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Anisatin modulation of the γ-aminobutyric acid receptor-channel in rat dorsal root ganglion neurons

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1 Anisatin, a toxic, insecticidally active component of Sikimi plant, is known to act on the GABA system. In order to elucidate the mechanism of anisatin interaction with the GABA system, whole-cell and single-channel patch clamp experiments were performed with rat dorsal root ganglion neurons in primary culture.

2 Repeated co-applications of GABA and anisatin suppressed GABA-induced whole-cell currents with an EC_{50} of 1.10 μM. No recovery of currents was observed after washout with anisatin-free solution.

3 However, pre-application of anisatin through the bath had no effect on GABA-induced currents. The decay phase of currents was accelerated by anisatin. These results indicate that anisatin suppression of GABA-induced currents requires opening of the channels and is use-dependent.

4 Anisatin suppression of GABA-induced currents was not voltage dependent.

5 Picrotoxinin attenuated anisatin suppression of GABA-induced currents. [^{3}H]-EBOB binding to rat brain membranes was competitively inhibited by anisatin. These data indicated that anisatin bound to the picrotoxinin site.

6 At the single-channel level, anisatin did not alter the open time but prolonged the closed time. The burst duration was reduced and channel openings per burst were decreased indicating that anisatin decreased the probability of openings.

Keywords: Anisatin; GABA receptor; picrotoxin; dorsal root ganglion; patch clamp; single channel; chloride channel

Abbreviations: DRG, dorsal root ganglion; EBOB, 1-(4-ethynylphenyl)-4-*n*-propyl-2,6,7-trioxabicyclo[2.2.2]octane; GABA, γ-aminobutyric acid

Introduction

The γ-aminobutyric acid$_A$ ($GABA_A$) receptor-channel complex is an important site of action of a variety of chemicals including barbiturates, benzodiazepines, picrotoxin, bicuculline, general anaesthetics, alcohols, heavy metals, and insecticides (Eldefrawi & Eldefrawi, 1987; Gant *et al*., 1987; Nakahiro *et al*., 1989; Nishio & Narahashi, 1990; Arakawa *et al*., 1991; Burt & Kamatchi, 1991; Yeh *et al*., 1991; Olsen *et al*., 1992; Ticku *et al*., 1992; Kurata *et al*., 1993; Ma & Narahashi, 1993; Marszalec *et al*., 1994; Nagata & Narahashi, 1994; 1995a,b; Nagata *et al*., 1994; Narahashi, 1996).

Sikimi plant, *Illicium anisatium*, also known as Japanese star anise, contains toxic substances. Early pharmacological studies on a crude crystalline sample of toxic components, named sikimin, indicated that sikimin caused picrotoxin- or bicuculline-like inhibitory effects on the cat spinal reflex (Curtis *et al*., 1973). A recent preliminary study indicated that crude extracts of Sikimi plant exhibited insecticidal activity against mosquito larvae (Ikeda *et al*., 1998). One of the toxic substances isolated from Sikimi plant is anisatin (Lane *et al*., 1952; Yamada *et al*., 1968). Neuropharmacological studies conducted with the frog spinal cord and crude synaptic membranes isolated from the rat whole brain showed that anisatin was a potent non-competitive GABA antagonist (Kudo *et al*., 1981; Shinozaki *et al*., 1981). However, little is known about the mechanism of action of anisatin on the GABA system at the ion channel level.

We performed whole-cell and single-channel patch clamp experiments with rat dorsal root ganglion (DRG) neurons in primary culture to elucidate the mechanism of action of anisatin on the GABA receptor-chloride channel complex at the ion channel level. Several important aspects of anisatin effects have been unveiled. Anisatin suppressed GABA-induced currents in a dose-dependent manner when co-applied with GABA, but not when preapplied alone. It accelerated the falling phase of GABA-induced currents. Picrotoxinin attenuated anisatin suppression of the current. These results indicate that anisatin blocks the GABA receptor-channel in its open state. In keeping with these whole-cell current data, anisatin decreased the probability of single-channel openings without changing the mean open time.

Methods

Culture of DRG neurons

The dorsal root ganglia were dissected from the lumbodorsal region of newborn rats (1–5 days postnatal) and were immediately placed into Ca^{2+} and Mg^{2+}-free phosphate buffered saline solution supplemented with 6 g l^{-1} of glucose. The ganglia were digested in phosphate buffered saline

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solution containing 2.5 mg ml^{-1} of trypsin (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 20 min at 37°C. The ganglia were then dissociated by repeated triturations using a fire-polished Pasteur pipette in Dulbecco's Modified Eagle Medium containing 0.1 mg ml^{-1} of foetal bovine serum and 0.08 mg ml^{-1} of gentamicin. The dissociated cells were placed on coverslips coated with poly-L-lysine. Neurons were maintained in Dulbecco's Modified Eagle Medium containing serum and gentamicin in a 90% air – 10% CO_2 atmosphere controlled at 37°C. Neurons cultured for 2 – 5 days were used for experiments.

Whole-cell current recording

Membrane currents were recorded using the whole-cell patch clamp technique at room temperature (22°C). Pipette electrodes were made from 0.8 mm (I.D.) borosilicate glass capillary tubes and fire-polished. The electrode had a resistance of 2 – 3 MΩ when filled with standard internal solution. The membrane was clamped at −60 mV, and a 5 min period was allowed following rupture of the membrane to equilibrate the cell interior with pipette solution. Currents through the electrode were recorded by an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, U.S.A.), filtered at 2 kHz, and stored in a microcomputer. Currents were continuously monitored by a chart recorder.

Single-channel current recording

The single-channel current recordings were made using the outside-out configuration of the patch clamp technique at room temperature (22°C). Pipette electrodes were prepared as described above. The electrodes had a resistance of 10 – 12 MΩ when filled with standard pipette solution. The membrane potential was clamped at −60 mV. The currents flowing through the electrode were recorded by an Axopatch 200B amplifier filtered at 2 kHz, and stored at 88 kHz on a video cassette recorder *via* an analogue-to-digital converter (VR10B, Instrutech, Elmont, NY, U.S.A.). For data analyses, the cut-off frequency of 1 kHz was used.

Solutions for patch clamp experiments

Internal and external solutions were designed to eliminate sodium and potassium currents. The standard internal solution contained (in mM): CsCl 140, $MgCl_2$ 1, EGTA 5, and HEPES 10. The pH was adjusted to 7.3 with tris(hydroxymethyl)-aminomethane (Tris base), and the osmolarity was adjusted to 290 mOsm. The standard external solution contained (in mM): choline chloride 136, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, and the pH was adjusted to 7.3 with Tris base.

