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Inhibition of delayed rectifier K^+ channels by phenytoin in rat neuroblastoma cells

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1 The action of the anticonvulsant drug phenytoin on K^+ currents was investigated in neuroblastoma cells by whole-cell voltage-clamp recording.

2 Neuroblastoma cells expressed an outward K^+ current with a voltage- and time-dependence which resembled the delayed-rectifier K^+ current found in other cells. When added to the standard external solution at concentrations ranging between 1 and 200 μM, phenytoin reduced the current ($n=65$). Inhibition was concentration-dependent with a half-maximal inhibitory concentration of 30.9 ± 0.8 μM.

3 The K^+ current inhibition by phenytoin was voltage-dependent with block by phenytoin being relieved by depolarization.

4 The times taken to reach steady-state inhibition and complete recovery from inhibition were about 20 s. Neither the activation and inactivation rates of the K^+ current nor the K^+ channel availability were significantly altered by the blocking drug. A use-dependent block was observed at phenytoin concentrations of 10, 25 and 50 μM.

5 These results suggest that phenytoin affects K^+ currents and that this effect might lead to a reduction in neuronal excitability.

Keywords: Anticonvulsant; phenytoin; K^+ currents; neuroblastoma cells; whole-cell voltage clamp

Introduction

We have recently carried out a series of experiments aimed at analysing the mechanisms of action of the anticonvulsant drug phenytoin, commonly used for the management of partial seizures and generalized tonic-clonic seizures (for a review, see Rogawski & Porter, 1990). Phenytoin is known to interact with the voltage-dependent Na^+ channels responsible for action potential generation. As in the early studies demonstrating a specific effect of phenytoin on repetitive firing in peripheral nerve preparations, more recent studies have confirmed that phenytoin inhibits high-frequency repetitive firing of action potential of mammalian central neurones in tissue cultures at clinically significant concentrations (≈ 10 μM) (Yaari *et al*., 1986; Macdonald, 1989). The effect on high-frequency firing is thought to be a consequence of the unique voltage- and frequency-dependent manner in which phenytoin binds to Na^+ channels (Ragsdale *et al*., 1991; Kuo & Bean, 1994). In addition to effects on Na^+ channels, phenytoin at relatively low concentrations can produce a voltage- and frequency-dependent block of low-threshold (T-type) Ca^{2+} channels in different cell types (Yaari *et al*., 1987; Twombly *et al*., 1988; Coulter *et al*., 1989). However, the relevance of this effect on Ca^{2+} channels to the anticonvulsant action of the drug is uncertain. Furthermore, Yaari *et al*. (1986) have emphasized the ability of high external K^+ to facilitate the frequency-dependent block produced by phenytoin. High K^+ depolarizes neurones and reduces the amplitude of K^+-dependent hyperpolarizing potentials, such as the spike after hyperpolarization, and it has been suggested that the more positive membrane potential facilitates the Na^+ channel block produced by phenytoin and acts to prevent removal of inactivation (Selzer *et al*., 1988). On the other hand, accumulation of extracellular K^+ during frequent neuronal firings appears to be an important factor in producing or promoting seizures (Heinemann & Louvel, 1983). Since it is well known that K^+ channels affect neuronal excitability by altering action potential durations, refractory periods and/or neuronal firing frequencies, a direct effect of phenytoin on voltage-dependent K^+ channels may suggest an important role for these channels in controlling seizures. Recently, the block of K^+ currents by some tricyclic antidepressants and the structurally related anticonvulsant compound, carbamazepine, was studied on rat sympathetic neurones (Wooltorton & Mathie, 1993). The authors showed that carbamazepine was ineffective at all concentrations tested, whereas Zona *et al*. (1990) suggested that the drug enhanced K^+ currents in rat cortical neurones. Other anticonvulsant drugs, valproate and U-54494A, have been shown to reduce K^+ currents in the frog node of *Ranvier* and in mouse neuroblastoma cells, respectively. It is of interest to compare the above-mentioned results obtained by structurally distinct but pharmacologically related compounds with the action of phenytoin on the K^+ currents. N2A cells were used because they only exhibit a delayed rectifier K^+ current and allow a better characterization of the effect of phenytoin on this component.

