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A discriminant block among K^+ channel types by phenytoin in neuroblastoma cells

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1 The action of the anticonvulsant drug phenytoin on K^+ channels was investigated in neuroblastoma cells (N2A) by using the single-channel patch-clamp technique.

2 N2A cells expressed three types of delayed rectifier K^+ channels, which were found to have a conductance of 10–20 pS in a 'physiological' K^+ gradient. When added to the external solution at concentrations ranging between 1 and 200 μM, phenytoin decreased single channel activity, whereas the unitary current amplitude was unaffected in all three types of channels.

3 The open probability of the biggest channel decreased, according to an exponential distribution of open and closed times, from 40% in control conditions to 10% in the presence of 50 μM phenytoin (Vm = 40 mv). The reduction in the open-channel probability was concentration-dependent with a $IC_{50} = 27.2 \pm 0.9$ μM.

4 A transient type of K^+ channel was identified that was affected by cumulative inactivation and had a conductance of a mean value equal to 26 pS. Finally, a voltage-and Ca^{2+}-dependent K^+ channel with a unitary conductance of 95 pS was recorded. Both the channel's amplitude and kinetics were unaffected by phenytoin.

5 These results confirm the phenytoin effect on K^+ currents and suggest that the drug may be considered a selective blocker of delayed rectifier K^+ channels.

Keywords: Anticonvulsant; phenytoin; K^+ channels; rat neuroblastoma cells; single channel

Introduction

The anticonvulsant phenytoin is widely used in the treatment of generalized to tonic-clonic seizures and partial seizures (for a review, see Rogawski & Porter, 1990). The effectiveness of phenytoin as an anticonvulsant is probably related to its ability to inhibit high-frequency action potential firing (Yaari *et al*., 1986; MacDonald, 1989). It has been demonstrated that phenytoin can interact with the voltage-dependent Na^+ channels that are responsible for the action potential upstroke in a specific voltage- and frequency-dependent manner (Ragsdale *et al*., 1991; Kuo & Bean, 1994). In addition to its effects on Na^+ channels, phenytoin can produce blocks of Ca^{2+} and K^+ channels (Yaari *et al*., 1987; Twombly *et al*., 1988; Nobile & Vercellino, 1997). Recently, we simulated the electrical behaviour of a neuron, including Ca^{2+} and various K^+ conductances, to test the ability of the anticonvulsant drugs to reduce high-frequency action potential firing. The results indicate that the drugs action on Ca^{2+} and delayed rectifier K^+ conductances other than Na^+ ones is important to determine their anticonvulsant effect on neurons at clinically significant concentrations. By contrast, other K^+ conductances included in the model, like transient and Ca^{2+}-activated K^+ conductances, seem to exert a smaller or no effect on the suppression of the action potential burst induced by a reduction in Na^+ conductance (Rauch *et al*., 1997). In an attempt to confirm these results on a biological preparation and to demonstrate a possible selective effect of phenytoin on delayed rectifier K^+ channels, we analysed the influence of the drug on different types of single K^+ channels in rat neuroblastoma cells (N2A). We worked on N2A cells at the single-channel level by using the patch-clamp technique in both the outside-out and inside-out configurations. In a previous paper, we reported results obtained by whole-cell experiments in which it was impossible to detect the transient K^+ current because repetitive stimulations at intervals shorter than 10 s produced cumulative channel inactivation and the Ca^{2+}-activated K^+ current was nullified by very low intracellular free Ca^{2+} concentration (Bertoli *et al*., 1996; Nobile & Vercellino, 1997). This allowed a better characterization of the effect of phenytoin on the delayed rectifier K^+ component. The data reported in the present paper show that N2A cells are characterized by three types of delayed rectifier K^+ channels, a transient K^+ channel and at least one type of Ca^{2+}-activated K^+ channel; they all showed differences in biophysical properties and pharmacological sensitivity (Prestipino *et al*., 1993).