Binding studies

The preparation of rat brain P_2 membranes and binding of [^{3}H]-1-(4-ethynylphenyl)-4-*n*-propyl-2,6,7-trioxabicyclo[2.2.2]octane ([^{3}H]-EBOB) were performed as described previously (Ozoe *et al*., 1998). Briefly, rat brain membranes (125 μg protein) were incubated with or without 370 nM anisatin and 0.5 – 30.5 nM [^{3}H]-EBOB in 1.0 ml of 10 mM sodium phosphate buffer (pH 7.5) containing 300 mM sodium chloride at 37°C for 90 min. After incubation, the mixtures were filtered through GF/B filters (Whatman International, Maidstone, U.K.) and were rapidly rinsed twice with 5 ml of the ice-cold buffer, using a Brandel M-24 cell harvestor (Biomedical Research & Development Labs, Gaithersburg, MD, U.S.A.). The radioactivity of [^{3}H]-EBOB that specifically bound to membranes on the filters was measured with a liquid scintillation counter. Nonspecific binding was determined in the presence of 5 μM unlabelled EBOB. Each experiment was performed in duplicate and repeated twice. [^{3}H]-EBOB (38.0 Ci $mmol^{-1}$) was purchased from NEN Life Science Products (Boston, MA, U.S.A.).

Chemicals

GABA was first dissolved in distilled water to make stock solutions. Anisatin and picrotoxinin were dissolved in dimethylsulphoxide. These stock solutions were then diluted with the standard external solution. The final concentration of dimethylsulphoxide in test solutions was 0.1% (v v^{-1}) or less, which had no adverse effect on GABA-induced currents.

Data analysis

EC_{50} values and their slope factors (Hill coefficients) were calculated from the equation:

$$I = I_{max} C^n / (C^n + EC_{50}{}^n)$$

where I is the amplitude of GABA-induced current, I_{max} the maximum current, C the drug concentration, and *n* the Hill coefficient. The nonlinear regression analysis was carried out using the least squares fitting method (Sigmaplot, Version 4.0) by a microcomputer.

Whole-cell and single-channel current records were analysed by the pClamp version 6.0 software (Axon Instruments). For the single-channel data, openings and closings of the channels were detected using the 50% threshold criterion (Colquhoun & Sigworth, 1995). Openings and closings shorter than a set duration (200 μs) were ignored in compiling event files for histograms. A cut-off frequency of 1 kHz was used. GABA has been shown to open chloride channels in at least four conductance states (Bormann *et al*., 1987). In the present study, only the main-conductance state (about 26 pS) was consistently observed. Currents with smaller conductances (11 and 19 pS) and a larger conductance (44 pS) were detected only rarely. These three types of currents were not analysed and only the main conductance currents were analysed in the present study. The burst was defined as repeated openings separated by a closure no longer than 5 ms as determined by the method of Colquhoun & Sakmann (1985).

Drug application

For the whole-cell experiments, test solutions were applied to the cell using a fast application system (Nagata & Narahashi, 1994). The application was controlled by a computer-operated magnetic valve. Using this application system, the external solution surrounding the cell could be completely changed within 100 ms. For the single-channel experiments, test compounds were dissolved in external bath solution and applied to the recording chamber.

Results

Co-application of anisatin and GABA

When the membrane potential was held at −60 mV in the normal external solution, bath application of GABA produced an inward current. At low GABA concentrations ranging from 3 – 30 μM, the inward current was maintained at a steady state

during GABA application. At higher GABA concentrations, the current comprised two components, an initial transient current and a lower sustained current. The GABA-induced inward current of rat DRG neurons was previously shown to be carried by chloride ions through open chloride channels (Ogata *et al*., 1988). Responses evoked by low concentrations of GABA were maintained at a stable level over a period of up to 60 min after rupture of the membrane (data not shown) indicating that no desensitization of the receptor occurred under these experimental conditions.

The currents induced by 30 μM GABA were suppressed when 1 μM anisatin was co-applied (Figure 1a). Figure 1b shows the time course of the changes in peak current amplitude during repeated co-applications of GABA and anisatin. The peak current amplitude gradually decreased to 58.3% of the control during repeated co-applications. No recovery of currents was observed after washout with anisatin-free solution (Figure 1a and b).

Concentration-response relationship for anisatin suppression

The concentration-response relationship for suppression of GABA-induced currents by co-application with anisatin is shown in Figure 2. The currents induced by 5 s co-applications of 30 μM GABA and anisatin were measured when they reached a steady-state level after repeated co-applications.

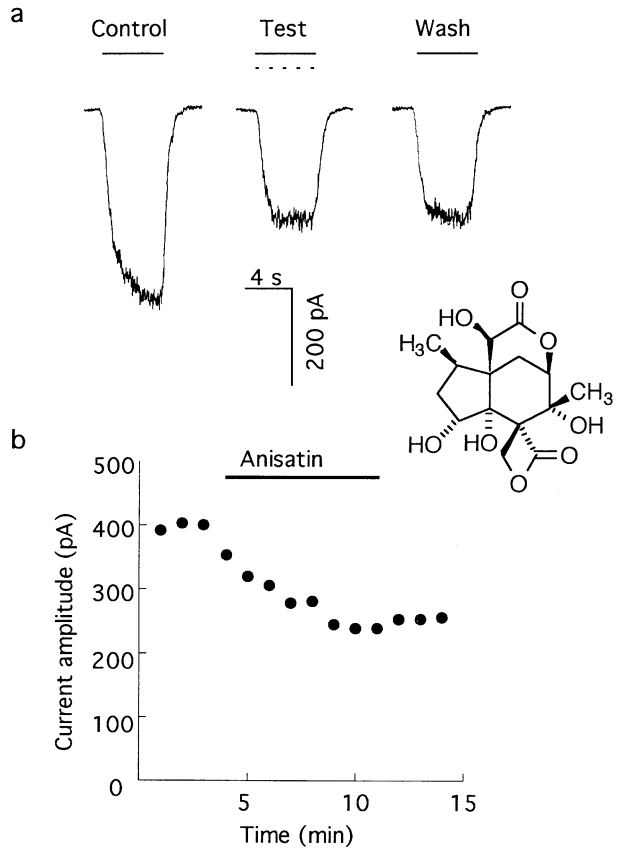

Figure 1 Suppression of GABA-induced currents by co-application of anisatin and GABA, and the structure of anisatin. (a) Current records in response to 5 s application of 30 μM GABA with and without anisatin. GABA alone (solid bar); co-application of GABA and anisatin (broken bar). (b) Time course of changes in peak current amplitude by repeated applications of GABA alone and co-applications of GABA and anisatin (solid line). The peak amplitude of current gradually decreased during repeated co-applications. No recovery was observed after washing with anisatin-free solution.

