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Accelerating effects of pentobarbitone on the inactivation process of the calcium current in *Helix* neurones

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1 The effect of pentobarbitone on Ca^{2+} current (I_{Ca}), separated from other ionic currents was studied under voltage clamp using a suction pipette technique in *Helix* neurones.

2 Pentobarbitone depressed the maximal peak amplitude (MPA) of I_{Ca} in a concentration-dependent manner without shifting the current-voltage (I-V) relationships along the voltage axis. Increases in external Ca^{2+}-concentration ($[Ca^{2+}]_o$) overcame the inhibitory action of the agent on MPA.

3 Pentobarbitone markedly accelerated the decay phase of I_{Ca} which took a distinctly different time course from that of the control. The accelerating action of the agent on the decay phase of I_{Ca} was not overcome by increases in $[Ca^{2+}]_o$. In the presence of internal EGTA (20 mM), pentobarbitone also accelerated the decay of I_{Ca}.

4 Changes in pH of the external perfusing solution altered the potency of pentobarbitone in depressing MPA; in the presence of pentobarbitone (3×10^{-4} M) at pH of 7.0, 8.0 and 9.0, fractional inhibition was approx. 46%, 21% and 4%, respectively.

5 Internal application of pentobarbitone (10^{-4}–10^{-3} M) inhibited MPA, but exerted no effect on the decay phase of I_{Ca}.

6 Pentobarbitone (10^{-4} M) markedly accelerated the decrease of MPA of I_{Ca} induced by repetitive stimuli applied at an interval of 150 ms, indicating a use-dependent depression of MPA.

7 Results provide evidence that pentobarbitone has a dual action on I_{Ca}, inhibiting MPA and accelerating the decay phase of I_{Ca}.

Introduction

Pancuronium (Yeh & Narahashi, 1977), N-alkylguanidines (Kirsch, Yeh, Farley & Narahashi, 1980) and gallamine (Smith & Schauf, 1981) are known to block the open-state of the voltage-dependent sodium channel, resulting in acceleration of the decay of sodium current without affecting the rising phase of the current. As to calcium current (I_{Ca}), the inactivation process (the decay phase of I_{Ca}) has been analysed in various tissues, and I_{Ca} after reaching its peak, is inactivated in a voltage- and current-dependent manner (Kostyuk, Krishtal & Shakhovalov, 1977; Akaike, Lee & Brown, 1978; Brehm & Eckert, 1978; Tillotson, 1979; Brown, Morimoto, Tsuda & Wilson, 1981; Plant & Standen, 1981). So far as we know, no drug has been found to accelerate the decay of I_{Ca}. We show here that pentobarbitone accelerates the decay of I_{Ca} in snail neurones. Preliminary results have been given elsewhere (Oyama, Nishi & Akaike, 1982).

Methods

The experimental method was essentially similar to that previously described (Lee, Akaike & Brown, 1980). Experiments were performed on an intracellularly-perfused identified giant neurone (Fl-cell) of *Helix* (Kerkut, Lambert, Gayton, Locker & Walker, 1975), using a suction pipette technique which allows voltage clamp and internal perfusion.

In brief, single neurones were isolated from circumoesophageal ganglia of *Helix aspersa*. The ganglion was removed and connective tissue was stripped off with fine forceps until clusters of neurones floated free in 'normal' snail Ringer. A part of an individual neurone (100–150 μm diameter) was aspirated under negative pressure of about −300 mmHg so as to occlude the tip of a suction pipette (20–30 μm diameter). The cell body was then isolated from residual connective tissue and the axon. Internal

Table 1 Ionic composition of external and internal solutions

External solution

	Tris Cl	TEA Cl	CsCl	$CaCl_2$	$CoCl_2$	$MgCl_2$	4-AP	Glucose	HEPES	pH
I_{Ca}	35	50	5	10		15	5	5.5	5	7.4
I_{NS}	35	50	5		10	15	5	5.5	5	7.4

Internal solution

	Cs aspartate	TEA OH	EGTA acid	HEPES	pH
I_{Ca}, I_{NS}	135	10	0.1	5	7.4

Tris: tris (hydroxymethyl) aminomethane; TEA: tetraethylammonium; HEPES: N-2-hydroxyethylpiperazine-N-2-ethylenesulphonic acid; 4-AP: 4-aminopyridine.
All values mM (except pH).

perfusion was preceded by disrupting part of the neuronal membrane aspirated into the tip of the suction pipette.