Methods

Cell cultures

Rat neuroblastoma cells (N2A) were grown in MEM medium (Sigma Chemical Company, St. Louis, MO, U.S.A.) supplemented with 10% foetal calf serum and 1% L-glutamine and kept at 37°C in a 5% CO_2 incubator, as previously described (Nobile & Vercellino, 1997).

Solutions

The composition of the standard external solution was the following (mM): NaCl 120, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, glucose 20 and HEPES 10. Na^+ and Ca^{2+} currents were nullified by adding 0.3 μM tetrodotoxin (TTX) and 50 μM Cd^{2+} to the bathing solution, respectively. The standard internal solution was (mM): KCl 120, EGTA 5, $CaCl_2$ 0.2, glucose 30 and HEPES 10 (5 nM free Ca^{2+}). As an alternative, the

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concentration of free Ca^{2+} in the solution bathing the intracellular surface of the membrane, $[Ca^{2+}]_i$, was 0.5 μM and was obtained by adding 4 mM Ca^{2+}. The pH was adjusted to 7.3 with NaOH and KOH for the external and internal solutions, respectively and osmolarity was set to 290 ± 10 mOsmol with mannitol.

Phenytoin ($C_{15}H_{11}N_2O_2Na$: Sigma) was applied by using a gravity perfusion system (≈ 1 ml min^{-1} flow). Every day stock solutions of phenytoin and ethanol were prepared by which the final concentrations were reached (Nobile & Vercellino, 1997). The highest ethanol concentration was $<0.5\%$. Control experiments showed that, at this concentration, ethanol did not affect the ionic membrane currents. The perfusion system tubings were treated with Sigmacote (Sigma) to minimize contamination. All the experiments were carried out at room temperature (20–22°C).

Electrophysiological measurements

Unitary channel currents were recorded by using the patch-clamp technique in both the outside-out and inside-out configurations, as in our previous studies (Acerbo & Nobile, 1994). Borosylicate glass electrodes were pulled and calibrated to have a tip resistance of 5–10 MΩ when filled with the aforesaid solutions. Current records were filtered at 3 kHz through an 8-pole low-pass Bessel filter. Voltage stimulation and data acquisition were performed by an IBM compatible 80486 personal computer through a 16-bit interface (Axon Instruments Inc., Foster City, Ca, U.S.A.). Currents were sampled at a frequency of 100 μs per point. Fast capacitance transient compensation was performed during the experimental procedure. Current traces were analysed by using Pclamp 5.7.1 (Axon Instruments Inc.) and Sigma Plot 1.02 (Jandel Scientific, Erkrath, Germany) software. Single-channel records were corrected for leakage by subtracting the average of null sweeps from all sweeps displaying channels recorded by using the same patch parameters. Data are given as mean values $\pm$ s.d., the Student's *t*-test for paired data was performed and $P \leqslant 0.01$ was regarded as being significant.

Results

In neuroblastoma cells (N2A) we found at least three distinct populations of K^+ channels characterized by differences in biophysical properties and pharmacological sensitivity. One of these, the family of delayed rectifier K^+ channels, could also be subdivided into three main kinetically distinct types. The sensitivities of K^+ channels to external applications of tetraethylammonium (TEA), 4-aminopyridine (4-AP) and Charybdotoxin (CTX) were examined. External application of 5 mM TEA reversibly blocked all types of K^+ channels. An application of 1 mM 4-AP drastically reduced the frequency of openings of the transient K^+ channel without affecting the single-channel current amplitude. CTX at 30 nM concentration appeared to reduce the activity of the Ca^{2+}-activated K^+ channel.