Each cell was tested for the degree of suppression caused by various concentrations of anisatin. Anisatin suppressed GABA-induced currents in a concentration-dependent manner with an EC_{50} estimated to be 1.10 μM and Hill coefficient 0.75 ± 0.13 ($n = 4-8$).

Effects of anisatin pre-application on GABA-induced current

To examine whether anisatin inhibition of GABA-induced currents requires the GABA-induced opening of channels, experiments were carried out by pre-application of anisatin without GABA. Application of 1 μM anisatin alone for 60 s had no effect on GABA-induced currents after washout of anisatin (Figure 3a and b). The ratio of current amplitudes after/before application of anisatin was calculated to be 104.2 ± 3.2 ($n = 5$). The results indicate that the anisatin-induced suppression of GABA-induced currents requires conformational changes associated with the opening of channels.

Time-dependent block by anisatin

The experiments shown in Figures 1 and 3 indicate that anisatin blocks GABA-induced currents only when the channels are opened repeatedly. This suggests that anisatin will suppress the current as induced by a prolonged single co-application of GABA and anisatin. This was actually the case as shown in Figure 4. When 1 μM anisatin and 30 μM GABA were co-applied for 30 s, the falling phase of current was accelerated, exhibiting current suppression only after a prolonged co-application. Similar results were obtained in three other experiments. When the falling phases of these currents were fitted to a single exponential with a constant residual, the time constant was shortened by 1 μM anisatin from 24.6 ± 8.6 to 17.9 ± 5.4 s (Figure 4b, $n = 4$). It is suggested that anisatin accelerates the desensitization of GABA-induced current, and that channel opening facilitates the anisatin suppression.

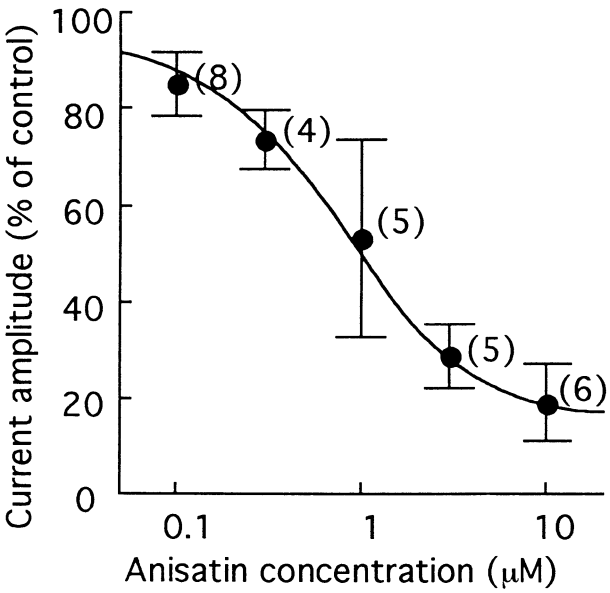

Figure 2 Concentration-response relationship for the suppression of GABA-induced peak currents by co-application of anisatin. Currents were induced by 5 s applications of 30 μM GABA, and suppressed by anisatin in a concentration-dependent manner with an EC_{50} of 1.10 ± 1.40 μM. The Hill coefficient was estimated to be $0.75 + 0.13$. Mean $\pm$ s.d. with the numbers of experiments in parentheses.

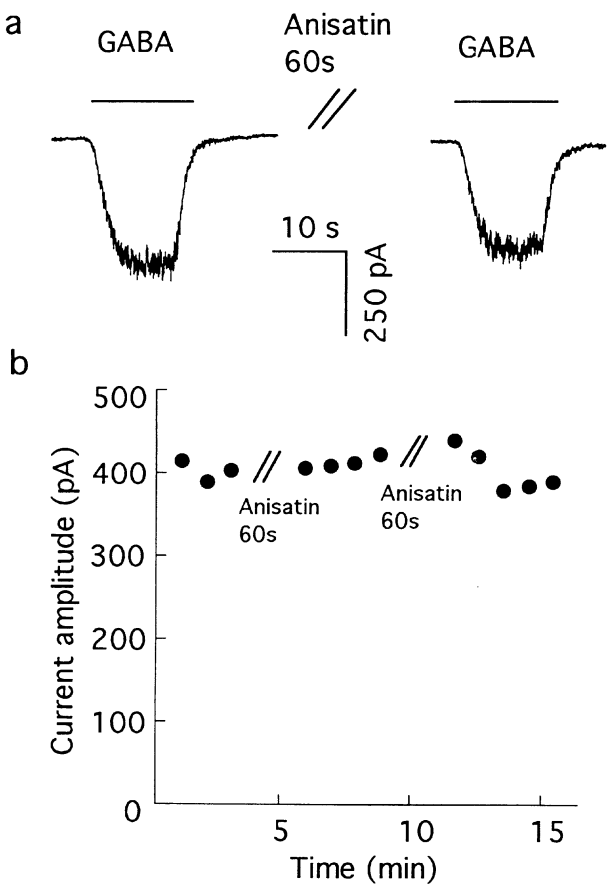

Figure 3 Pre-application of anisatin alone does not suppress the 30 μM GABA-induced currents. (a) Current records before and after pre-application of 1 μM anisatin alone for 60 s. (b) Time course of the changes in current amplitude during the experiment shown in (a). The ratios of after (pre-application of anisatin)/before (control) is 104.2 ± 3.2 ($n = 5$).

