockers to reach the inactivated state (Courtney, 1988).

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