The Ca^{2+} current (I_{Ca}) was separated by blocking the K^+ current (I_K) and Na^+ current (I_{Na}) by substitution of $Tris^+$ for Na^+ and Cs^+ for K^+ in the internal and external solutions. The compositions of all test solutions are listed in Table 1. I_{Ca} and nonspecific outward current (I_{NS}) (Byerly, Hagiwara, Matsuda & Yoshii, 1979) were recorded under voltage clamp conditions in the neurone perfused with the relevant external and internal solutions for each current. Leakage and capacitive currents were substracted by adding the currents produced by each of a pair of depolarizing and hyperpolarizing pulses of equal amplitude. Pentobarbitone (Tanabe Pharmac. Co., Japan) was dissolved in the test solution at appropriate concentrations just before use.

All experiments were carried out at room temperature (23°–24°C).

Results

Effects of pentobarbitone on the calcium-dependent action potential

In a neurone perfused with the specific external and internal solutions for isolating I_{Ca}, a depolarizing pulse produced a Ca^{2+}-dependent action potential (Ca-AP) with a markedly prolonged plateau, as shown in Figure 1a. Once such a Ca-AP was elicited, the neurone could not generate the same Ca-AP within 5 to 7 min after repolarization. We examined effects of pentobarbitone on the Ca-AP following the experimental protocol illustrated in Figure 1; 15 min after the cessation of a previous Ca-AP, the drug was applied and 5 min after the drug application, the neurone was stimulated by passing a depolarizing pulse. Pentobarbitone at a concentration of 1×10^{-3} M decreased the Ca-AP duration greatly (66–83%) without affecting the Ca-AP amplitude

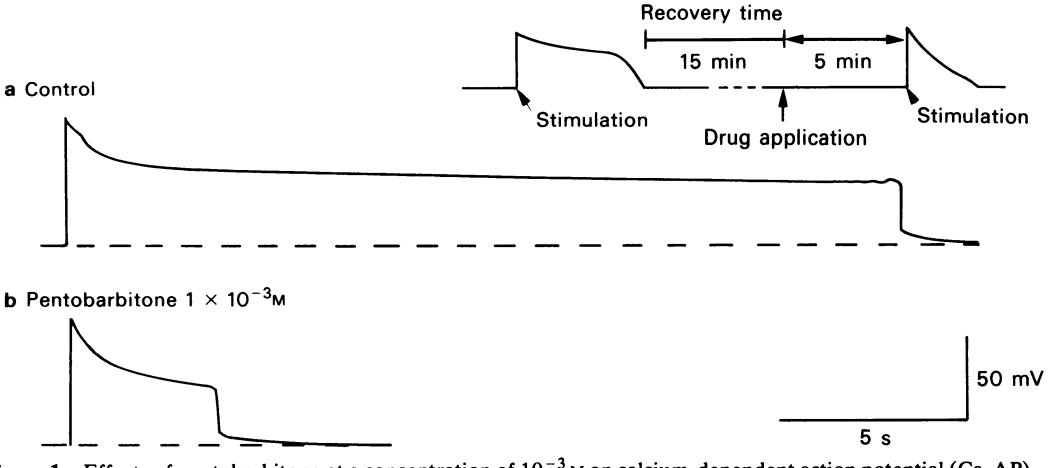

Figure 1 Effects of pentobarbitone at a concentration of 10^{-3} M on calcium-dependent action potential (Ca-AP). (a) Ca-AP during the control period. (b) Ca-AP recorded 20 min after the cessation of the Ca-AP in (a); the cell was exposed to pentobarbitone 10^{-3} M for 5 min. Inset illustrates experimental sequence.