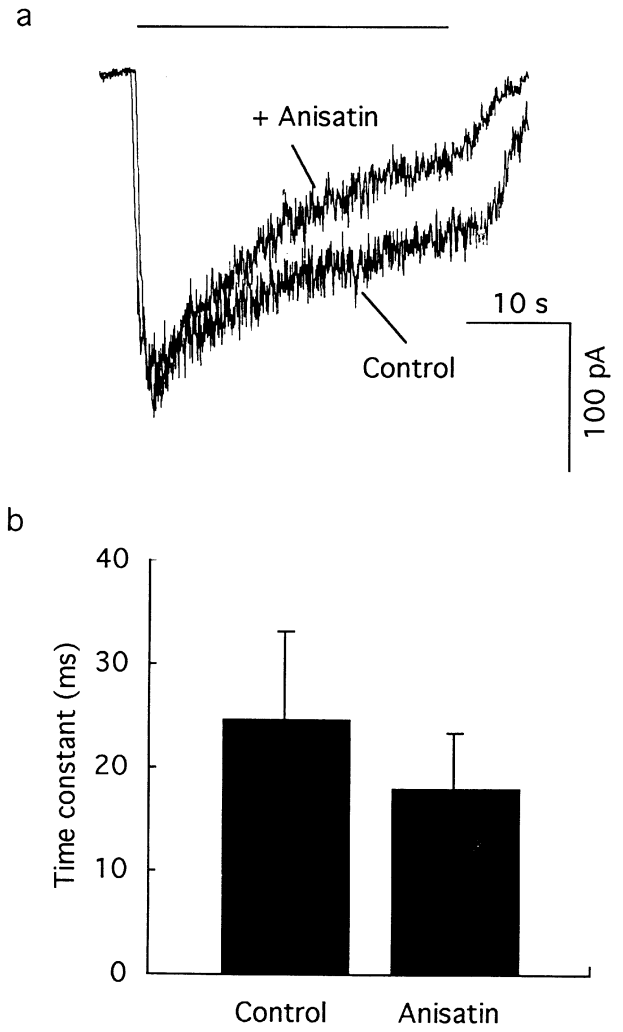

Figure 4 Prolonged co-application (30 s) of 1 μM anisatin and 30 μM GABA. (a) Currents induced by GABA alone and co-application of GABA and anisatin. Co-application caused accelerated desensitization of current. (b) The time constant of desensitization was shortened by 1 μM anisatin from 24.6 ± 8.6 to 17.9 ± 5.4 s ($n = 4$).

Anisatin effect on whole-cell current-voltage relationship

Whole-cell current-voltage relationships were constructed by applying voltage step pulses during application of 30 μM GABA or co-application of 30 μM GABA plus 1 μM anisatin. Although anisatin reduced the amplitude of GABA current in all cells examined, no change was observed in the shape of the current-voltage relationship over the voltage range of +30 to −120 mV (four cells) (Figure 5). When the data were normalized, the current-voltage relationships were virtually superimposable (data not shown). The reversal potentials in the absence and presence of anisatin were estimated to be -7.5 ± 13.3 and 0.0 ± 12.75 mV, respectively (mean ± s.d., four cells). Thus, anisatin suppression of GABA-induced current was not voltage dependent.

Interaction of anisatin and picrotoxinin

Previous studies suggested that the apparent neuropharmacological actions of anisatin were similar to those of picrotoxin (Curtis *et al*., 1973; Kudo *et al*., 1981; Shinozaki *et al*., 1981; Walker & Roberts, 1984; Scott & Duce, 1987). Thus, the site of action of anisatin may be the same as that of picrotoxin. In order to test this hypothesis, competition experiments were performed using picrotoxinin (Figure 6). Picrotoxin is an equimolar mixture of picrotin and picrotoxinin. In the present study, we used picrotoxinin because the effects of picrotoxin are mainly ascribed to picrotoxinin (Yoon *et al*., 1993). Anisatin was used at a concentration of 1 μM which was close to its EC_{50} value (see Figure 2). When co-applied with 30 μM GABA, picrotoxinin suppressed the current in a concentration-dependent manner (Figure 6, closed circles). Picrotoxinin suppressed GABA-induced currents in a concentration-dependent manner with an EC_{50} value of 0.42 ± 0.04 μM and Hill coefficient of 0.89 ± 0.05 ($n = 5$). Co-application of picrotoxinin and anisatin suppressed GABA-induced currents in a concentration-dependent manner with an EC_{50} of 0.38 ± 0.21 μM and Hill coefficient of 0.52 ± 0.12 ($n = 5$) (Figure 6, open circles). When low concentrations of picrotoxinin (0.03–0.3 μM) were co-applied with 1 μM anisatin, GABA-induced currents were suppressed to about 50% of the level achieved in the presence of picrotoxinin alone, indicating no interaction between picrotoxinin and anisatin (Figure 6, open circles). However, when the picrotoxinin concentration was high (3–10 μM), the suppressive effect was similar to that achieved by the co-application of picrotoxinin and 1 μM anisatin (Figure 6, open circles). Thus it was concluded that anisatin and picrotoxinin shared a common binding site on the GABA receptor-channel complex.