Methods

Cell cultures

Rat neuroblastoma cells (N2A) were grown in storage flasks, maintained with 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. Confluent cells were treated with 1 mM EDTA and plated in 35 mm Petri dishes with a density of about 3×10^5 per dish.

Solutions

The composition of the standard external solution was (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 3×10^{-7} M tetrodotoxin (TTX) and 5×10^{-5} M 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. The pH was adjusted to 7.3 with NaOH and KOH for the external and the internal solutions, respectively, and their osmolarity

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was set to 290±10 mOsmol with mannitol. Phenytoin ($C_{15}H_{11}N_2O_2N_a$; Sigma) was applied by means of a gravity perfusion system (≈1 ml min^{-1} flow). Stock solutions of phenytoin and ethanol were prepared each day from which the final concentrations were made (Schwarz & Grigat, 1989). The maximal ethanol concentration was <0.5%. Control experiments showed that ethanol at this concentration did not affect the ionic membrane currents. The perfusion system tubings were treated by Sigmacote (Sigma) to minimize contamination. All the experiments were carried out at room temperature (20–22°C).

Electrophysiological measurements

Electrophysiological experiments were performed by using the patch-clamp technique in whole-cell configuration, as in our previous studies (Nobile *et al.*, 1994). Borosylicate glass electrodes were pulled and calibrated to have a tip resistance of ≈5 MΩ when filled with the standard solution. Current records were filtered at 2–5 kHz with 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). Currents were sampled as a frequency of 50–100 μs per point. The stimulation patterns were normally applied every 10 s from holding potentials (HPs) of −50 or −70 mV. Whole-cell current records were usually corrected for leakage and capacitive transients by using the P/4 protocol from an HP of −90 mV. Series resistance was about 10 MΩ and series resistance compensation was used.

Current traces were analysed by Pclamp (Axon Instruments Inc). and Sigma Plot (Jandel Scientific, Erkrath, Germany) software. The current values were obtained by averaging over 20 points at the steady-state level. Data are given as mean±s.d., Student's *t* test for paired data was performed and $P \leqslant 0.01$ was regarded as being significant.

Results

Characterization of K^+ currents in N2A cells

Whole-cell recordings from N2A cells, under control conditions and in the presence of 3×10^{-7} M TTX and 5×10^{-5} M Cd^{2+}, revealed outwardly rectifying currents. In Figure 1, a family of outward currents in response to a set of test potentials that ranged from −20 to 60 mV from an HP of −60 mV and the relative current vs. membrane potential relationship are illustrated. The activation time constants estimated by fitting experimental data with an exponential function turned out to be voltage-dependent and became faster with increasing depolarizations (0 to 60 mV) with values ranging from 107±10 to 7±1 ms ($n=3$), respectively. The outward current showed limited inactivation over a 130 ms pulse duration. The ionic selectivity of the channels was determined from reversal potential measurements. Tail currents elicited by repolarizations to various voltages after stepping to 50 mV from an HP of −60 mV were recorded in a bath K^+ concentration of 10 mM. Tail currents (Figure 2a) reversed polarity at about −55 mV (Figure 2b). In standard internal and external solutions, the reversal potential was −82±4 mV ($n=6$) and shifted to 54±2 mV ($n=4$) with 10 mM external K^+, thus indicating that the channel is selective for K^+ over Na^+ and Cl^-. The steady-state voltage-dependence of K^+ channel inactivation (Figure 2c) was studied by using a pulse protocol in which the test voltage step to 40 mV, 100 ms long, was preceded by 3 s preconditioning pulses from −60 to 20 mV, stepped by 10 mV. K^+ channel inactivation was evaluated as the ratio I/I_{max}, namely, the ratio between each current, dependent on different preconditioning potentials, and the maximum current corresponding to a −60 mV preconditioning pulse. Experimental points were fitted by a Boltzmann distribution as follows:

$$I/I_{max} = 1/(1+\exp((V_m - V_{0.5})/k)) \quad (1)$$

where V_m is the preconditioning potential, $V_{0.5}$ is the midpoint potential, and k is the steepness of the curve. $V_{0.5}$ was −16.1 mV and k was 8.2. The conductance-voltage relationship was determined from tail currents measured at −60 mV after stepping the depolarizing voltage from −20 to 60 mV (Figure 2c). Relative conductance values were normalized according to the relationship $g = I_k/I_{Kmax}$, where I_{Kmax} is the maximum amplitude of the instantaneous K^+ tail currents and I_K is the value relative to i_{Kmax}. Instantaneous currents were determined by extrapolation from single exponential fits of tail currents and plotted as a function of the depolarizing pulses. Experimental points were fitted by a Boltzmann relation:

$$G/G_{max} = 1/(1+\exp((V_{0.5} - V_m)/k)) \quad (2)$$

where V_m is the membrane potential during the test pulse, $V_{0.5}$ is the voltage at which the conductance was half-maximal and k is the steepness of the curve. In five experiments, $V_{0.5}$ was 23±2 mV and k was 10±2. The sensitivity of the outwardly

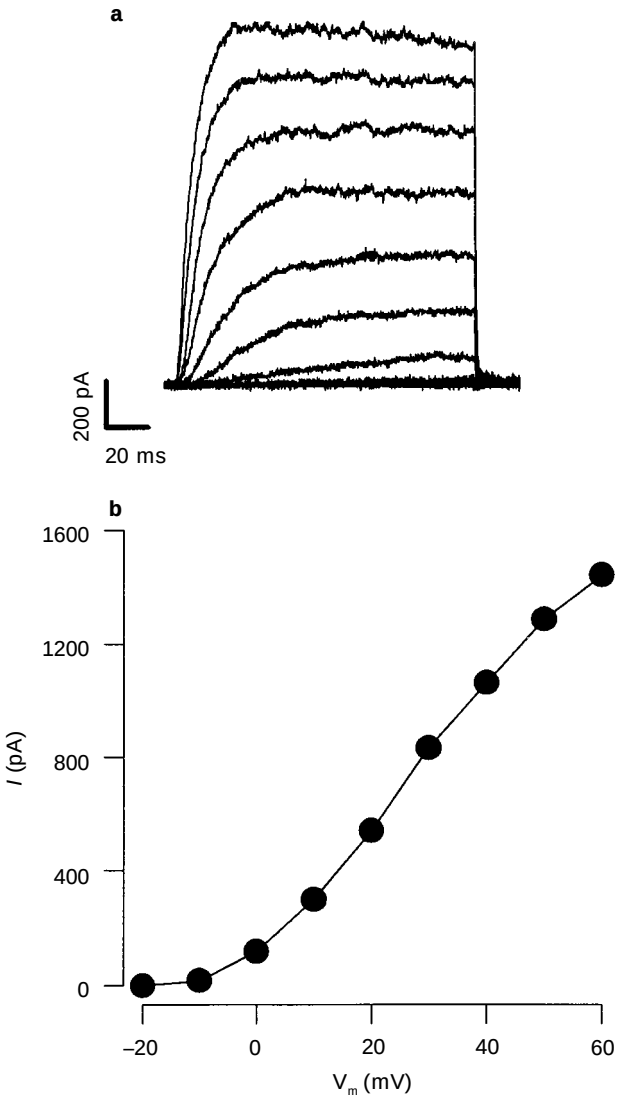

Figure 1 Outwardly rectifying currents in N2A cells. (a) Outward currents evoked from an HP of −60 mV in response to depolarizing voltage steps ranging from −20 to 60 mV in 10 mV increments. (b) Steady-state current values from traces in (a) plotted as a function of membrane potential.