Phenytoin effect on delayed rectifier K^+ channels

Figures 1–3 illustrate the sensitivity to phenytoin of a type of delayed rectifier K^+ channel. Figure 1A shows representative leak-subtracted unitary-current traces in control conditions, in the presence of extracellular 50 μM phenytoin and after wash-out of the drug. Traces were recorded consecutively, during a series of 200 ms long depolarizing pulses to 40 mV from a holding potential (HP) of -50 mV. Pulses were repeated every 5 s and phenytoin was perfused during stimulation. Phenytoin decreased the single-channel activity, whereas the unitary-current amplitude was unaffected. This blocking effect of the drug was not completely reversed by 4-min perfusion of the external membrane with standard solution. In Figure 1B, single-channel current values are given that were induced by depolarizing potentials ranging between 0 and 60 mV from an HP of -50 mV in control conditions. The unitary slope conductance was 18 ± 1 pS ($n=4$). At 40 mV membrane potential the channel open probability (P_o) was 0.4 ± 0.05 and the mean open- and close-times were 11.7 ± 1.7 and 17.9 ± 4 ms, respectively. The distribution of channel open-time durations of the cells in control conditions and after application of phenytoin displayed time courses best fitted by a single exponential function τ_o, whereas the closed durations required at least two exponential functions (time constants τ_{c1} and τ_{c2}). These and the following analyses of the channel kinetics were made, if not otherwise stated, on data obtained by voltage pulses to 40 mV from an HP of -50 mV. From Figure 2, it appears evident that the presence of extracellular phenytoin caused a shift in the opening-time constants towards shorter durations of about 60%, whereas the closed-duration components τ_1 and τ_2 increased by about 100% and 90%, respectively ($P<0.01$). The current amplitudes were instead unaffected and distributed in a Gaussian fashion centred around values of 1.28 and 1.25 pA, respectively ($P>0.05$). The data shown are representative of four cells. Phenytoin affected the delayed rectifier K^+ channel open probability in a concentration-dependent way. Figure 3 shows the percent effects on P_o (*block*) produced by different concentrations of phenytoin at a membrane potential of 40 mV. Experimental data were fitted according to the equation:

$$P_{o\,Ctl} \quad P_{o\,PHT} \quad P_{o\,Ctl}$$

$$Block_{max}\, C^{n_H} \quad C^{n_H} \quad IC_{50}{}^{n_H} \qquad 1$$

where C is the concentration of phenytoin, IC_{50} is the concentration value producing a half-inhibition and n_H is the Hill coefficient. The best fit gave $IC_{50} = 27.2 \pm 0.9$ μM, $n_H = 2$ and a phenytoin $block_{max} = 1$. The n_H value indicates that two

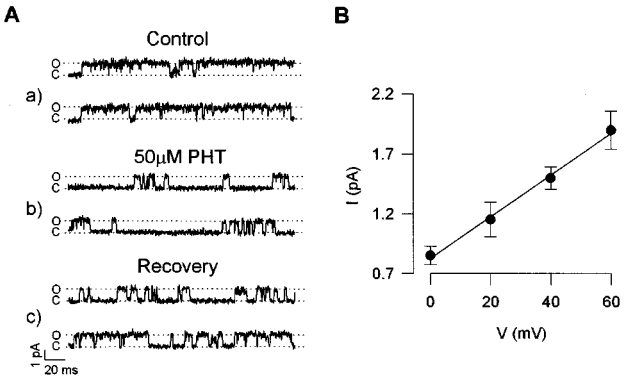

Figure 1 **(A)** Effect of extracellular application of phenytoin on delayed rectifier K^+ channels in an N2A cell. Consecutive unitary current records before addition of the drug (*a*), in the presence of 50 μM phenytoin (*b*) and after wash-out of the drug (*c*). The membrane was depolarised to 40 mV from a holding potential (HP) of -50 mV. The channel activity was not completely recovered after 4 min. (B) Current-voltage relationship (I/V) of the single channel current amplitude in control conditions. The unitary slope conductance was 18 ± 1 pS. Data points and bars are the mean $\pm$ s.d. ($n=4$) of the unitary current recorded at the indicated potentials. All records were filtered at 1 kHz.