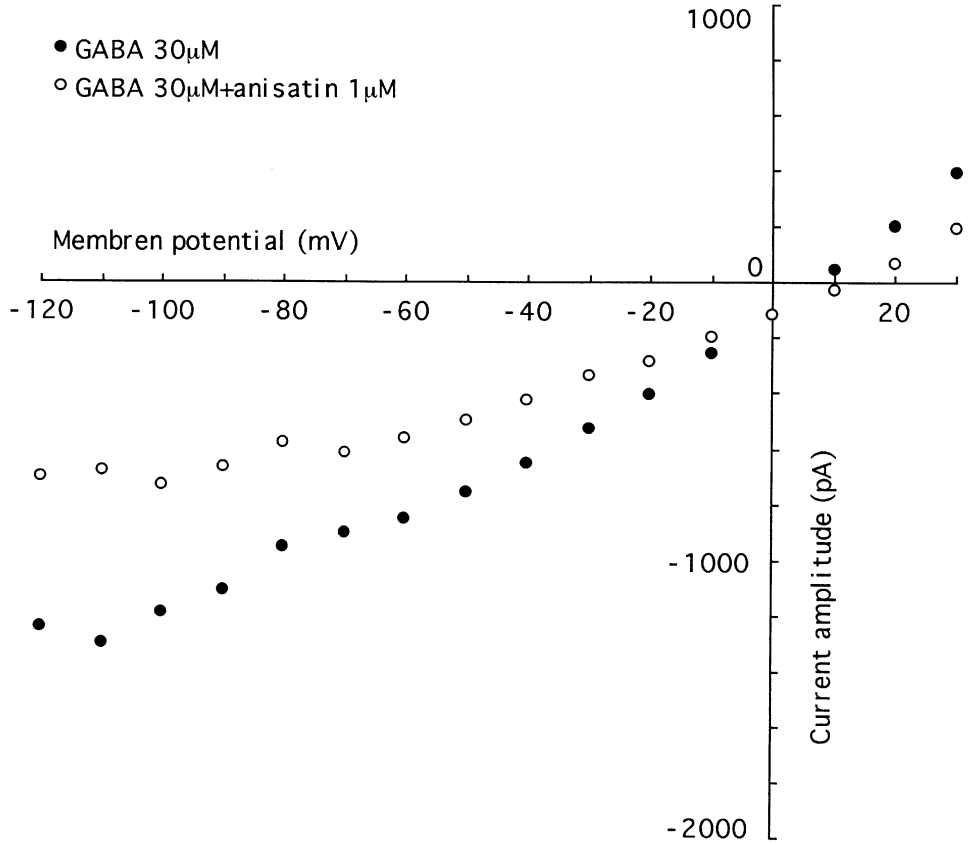

Figure 5 GABA-induced whole-cell current-voltage relationships with 30 μM GABA alone (●) and co-application of 30 μM GABA and 1 μM anisatin (○) at different holding membrane potentials. The reversal potentials in the absence and presence of anisatin were estimated to be -7.5 ± 13.3 and 0.0 ± 12.8 mV, respectively (mean $\pm$ s.d., $n=4$). Anisatin suppression of GABA-induced currents is voltage-independent.

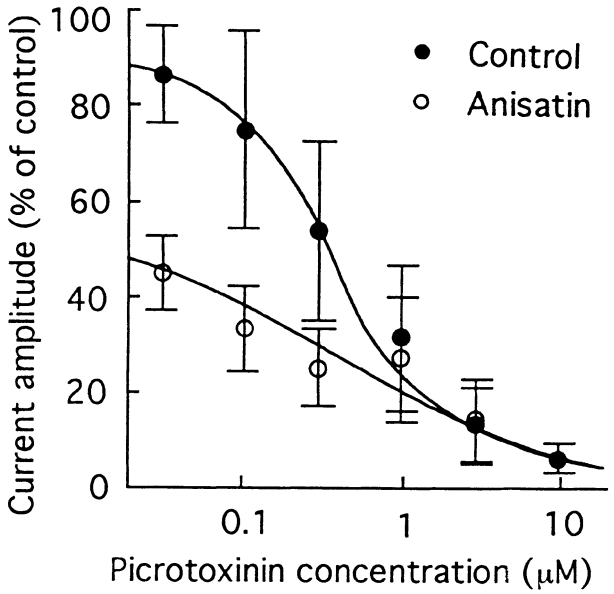

Figure 6 The effect of 1 μM anisatin on picrotoxinin suppression of 30 μM GABA-induced currents. Picrotoxinin suppressed GABA-induced currents in a concentration-dependent manner with an EC_{50} of 0.42 ± 0.04 μM and a Hill coefficient of 0.89 ± 0.05 ($n=5$). When the concentration of picrotoxinin was low (0.03–0.3 μM), anisatin suppressed the currents to about 50% of the level achieved by picrotoxinin alone. However, at higher concentrations of picrotoxinin (1–10 μM), anisatin did not suppress the current beyond the level achieved by picrotoxinin alone indicating that anisatin and picrotoxinin share a common binding site ($n=3$–7).

Binding experiments

The validity of the aforementioned conclusion was further demonstrated by binding experiments using [^{3}H]-EBOB, a high-affinity radioligand for the picrotoxinin binding site of GABA receptor channels. Anisatin inhibited specific [^{3}H]-EBOB binding to rat brain membranes, with an IC_{50} value of 0.43 μM. Scatchard analysis of this inhibition over a wide range of [^{3}H]-EBOB concentration indicated that anisatin bound to the picrotoxinin site (Figure 7).

Single-channel currents recorded from excised membrane patches

In outside-out membrane patches held at a holding potential of -60 mV, channel openings were rarely observed in the absence of GABA (data not shown). Following application of 10 μM GABA to the bath, inward single-channel currents occurred either singly or in the form of bursts (Figure 8a). Currents evoked by GABA from a membrane patch were often reproducible and could be evoked for as long as 20 min. Occasionally the current activity decreased significantly or stopped after repeated applications of 10 μM GABA. Only membrane patches with reproducible activity were used for the kinetic analysis. GABA-induced currents are represented by the main conductance state and subconductance state currents (Sakmann *et al*., 1983; Bormann *et al*., 1987; Macdonald *et al*., 1989; Macdonald & Twyman, 1992; Ma *et al*., 1994). The subconductance state currents occurred much less frequently

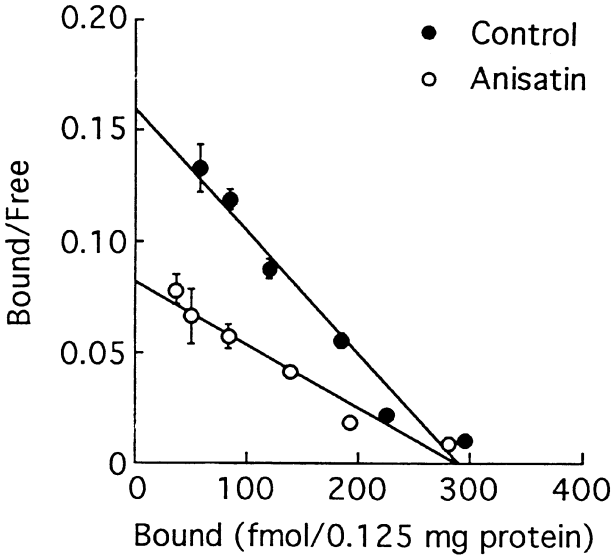

Figure 7 Scatchard plot of [3H]-EBOB binding to rat brain membranes in the absence and presence of 370 nM anisatin.

than the main conductance state currents. The main conductance was estimated to be 26 pS, and a smaller subconductance state current had a conductance of about 19 pS.

Effects of anisatin on GABA-induced single-channel currents

Single-channel currents in the presence of 10 μM GABA and 1 μM anisatin are illustrated in Figure 8b. The mean value for the main conductance in the presence of 10 μM GABA plus 1 μM anisatin was estimated to be 26 pS which was not significantly different from the corresponding value in the presence of 10 μM GABA alone. Subconductance state currents were observed only rarely. Thus, anisatin does not affect the current amplitude and the distribution of conductance states.