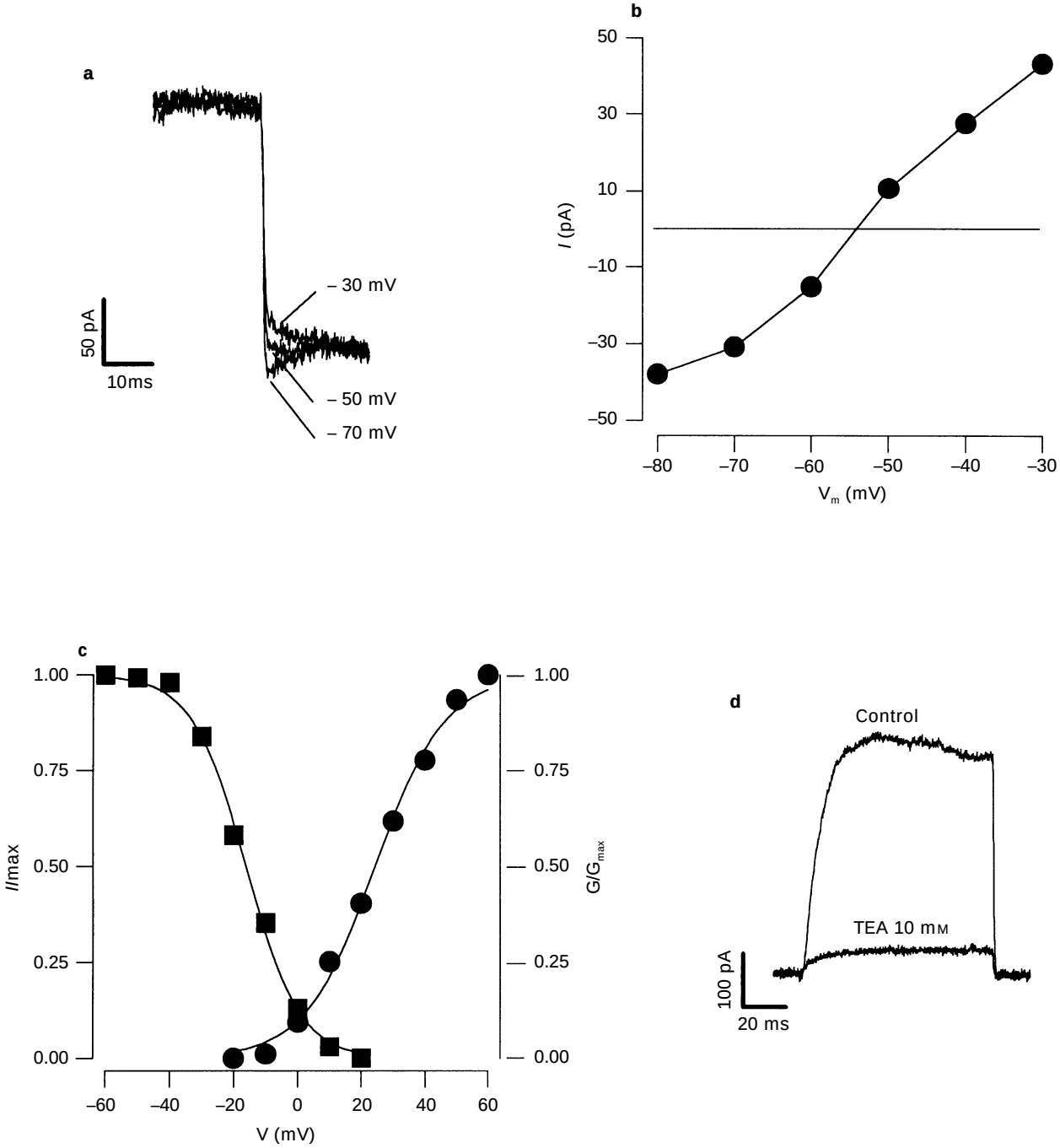

Figure 2 (a and b) Outward currents in N2A cells are carried by K^+ ions. (a) Tail currents, recorded in the presence of 10 mM K^+ in the external bath, were evoked by a depolarization to 50 mV followed by repolarizing voltage steps to the values indicated. (b) Tail currents from (a) plotted as a function of test potentials. Tail currents reversed near −55 mV. (c) Steady-state inactivation and conductance-voltage characteristics of N2A cells. The steady-state current was measured during a 100 ms-long depolarizing test pulse to 40 mV, following a 3 s-long conditioning prepulse to various potentials. Current values (■) normalized to the value obtained by a conditioning prepulse of −60 mV were fitted by the Boltzmann equation (1) given in the text. Relative conductance values (●) were normalized according to the relationship $g = I_K/I_{Kmax}$, where I_{Kmax} is the maximum amplitude of the instantaneous K^+ tail current and I_K is the value relative to I_{Kmax}. Data points were fitted by the Boltzmann equation (2). The voltage at which the conductance was half-maximal, $V_{0.5}$, was 24.2 mV and the steepness of the curve k was 11. (d) Outward K^+ current recorded at 50 mV in the absence and in the presence of 10 mM tetraethylammonium (TEA).