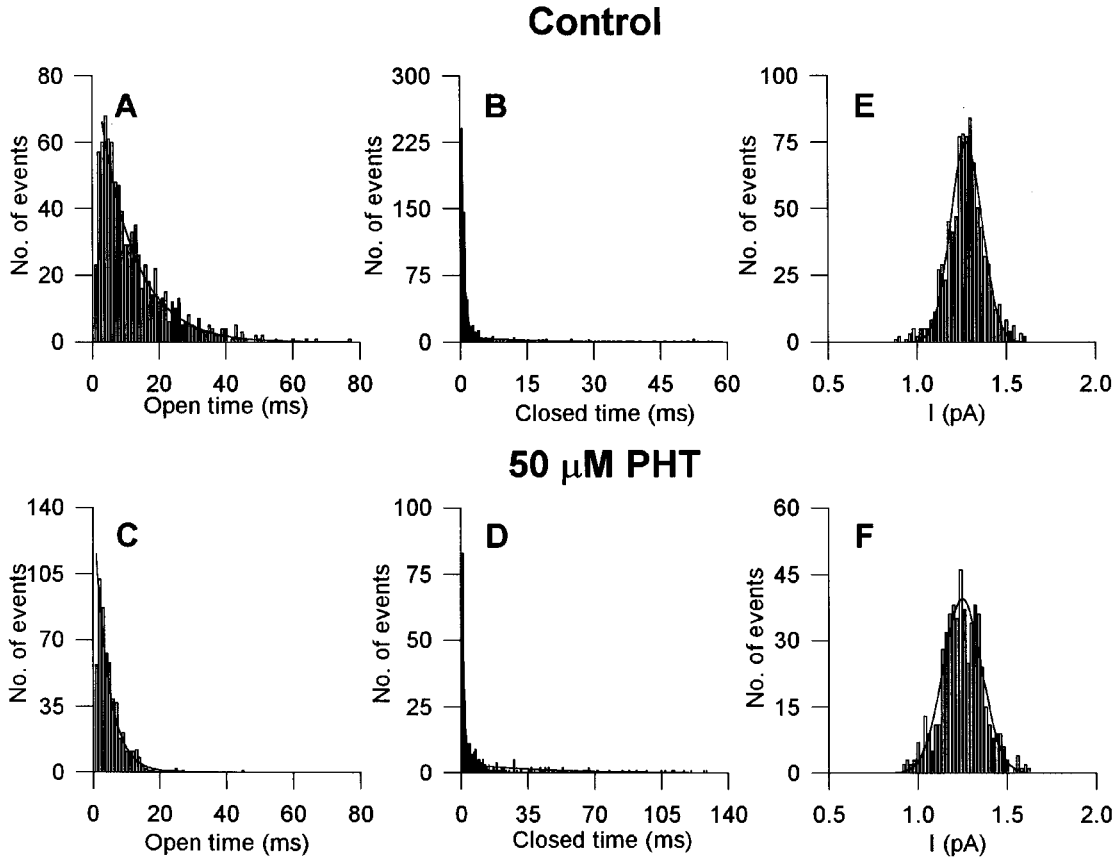

Figure 2 Effect of phenytoin on delayed rectifier K^+ channels kinetic and amplitude characteristics. Open- and closed-time distributions in control conditions (A, B) and in the presence of 50 μM phenytoin (C, D) at 40 mV. The distribution of open times was fitted by a single exponential, with $\tau_o = 10.4 \pm 1.5$ ms in control conditions and $\tau_o = 4.2 \pm 0.8$ ms in the presence of the drug. The histograms of closed times were fitted by the sum of two exponential functions. The time constants of the exponential components were $\tau_{c1} = 0.6 \pm 0.1$ ms, $\tau_{c2} = 18.3 \pm 3$ ms and $\tau_{c1} = 1.2 \pm 0.2$ ms, $\tau_{c2} = 41.2 \pm 4$ ms, respectively. (E, F) Histograms of the single-channel current amplitude from the same cell. Peaks occurred at 1.3 ± 0.9 pA in control conditions and at 1.2 ± 0.1 pA in the presence of phenytoin.