(Figure 1b). The falling phase of the Ca-AP was steeper in the presence of pentobarbitone. The recovery was complete within 25 min after washing the preparation.

Effects on the transient peak amplitude of I_{Ca}

The membrane potential of the neurone was clamped at a constant holding potential of −50 mV and depolarizing voltage steps of 10 mV were applied to the preparation. The maximal peak amplitude (MPA) of I_{Ca} was attained at membrane potentials of +10 to +20 mV in the control (Akaike *et al.*, 1978; Akaike, Brown, Nishi & Tsuda, 1981). Effects of pentobarbitone on MPA were examined 5 min after the start of superfusion with test solutions containing various concentrations of the agent, since the drug-effect reached the steady state within 5 min. Pentobarbitone at a concentration of 10^{-6} M slightly reduced MPA, and an increase in the concentration of the agent (10^{-4}–10^{-3} M) depressed MPA to 60–80% of control. Dose-response curves were obtained by calculating percentage inhibition of MPA in the presence of pentobarbitone at various concentrations relative to the control (Figure 2b). The current-voltage (I-V) relationships for I_{Ca} in the presence of pentobarbitone are shown in Figure 2c; the agent induced a dose-dependent depression of the transient peak amplitude of I_{Ca} without shifting of the I-V relationship.

Effects of pentobarbitone on activation and inactivation processes of I_{Ca}

In the control solution, the onset of I_{Ca} occurred without a detectable delay and the rising phase (acti-