rectifying K^+ current to the K^+ channel blocker tetraetylammonium (TEA) was examined. External perfusion of 10 mM TEA practically blocked the outward current in N2A cells (Figure 2d). In six cells tested, TEA (10 mM) reversibly reduced the outward current at 60 mV from an HP of −60 mV by 90±5%. On the whole, the results show K current properties of the delayed rectifier type (Prestipino *et al.*, 1993).

Effect of phenytoin on K^+ currents of N2A cells

Phenytoin externally applied reduced K^+ currents evoked by depolarizing pulses from −20 to 60 mV, as illustrated in Figure 3. Figure 3a shows current traces elicited by test pulses from an HP of −60 mV to 50 mV, in a cell held in standard solution and in the presence of 25 μM phenytoin. The drug reversibly reduced the K^+ current amplitude, whereas the ac-

tivation and inactivation time courses were unaffected. Figure 3b shows the steady-state current values as a function of membrane potential. The steady-state values were obtained by averaging over 20 points at the end of the 130 ms-long voltage pulse. At a drug concentration of 25 mM, the inhibition value and the complete recovery after washing phenytoin out of the bath were rapidly reached in 20–30 s (Figure 3c). The time-dependence of the decrease in the K^+ current and the recovery time were about the same at each concentration tested. The time-dependent effect of phenytoin is consistent with an action on open channels during the voltage steps and on closed channels between voltage steps. To determine the percentage of closed channel block, experiments with bath perfusion of phenytoin in the absence of voltage steps were performed. After a 2 min application of 100 μM phenytoin the first pulse showed a closed channel block of $50\pm5\%$ and a subsequent open channel block of $12\pm8\%$ ($n=3$). In Figure 3d, the % block induced by phenytoin 25 μM is plotted against depolarizing pulses between 0 and 60 mV. The current inhibition by phenytoin averaged over four cells was voltage-dependent ($P=0.01$) except for limited depolarizations. The voltage-dependence of the steady-state inactivation of K^+ currents was studied by using the same pulse protocol as in Figure 2c, both under control conditions and after application of 50 μM phenytoin; the results are shown in Figure 4a. Experimental data were fitted by a smooth curve from the Boltzmann relation (1). The best fits gave $V_{0.5}=-13.3$ mV and $k=7.4$ in control conditions, and $V_{0.5}=-17.3$ and $k=6.6$ in the presence of phenytoin, thus indicating that phenytoin does not significantly affect the K^+ channel availability ($P>0.05$). To verify if phenytoin affects K^+ channels by inducing a use-dependent block, trains of 130 ms-long pulses to 50 mV from an HP of -60 mV, at pulse intervals of 0.5 s and 1 s (2 and 1 Hz), after a 4 min rest period, were delivered. In control