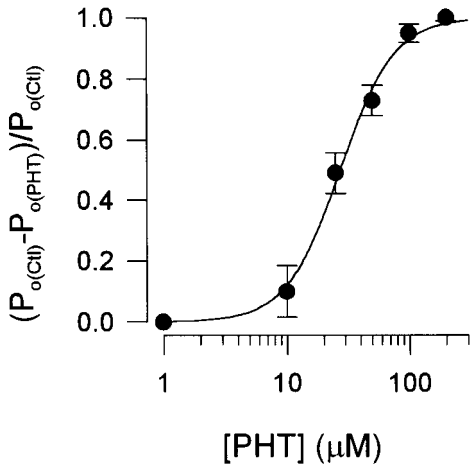

Figure 3 Concentration-dependence effect of phenytoin on open channel probability. Experimental points were obtained from an HP of -50 mV to 40 mV. Each value of $(P_{o(Ctl)} - P_{o(PHT)})/P_{o(Ctl)}$ was averaged over three to five different cells and best approximated by Equation 1. The half-effective dose was 27.2 ± 0.9 μM.

phenytoin molecules must bind to a site in order that the channels may be inhibited. Each point represents the mean value $\pm$ s.d. over three to five different cells. In Figure 4, the unitary-current traces and the I/V relationships of the other two types of delayed rectifier K^+ channels are displayed; their conductances, as assessed by I/V curve slopes, were found to be 12 ± 1 pS (●; $n = 3$) and 15 ± 1.2 pS (■; $n = 4$). Because of the high K^+ channels density in the N2A cells, it was difficult to isolate single-channel activity. In any case, different opening events were considered to be due to three types of delayed rectifier K^+ channels and not to substates of the same channel, as they were also observed separately in different patches. Moreover, the channels open probability and the mean open- and close-times were significantly different. At 40 mV the P_o of the channel with conductance of 12 pS was 0.1 ± 0.02 and the mean open- and close-times were 5.5 ± 1.1 and 116 ± 20 ms, respectively. The P_o of the channel with conductance of 15 pS was 0.7 ± 0.1 and the mean open- and close-times were 442 ± 30 and 231 ± 25 ms, respectively. Both K^+ channel types were affected by phenytoin, like the delayed rectifier K^+ channel previously described. In five records, at a 40 mV membrane potential, the presence of 100 μM phenytoin reduced the opening probability of the intermediate conductance channel from $68 \pm 10\%$ to $10 \pm 5\%$, whereas the opening probability of the smaller conductance shifted from $15 \pm 3\%$ to an undetectable value ($P < 0.01$).

Phenytoin effect on transient K^+ channels

A transient K^+ channel was detected in 30% of excised outside-out patches ($n = 125$). We found that repetitive

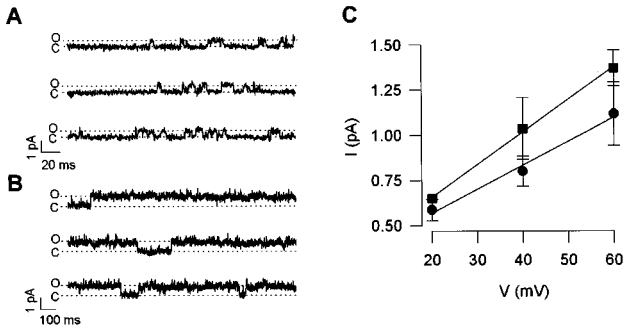

Figure 4 (A, B) Single-channel traces of two other types of delayed rectifier K^+ channels. The activity of the smaller channel (12 ± 1 pS) consisted of short open events, whereas the intermediate conductance channel (15 ± 1 pS) resided mainly in the open state, occasionally returning to the closed state. (C) Current-voltage relationships of the single-channel current amplitude in control conditions ($n=4$). Records filtered at 0.5 kHz.