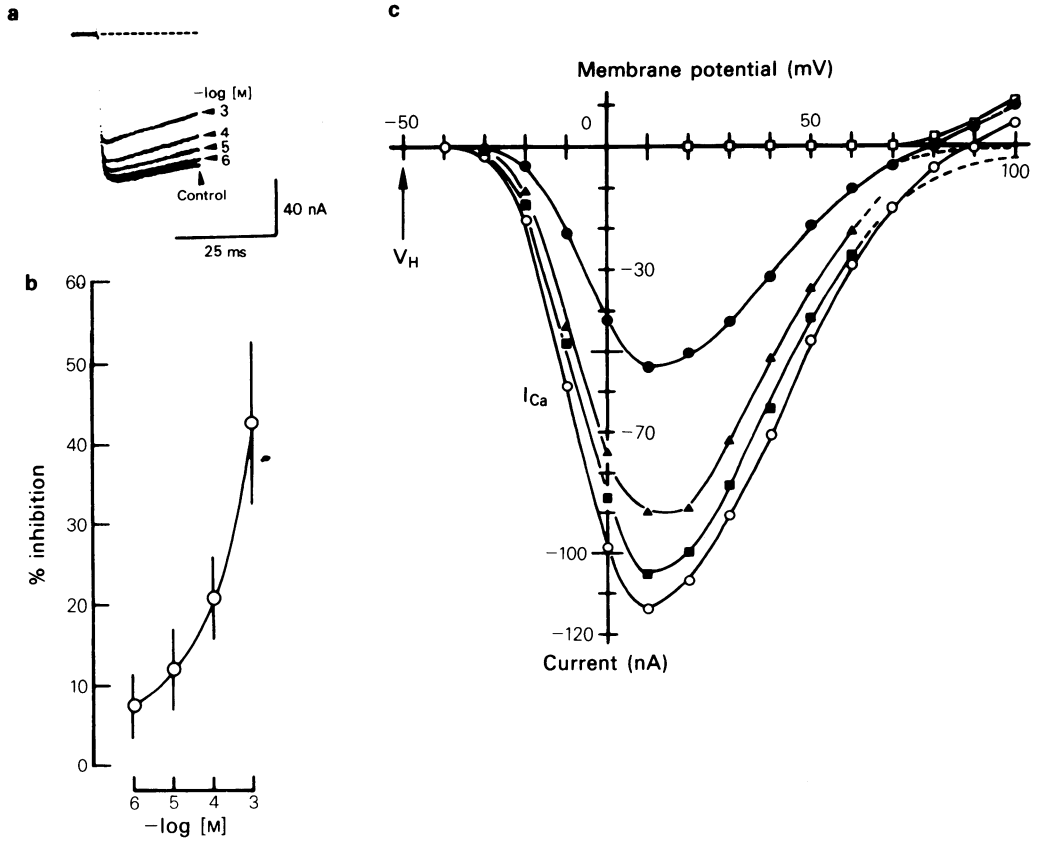

Figure 2 Effects of pentobarbitone on maximal peak amplitude (MPA) of calcium current (I_{Ca}). (a) Superimposed records of I_{Ca} before and 5 min after external application of pentobarbitone at various concentrations (10^{-6}–10^{-3} M). (b) Dose-response curve for inhibition of MPA of I_{Ca} after application of pentobarbitone at various concentrations. Vertical bars at each point indicate s.e.mean. (c) Effects of pentobarbitone on current-voltage relationships of I_{Ca}. (○) Control; (■) 5 min–7 min after application of pentobarbitone at 10^{-5} M; (▲) 10^{-4} M; (●) 10^{-3} M; (□) I_{NS}. V_H, holding potential.