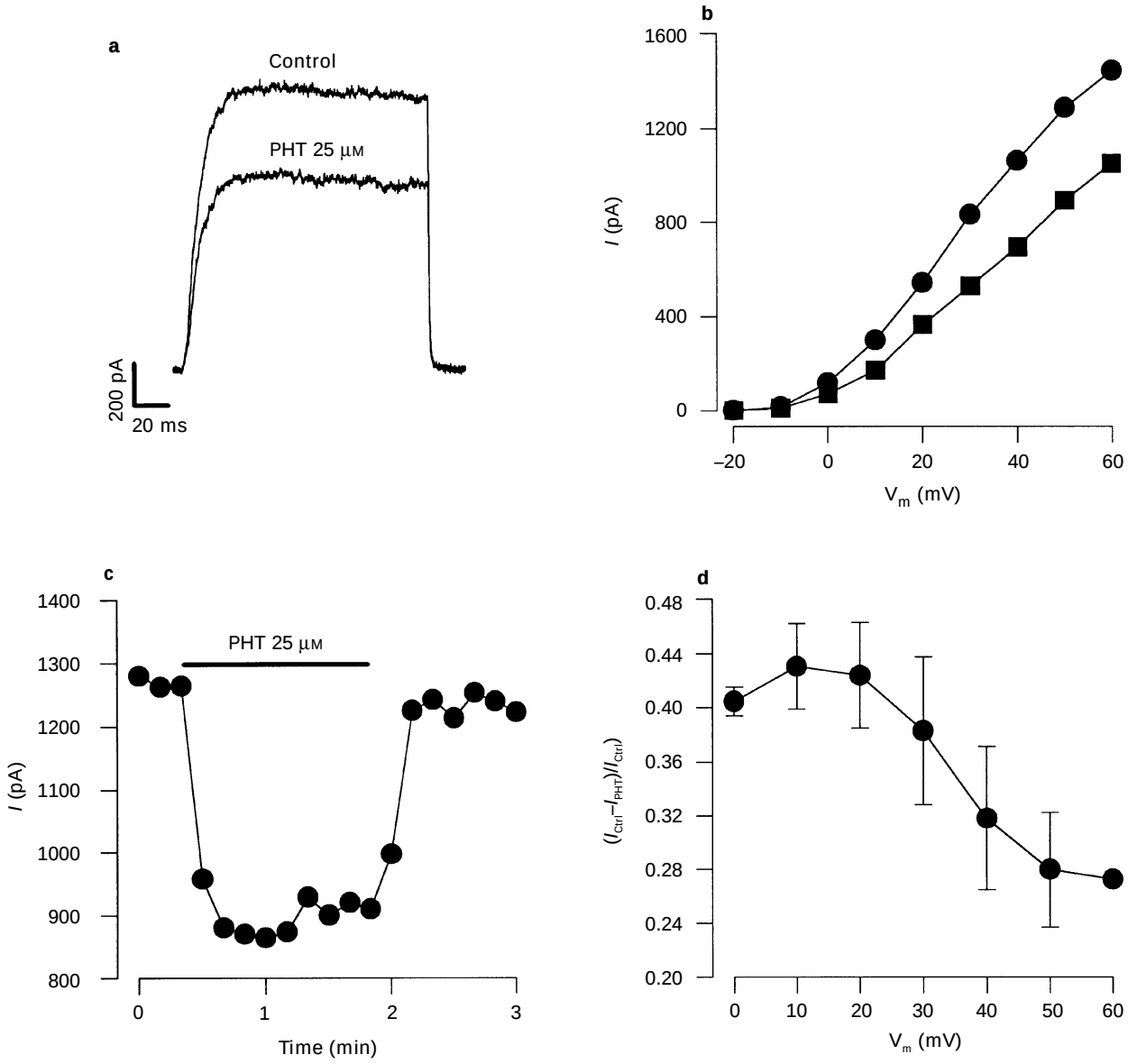

Figure 3 Phenytoin affects the delayed rectifier K^+ current in N2A cells. Current traces recorded at a 50 mV test pulse from an HP of -60 mV in control conditions and in the presence of 25 μM phenytoin. (b) Current-voltage relationships for steady-state values at the end of 130 ms-long voltage pulses. The current amplitudes are plotted against the voltage values before (●) and during the application of phenytoin (■). (c) Time course of the phenytoin response and the current recovery after washout. Depolarizing pulses to 50 mV (HP $=-60$ mV) were repeated every 10 s. Phenytoin was applied for the duration of the bar. The maximum response and current recovery were obtained in about 20 s. (d) Voltage-dependence of steady-state K^+ current inhibition induced by 25 μM phenytoin. Mean values from 6 cells with vertical lines showing s.d.