Open time distributions for GABA-induced currents in the absence and presence of anisatin are shown in Figure 9. The time axis is drawn on the logarithmic scale so that the effective bin width increases exponentially from left to right. This displays a multi-exponential distribution as a series of skewed bell-shaped curves whose peaks overlie the time constants of

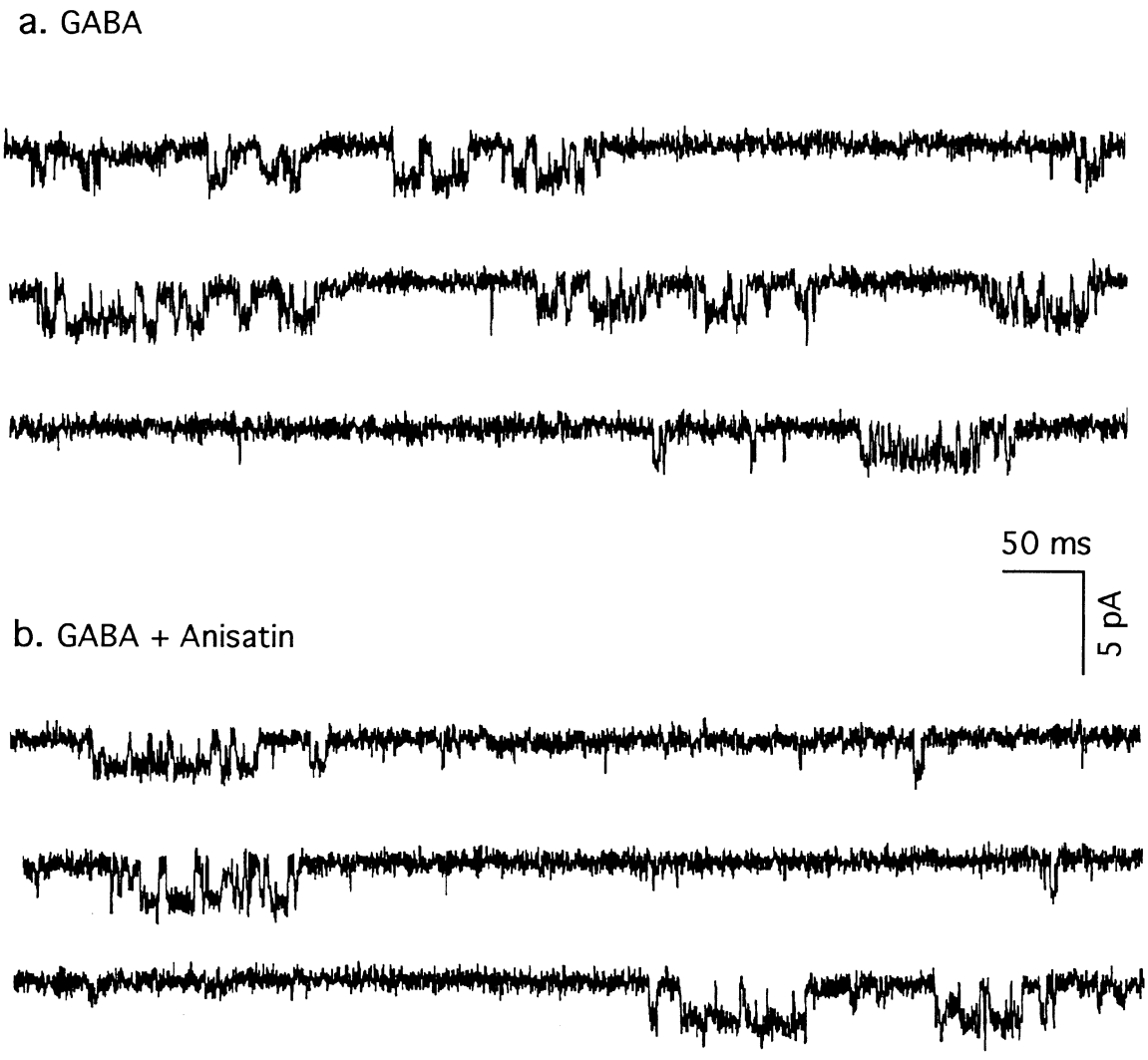

Figure 8 Single-channel currents induced by application of 10 μM GABA and co-application of 10 μM GABA and 1 μM anisatin to outside-out membrane patches clamped at a membrane potential of -60 mV. Currents were filtered at 1 kHz. (a) Currents induced by 10 μM GABA occurred during brief isolated openings or during longer openings interrupted by short closures or gaps. (b) Currents induced by co-application of GABA and anisatin.

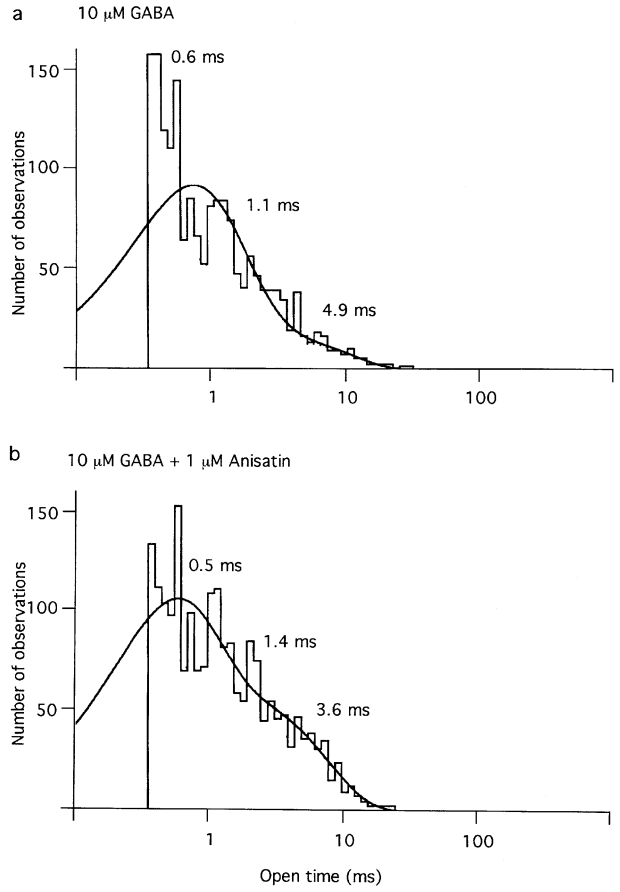

Figure 9 Open time distributions for currents induced by 10 μM GABA and co-application of 10 μM GABA and 1 μM anisatin. The best fit of three exponential functions is shown. Three time constants in GABA and in GABA plus anisatin are given in (a) and (b), respectively. See text for further explanation.