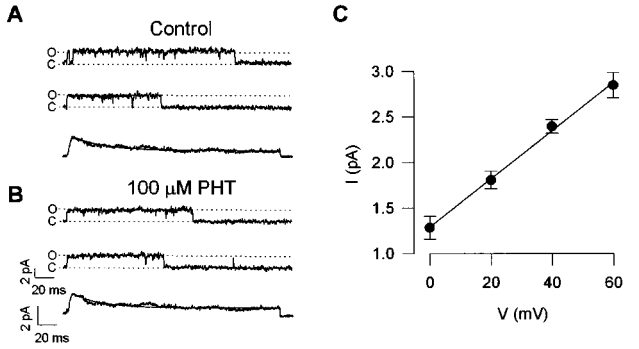

Figure 5 Effect of phenytoin on transient K^+ channels. Representative unitary current records before (A) and after addition of 100 μM phenytoin to the bath (B). The average currents resulting from the sum of 25 current traces are shown below the single-channel records. The best fits of the mean current inactivation gave time constants of 34.6 ± 2.2 ms in control conditions and of 31.5 ± 3.1 ms in the presence of phenytoin. (C) I/V relationship of the channel amplitude in control conditions. The unitary slope conductance was 26 ± 3 pS ($n=7$). Records filtered at 1 kHz.

stimulations at intervals shorter than 10 s produced a cumulative inactivation of the channel. This phenomenon could be minimized by using stimulation patterns with pulses applied every 40 s from an HP of -70 mV. Traces in control conditions and in the presence of 100 μM phenytoin together with the unitary I/V relationship in control conditions are shown in Figure 5. The reconstituted whole currents displayed at the bottoms of Figure 5A and Figure 5B were given by the averages of 25 single-channel traces. K^+ channels of this type revealed the occurrence of openings in bursts and time-dependent activation and inactivation. Neither the activity nor the conductance of the transient K^+ channel was affected by phenytoin. The slope conductances were found to be 26 ± 3 pS and 25 ± 3 pS in control conditions and in the presence of phenytoin, respectively ($P>0.05$; $n=7$). The inactivation of the mean currents was fitted by an exponential function and the time constants were 34.6 ± 2.2 ms and 31.5 ± 3.1, respectively ($P>0.05$; $n=5$).

Phenytoin effect on Ca^{2+}-activated K^+ channels

The large-conductance K^+ channel observed in the N2A cells was characterized as a Ca^{2+}-activated K^+ channel. The traces in control conditions, at two different concentrations of calcium ions at the intracellular membrane surface, $[Ca^{2+}]_i$, and in the presence of extracellular 100 μM phenytoin, are shown in Figure 6A and 6B, whereas the L/V relationship in control conditions is plotted in Figure 5C. The channel activity at a 40 mV membrane potential and 2 nM $[Ca^{2+}]_i$ consisted typically of very short open events. As the $[Ca^{2+}]_i$ was increased to 0.5 μM, the channel resided mainly in the open state with few closure flickers, occasionally returning to the closed state for longer periods. Phenytoin affected neither the unitary conductance nor the channel activity. The slope conductances, at a physiological K^+ gradient, were found to be 95 ± 5 pS in control conditions and 93.6 pS in the presence of phenytoin ($P>0.05$). Current values were averaged over eight cells. The distribution of channel open- and closed-time durations of the cells in control conditions and their application of phenytoin displayed time courses best fitted by two exponential functions. The long periods in the closed state were omitted in the analysis. The open-time constants τ_{o1} and τ_{o2} were 0.7 ± 0.2 ms and 12.4 ± 2.1 ms in control conditions and 0.5 ± 0.3 ms and 13.2 ± 3.5 ms in the presence of phenytoin. The closed time constants τ_{c1} and τ_{c2}, were 0.4 ± 0.1 ms and 2 ± 0.5 ms in control conditions and 0.6 ± 0.2 ms and 1.9 ± 0.4 ms in the presence of phenytoin, respectively ($P>0.05$). Data were averaged over five cells.