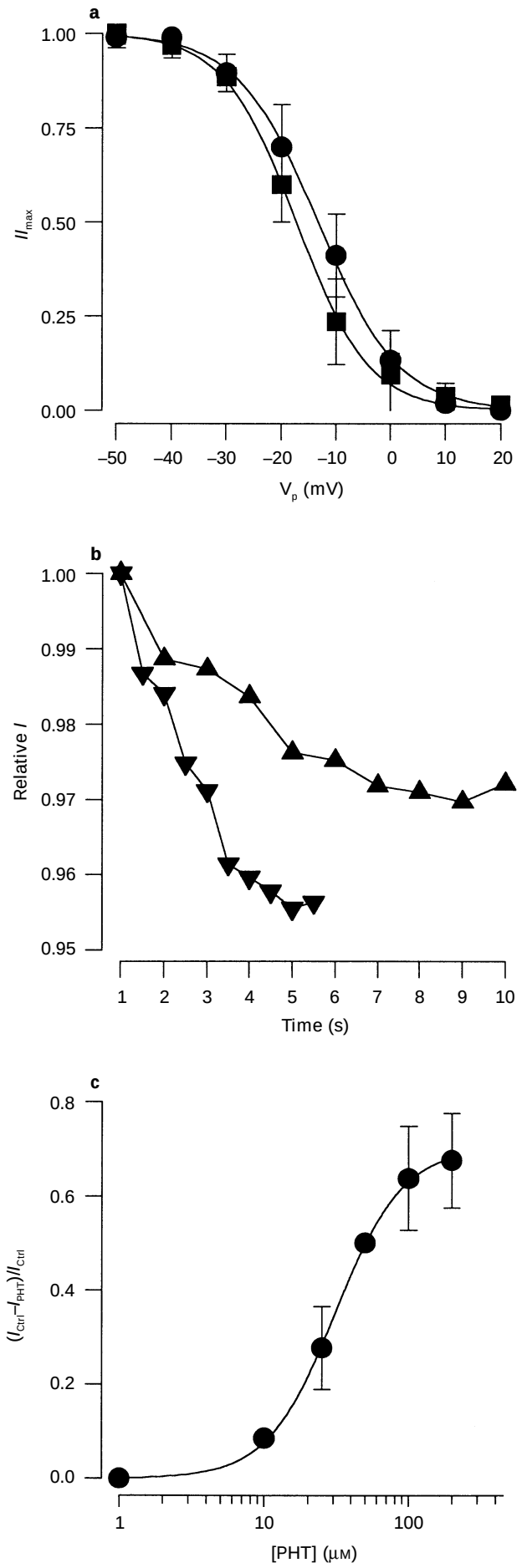

Figure 4 (a) K^+ current steady-state inactivation was not significantly affected by phenytoin (PHT). Same stimulation protocol as described in Figure 3. Current values normalized to the values at −50 mV were fitted by a Boltzmann distribution (Eq. 1). Midpoint potentiated $V_{0.5}$ and k values were −13.4 mV and 7.4 in control conditions (●) and −17.2 mV and 6.6, respectively, in the presence of 50 μM phenytoin (■). Data were averaged over 8 cells; vertical lines show s.d. (b) Use-dependence of the block by phenytoin of the K^+ current. Trains of test pulses 50 ms-long from an HP of −60 mV to 50 mV at different time intervals were applied. Symbols (▲) and (▼) indicate recordings in the presence of 10 μM phenytoin at 1 and 2 Hz, respectively. Points were normalized to the tonic block of phenytoin (10%). (c) Concentration-dependence of the effect of phenytoin. Experimental points were obtained from an HP of −60 mV to 50 mV. Values of $(I_{Ctrl}-I_{PHT})/I_{Ctrl}$ wre averaged over 3 to 12 cells and best approximated by Eq. 3 The half-effective dose was 30.9 μM.

conditions, the K^+ current was almost constant during each train at pulse intervals of 0.5 s or 1 s. Phenytoin 10 μM reduced ($P<0.01$) the current evoked by the first depolarizing pulse by about 10% (tonic block) and a further reduction was observed during the pulse trains and after increasing the stimulation frequency from 1 Hz (▲) to 2 Hz (▼) (Figure 4b), thus indicating that phenytoin affects K^+ channels in a use-dependent way. The values in the presence of the drug were normalized to the tonic block of phenytoin. The same results were obtained for eight other cells at phenytoin concentrations ranging from 10 to 50 μM.