several exponential components (Sigworth & Sine, 1987). The open time distributions of main conductance currents in the presence of 10 μM GABA (Figure 9a) and in the presence of 10 μM GABA plus 1 μM anisatin (Figure 9b) clearly indicate multi-exponential components. There were at least three components. The slowest component of the open time distribution for 10 μM GABA-induced currents had a time constant of 4.9 ms (13.0% of total 1834 events), the next component had a time constant of 1.1 ms (30.8%), and the fastest component had a time constant of 0.6 ms (56.2%). The mean open time was estimated to be 2.1 ms. For the open time distribution of currents induced by co-application of 10 μM GABA and 1 μM anisatin, the slowest component had a time constant of 3.6 ms (27.8% of total 2104 events), the next component had a time constant of 1.4 ms (14.6%), the fastest component had a time constant of 0.5 ms (57.6%), and the mean open time was 2.2 ms. These results indicate that anisatin does not change the open time parameters.

The closed time distributions for the main conductance state currents in the presence of 10 μM GABA and of 10 μM GABA plus 1 μM anisatin clearly show multi-exponential components (Figure 10). There were at least four components. The slowest component of the closed time distribution for 10 μM GABA-induced currents had a time constant of 37.0 ms (12.7% of total 2027 events), the second slowest component had a time constant of 3.6 ms (10.7%), the third slowest component had a time constant of 1.1 ms (15.1%), and the fastest component had a time constant of 0.2 ms (61.5%). The mean closed time was estimated to be 14.3 ms (Figure 10a). For the close time distribution of the currents in the presence of 10 μM GABA and 1 μM anisatin, the time constants of the four components were 43.8 ms (18.4% of total 2128 events), 13.7 ms (13.8%), 1.4 ms (19.0%), and 0.2 ms (48.7%); the mean closed time was 19.5 ms (Figure 10b). Thus, the two slower time constants were much longer in the presence of anisatin than in the control. The mean closed time in anisatin (19.5 ms) was also longer than that in the control (14.3 ms).

The distributions of burst durations of the main conductance state currents in 10 μM GABA and in 10 μM GABA plus 1 μM anisatin clearly show multi-exponential components (Figure 11). The burst was defined as repeated openings separated by a closure no longer than 5 ms. There were at least three components. The slowest component for 10 μM GABA-induced main conductance state currents had a time constant of 37.5 ms (8.7% of total 626 events), the next component had a time constant of 8.4 ms (23.8%), and the fastest component had a time constant of 0.3 ms (67.5%) (Figure 11a). The mean burst duration was 9.7 ms. The mean number of openings per burst was 4 and the mean open time within a burst was 0.8 ms. For the currents induced by co-application of 10 μM GABA and 1 μM anisatin, the slowest component of bursts had a time constant of 18.7 ms (18.0% of total 1003 events), the next component had a time constant of 3.6 ms (19.7%), and the fastest component had a time constant of 0.4 ms (62.3%); the mean burst duration was 6.6 ms (Figure 11b). The mean number of openings per burst was 2 and the mean open time within a burst was 0.8 ms. Thus, two slower time constants were smaller in anisatin than in the control. The mean burst duration in GABA plus anisatin (6.6 ms) was shorter than that

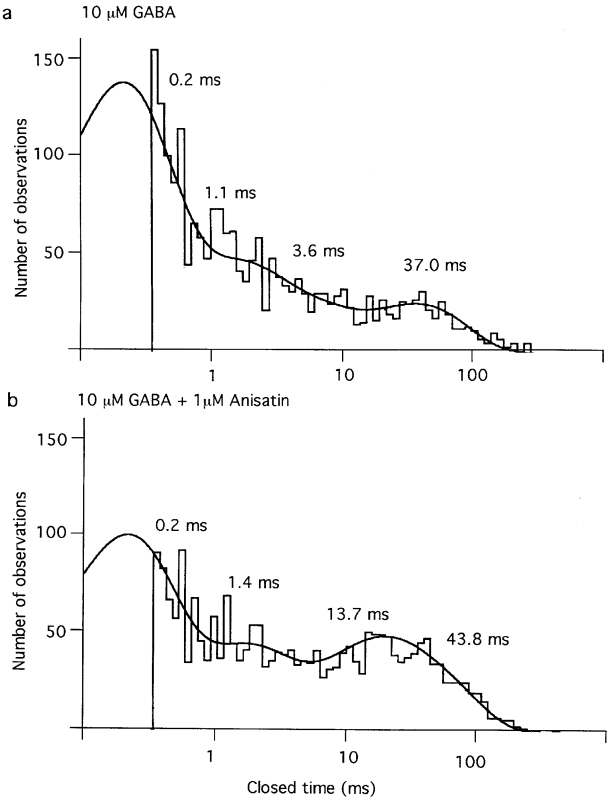

Figure 10 Closed time distributions for currents induced by 10 μM GABA and co-application of 10 μM GABA and 1 μM anisatin. The best fit of four exponential functions is shown. Four time constants in GABA and GABA plus anisatin are give in (a) and (b), respectively. See text for further explanation.

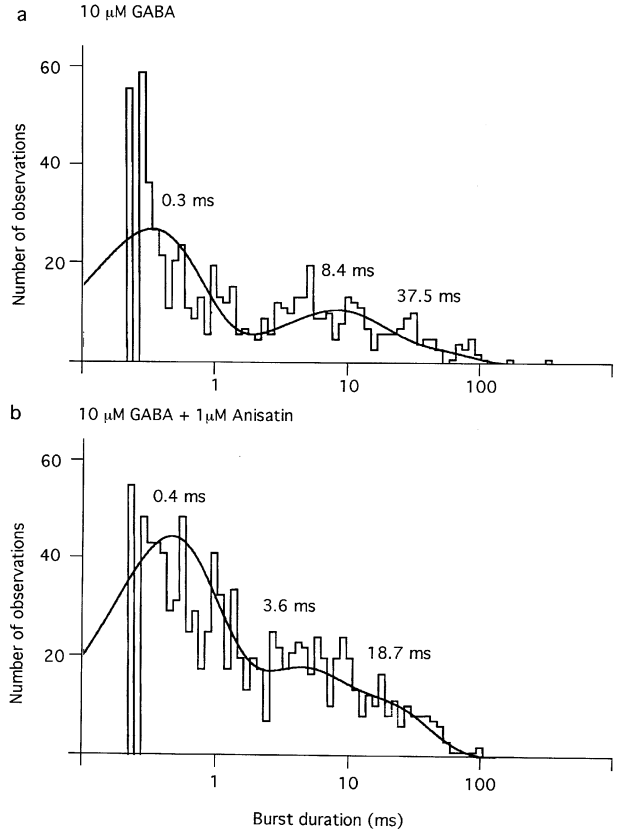

Figure 11 Distributions of burst durations for currents induced by 10 μM GABA and co-application of 10 μM GABA and 1 μM anisatin. The burst was defined as repeated openings separated by a closure no longer than 5 ms. The best fit of three exponential functions is shown. Three time constants in GABA and GABA plus anisatin are given in (a) and (b), respectively. See text for further explanation.

in the control (9.7 ms). The mean open time within a burst in anisatin was similar to that of the control. However, the mean burst duration was decreased by anisatin because openings per burst were much reduced by anisatin.