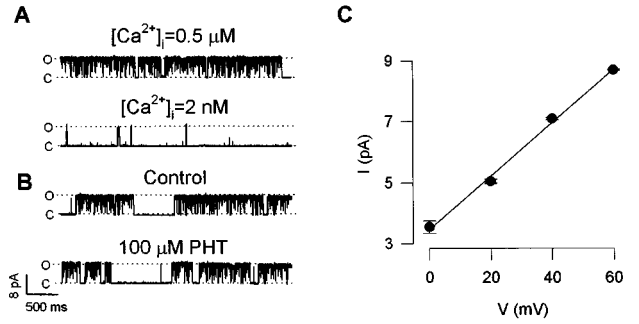

Figure 6 Effect of phenytoin on Ca^{2+}-activated K^+ channels. (A) The sweeps show events recorded from a single inside-out patch at the indicated intracellular free calcium concentrations. (B) Representative traces from an outside-out experiment (0.5 μM $[Ca^{2+}]_i$) in control conditions and after application of 100 μM phenytoin. The drug affected neither the unitary conductance nor the channel activity. (C) I/V relationship of the channel amplitude in control conditions. The unitary slope conductance was 95 ± 5 pS ($n=8$). Records filtered at 0.5 kHz.

Discussion

In order to explore qualitative aspects of the K^+ conductance block by phenytoin, single-channel experiments were performed. Three different types of delayed rectifier K^+ channels with conductance values of approximately 12, 15 and 18 pS and different kinetic behaviours were found in patches held at an HP of -50 mV. K^+ channels with similar conductances were observed in other preparations (Cook, 1988; Magnelli *et al.*, 1992). The experiments reported in this paper show that phenytoin described the single-channel activities of the three types of K^+ channels, whereas the unitary-current amplitudes were unaffected. The open-probability reduction of the delayed rectifier K^+ channels was concentration-dependent. For the biggest channel, the half-maximal inhibitory concentration was ≈27 μM. This value is similar to that reported in our previous paper (Nobile & Vercellino, 1997) and is comparable to that obtained with the anticonvulsant U-

54494A in similar experimental conditions (Zhu *et al*., 1992). A transient K^+ channel affected by cumulative inactivation was characterized by using stimulation patterns with pulses applied every 40 s from an HP of −70 mV (Bertoli *et al*., 1996). Neither the activity nor the conductance (≈26 pS) of the channel was influenced by 100 μM phenytoin. Finally, a large-conductance K^+ channel (≈95 pS in a physiological K^+ gradient) was found, the activity of which was strongly dependent on the intracellular Ca^{2+} concentration (Smart, 1987). Phenytoin affected neither the unitary conductance nor the channel activity. The blocking characteristics of phenytoin were quite similar to those of the anticonvulsant U-54494A (Zhu *et al*., 1992). However, the analysis by Zhu *et al*. (1992) was limited to one type of delayed rectifier K^+ channel and the current inhibition was voltage-dependent in a different manner. Of great interest is the fact that phenytoin selectively blocks delayed rectifier K^+ channels. Several drugs, currently in use or undergoing clinical trials, appear to act by opening or closing K^+ channels. These agents constitute a chemically heterogeneous group and their effects are presently being investigated because of their therapeutic potential (Prestipino *et al*., 1993). Finally, other aspects deserve consideration. First, the selective effect of phenytoin can be of importance as a pharmacological tool for isolating K^+ channels in biophysical studies. Second, a mutational analysis of voltage-gated K^+ channels in which the aminoacid composition of the pore is altered could suggest the position of the binding site for phenytoin in respect to the other K^+ channel blockers as TEA or 4-AP (Kirsch *et al*., 1993; Taglialatela *et al*., 1993).

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