Phenytoin also affected K^+ conductance in a concentration-dependent way. Figure 4c shows the percentage block produced by different concentrations of phenytoin at a membrane potential of 50 mV. Experimental data were fitted according to the equation:

$$(I_{Ctr} - I_{PHT})/I_{Ctr} = Block_{max}[C^{n_H}/(C^{n_H} + IC_{50}{}^{n_H})] \quad (3)$$

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 $IC_{50} = 30.9 \pm 0.8$ μM (mean ± s.e.), $n_H = 1.9$ and a phenytoin $block_{max} = 0.71$. The n_H value indicates that two phenytoin molecules must bind to a site in order for the K^+ current to be inhibited. Data were averaged over three to twelve cells.

Discussion

The present results show that, when externally perfused, phenytoin affects the amplitude of K^+ currents in neuroblastoma cells (N2A). In the presence of extracellular Cd^{2+} to nullify the Ca^{2+} currents and reduce internal Ca^{2+} free concentration (5×10^{-9} M), the outward currents displayed a threshold of activation, time course of activation and inactivation and voltage-dependence typical of the 'delayed rectifier' K^+ channels identified in excitable and nonexcitable cells (Rudy, 1988; Prestipino *et al.*, 1993).

In N2A cells, the steady-state reduction of K^+ current by phenytoin was partially relieved at high depolarizations. The K^+ current inhibition was concentration-dependent with a half-maximal inhibitory concentration of ≈30 μM. This value can be considered to be within the range of its anticonvulsant dose. At normal therapeutic serum concentrations of 10–20 μg ml^{-1} (40–80 μM), phenytoin protects against seizures. The inhibition of K^+ current by phenytoin was markedly dependent upon the frequency of stimulation. Phenytoin did not bind exclusively to open channels, but a considerable amount of block occurred to closed channels between the stimulating voltage steps. Furthermore, our results indicate that activation and inactivation kinetics of K^+ current are unaffected by phenytoin. The observed phenomenon resembling a voltage-dependent removal of K^+ current block together with the use-dependence suggests a mechanism of block partially similar to that induced by 4-aminopyridine (4-AP) on squid giant axon (Yeh *et al.*, 1976). These authors assumed that 4-AP molecules

interact with K^+ channels by binding to a site within the channel. Channels in which 4-AP molecules occupy the binding site are blocked and this cannot conduct K^+ ions, regardless of the state of the activation gating machinery. Further biophysical analysis of delayed rectifier K^+ channels expressed in mammalian cells and *Xenopus laevis* oocytes support the hypotheses that 4-AP blocks the intracellular mouth of the channels in both the open and closed states (Kirsch *et al*., 1993; Stephens *et al*., 1994).

Phenytoin also blocks the voltage-dependent Na^+ channels, but with a mode of interaction different from that of the delayed rectifier K^+ channels (Schwarz & Grigat, 1989; Kuo & Bean, 1994). Phenytoin preferentially interacts with the inactivated state of the Na^+ channel by stabilizing the channel in the inactivated state and preventing its transition to the resting state that it must re-enter before it can open. Furthermore, its inhibition of Na^+ channels is use-dependent and dependent on the holding potential but not the membrane potential.

As mentioned in the introduction section, other anti-convulsant drugs have been shown to interact with K^+ channels. It has been suggested that carbamazepine does not block the delayed rectifier K^+ current in sympathetic neurones, whereas it enhances K^+ current in cortical neurones (Zona *et al*., 1990; Wooltorton & Mathie, 1993). Valproato and U-54494A have been shown to suppress neuronal delayed rectifier K^+ current (Van Erp *et al*., 1990; Zhu *et al*., 1992). Considering that these compounds are pharmacologically related but structurally distinct, the blocking characteristics of phenytoin are quite similar to those of the last two drugs, even though the voltage- and use-dependence are different and the time course profile for inhibition of K^+ currents by phenytoin is faster. The block of the delayed rectifier K^+ channels by phenytoin may have pharmacological relevance to its anticonvulsant activity, not only because it would delay the repolarization in neurones, but also because it would augment its Na^+ channels blocking action; both effects might lead to a reduction in the neuronal excitability. Moreover, the accumulation of extracellular K^+ concentration often occurs during long-lasting seizures and is known to be responsible for the continuation of spike trains. By blocking the delayed rectifier K^+ channels, phenytoin may reduce the increase in the extracellular K^+ concentration, thereby preventing a continuation of spikes.

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