Discussion

Anisatin suppression of GABA-induced current

Anisatin is known to be a noncompetitive GABA antagonist (Kudo *et al*., 1981; Shinozaki *et al*., 1981; Walker & Roberts, 1984; Scott & Duce, 1987). When anisatin was repeatedly co-applied with GABA for 5 s, the current was suppressed gradually (Figure 1). No recovery from suppression was observed after a prolonged washout with anisatin-free solution. The pre-application of anisatin alone did not suppress the currents as tested by subsequent GABA applications (Figure 3). During anisatin suppression, the desensitization of GABA-induced current was accelerated (Figure 4). These results indicate that the anisatin suppression of GABA-induced currents was use-dependent, being accelerated by frequent openings of the channels.

Site of action of anisatin

The GABA receptor-channel complex comprises several binding sites including those for GABA, barbiturates, benzodiazepines, and picrotoxin (Olsen *et al*., 1991). In the present experiment, interactions were clearly demonstrated between anisatin and picrotoxinin (Figure 6). Anisatin did not cause additional suppression of GABA-induced currents in the presence of high concentrations of picrotoxinin. These data lead to the conclusion that anisatin and picrotoxinin share a common binding site. This conclusion is also supported by [^{3}H]-EBOB binding experiments (Figure 7). The picrotoxin block of GABA-induced currents is use-dependent and voltage-independent (Newland & Cull-Candy, 1992; Yoon *et al*., 1993). The site of picrotoxin block is exposed by the conformational change initiated by GABA binding to the receptor (Yoon *et al*., 1993). Anisatin block of GABA-induced currents was also use-dependent and voltage-independent. It is likely that the anisatin and picrotoxinin binding site is associated with or allosterically linked to the chloride channel of the GABA receptor.

Single-channel experiments

Single-channel currents were recorded from the GABA receptor using outside-out membrane patches excised from rat DRG neurons. Channel openings with at least two conductance state currents were detected in the present study. A larger component (main conductance state current) has a conductance of about 26 pS in symmetrical chloride concentrations outside and inside the membrane. A smaller component (subconductance state current) has a conductance of about 19 pS. The main conductance state currents were observed more frequently and analysed in the present study. The amplitude of main conductance state current was not altered by anisatin.

Three exponential functions were required to fit the frequency histogram of open time of GABA-activated channels, suggesting that the channel can be in at least three open states (O1, O2 and O3). In the presence of anisatin and GABA, three open time constants and the mean open time were not significantly different from those in the presence of GABA alone. Multiple closed time constants were found suggesting the presence of multiple closed states. Four exponential functions were required to fit the frequency histogram of closed time, suggesting that the channel can be in at least four closed states. The two briefest time constants may be associated with a closed but ligand bound receptor (Macdonald & Twyman, 1992), and were not changed after application of anisatin. In the presence of GABA and anisatin, two other slower time constants became longer compared to those in GABA alone. This is consistent with the observed increase in the mean closed time. Thus, anisatin prolongs slower closed times indicating that the anisatin suppression of GABA-induced whole-cell currents is caused in part by reducing the frequency of channel openings.

The frequency histograms for burst durations with and without anisatin could be fitted by three exponential functions (B1, B2 and B3), suggesting that the channel can be in at least three burst states. Since the B1 state time constant was similar to that of the O1 state, the B1 state appears to be produced by a single opening to the O1 state. B2 and B3 were composed of two or more openings to O2 and O3 states. The burst duration was shortened by anisatin. The mean open time within a burst was not significantly different between the control and anisatin. However, the B2 and B3 time constants were shorter in the presence of GABA and anisatin than in the presence of GABA alone, because the mean closing per bursts was decreased by anisatin. Thus, the burst duration shortened by anisatin could be accounted for by the decrease in the mean number of openings per burst.

Several single-channel experiments have been performed for picrotoxin. Picrotoxin shortens bursts by reducing the mean duration and the number of intraburst openings (Twyman *et al*., 1989). Picrotoxin has little or no effect on open time and burst duration but reduces the frequency of channel openings (Newland & Cull-Candy, 1992). We have also recently found that picrotoxinin does not alter open time and burst duration but prolongs closed time (Ikeda *et al*., 1998). In our experiments, the mechanisms of prolongation of closed time and shortening of burst duration caused by anisatin may be similar to those caused by picrotoxin. Some discrepancies in the past studies may be due to different experimental conditions, different sources of the GABA receptor, and/or different combinations of the GABA receptor subunits.

Penicillin is known as an open channel blocker of the GABA receptor-channel complex (Chow & Mathers, 1986). In single-channel experiments, penicillin reduced the mean channel open time and increased the mean burst duration without altering the single-channel conductance (Twyman *et al*., 1991). In contrast, anisatin did not change the mean channel open time and decreased the mean burst duration in the present study. Thus, both anisatin and penicillin appear to be open channel blockers, but their mechanisms of action on the GABA receptor-channel complex are dissimilar.

The insecticide dieldrin acts on the $GABA_A$ system. It has been demonstrated that dieldrin and picrotoxin share a common binding site (Matsumura & Ghiasuddin, 1983; Eldefrawi & Eldefrawi, 1987; Bloomquist *et al*., 1992; Nagata & Narahashi, 1994). Noise analysis conducted by Bermudez *et al*. (1991) with cockroach neurons has shown that dieldrin decreases the frequency of GABA channel openings without changing the mean open time. Thus, there are similarities in the actions of dieldrin and anisatin on the GABA system. More detailed analyses of these two chemicals on the $GABA_A$ receptor-channel complex are warranted.

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