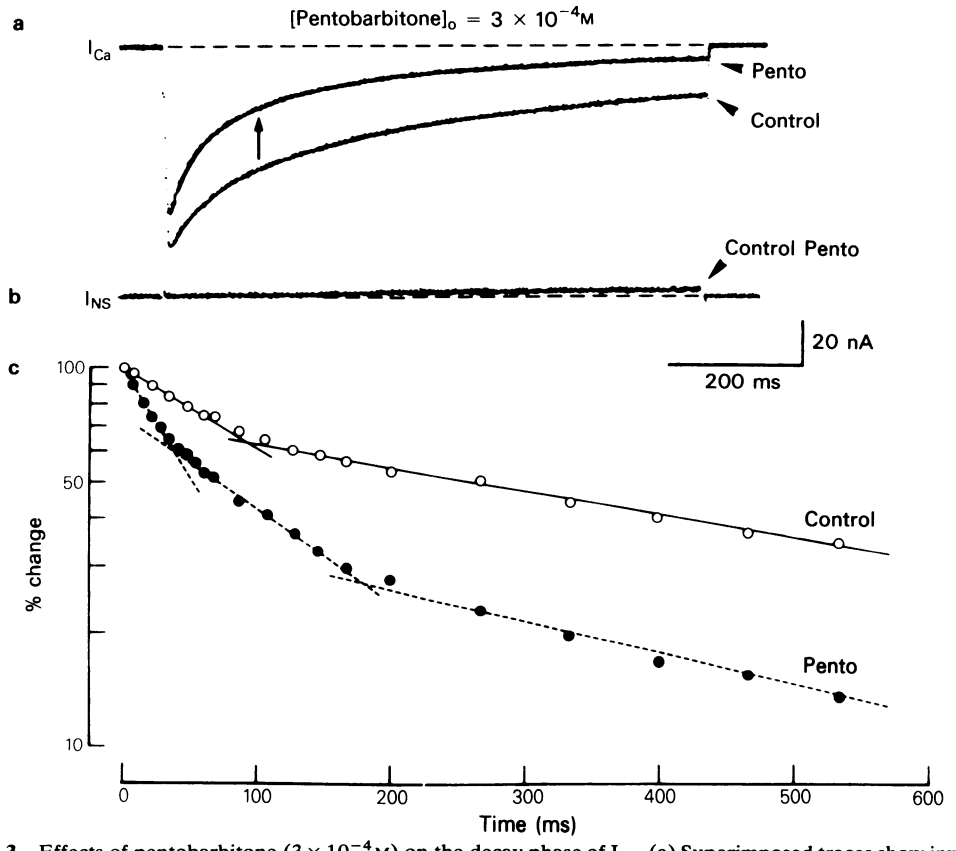

Figure 3 Effects of pentobarbitone (3×10^{-4} M) on the decay phase of I_{Ca}. (a) Superimposed traces show inward currents elicited on depolarizing the membrane to +15 mV from the holding potential of −50 mV. Pento = I_{Ca} recorded 5 min after perfusion with external solution containing pentobarbitone. (b) Superimposed traces show non-specific outward current (I_{NS}) elicited during the same depolarizing pulse as in (a). The I_{NS} was recorded from the same neurone as in (a). (c) Semilogarithmic plots of the relative decay of I_{Ca} of neurone externally perfused with solutions without (Control) and with (Pento) pentobarbitone. The plots were obtained after subtraction of I_{NS} from recorded I_{Ca} by using a graphic technique. Lines were drawn by eye.

vation) of I_{Ca} was fitted by a single exponential function, while the falling phase (inactivation) consisted of fast and slow components. Pentobarbitone at concentrations of 10^{-4}–10^{-3} M did not affect the rising phase (Figure 2a), but greatly accelerated the decay phase of I_{Ca}. I_{Ca} took a distinctly different time-course from the control (Figure 3a). The accelerating effect of pentobarbitone on the decay phase started to appear at a concentration of 3×10^{-5} M. At the end of a depolarizing pulse lasting 800 ms, pentobarbitone (10^{-4}–3×10^{-4} M) reduced I_{Ca} to about 10% of the control. Thus, the agent exerted rather selective actions on the decay phase of I_{Ca}, accelerating the inactivation process.

Effects on non-specific outward currents There is a possibility that non-specific outward current (I_{NS}) (Byerly *et al.*, 1979) might be augmented by pentobarbitone, which in turn, would have resulted in acceleration of the decay phase of the recorded inward current. To examine this possibility, we observed the effects of pentobarbitone on I_{NS} after substituting Ca^{2+} for Co^{2+}. Results are shown in Figure 3b, in which I_{NS} was not affected by pentobarbitone even at a high concentration of the agent (3×10^{-4} M). Thus, the possibility mentioned above was unlikely. For kinetic analysis of the effects of pentobarbitone on the inactivation process of I_{Ca}, I_{NS} should be subtracted from the recorded inward currents. Figure 3c shows semilogarithmic plots of the relative decay of actual I_{Ca} in the presence or absence of pentobarbitone, obtained after subtraction of I_{NS} using a graphic technique. Both fast and slow components of the decay phase of I_{Ca} were accelerated by pentobarbitone; in the presence of pentobarbitone, I_{Ca} seemed to decay with three different time courses.

It has been reported elsewhere that the decay phase of I_{Ca} may be fitted by a least, two exponential functions (Kostyuk, *et al.*, 1977; Brown *et al.*, 1981; Byerly & Hagiwara, 1982; Oyama, Nishi & Akaike, unpublished observation). At this moment, however, the slower components of the decay phase of I_{Ca} have yet to be characterized in more detail. Therefore, in the present experiments, effects of pentobarbitone on the fast component of the decay phase were analyzed. The time constant of the fast component was voltage-dependent. In the presence of pentobarbitone (3×10^{-4} M), the values at any given potential were markedly lower than controls (Figure 4b). On the other hand, the time constant of activation of I_{Ca} was not much affected by the agent, although values were slightly lower than controls at potentials below -10 mV.

Internal perfusion with EGTA Intracellular calcium ions are known to reduce I_{Ca} (Kostyuk *et al.*, 1977; Akaike *et al.*, 1978). If pentobarbitone, acting on the Ca^{2+} sequestering processes of the cell, were to block the uptake of intracellular Ca^{2+}, the phenomena so far described would be seen. We examined this possibility by perfusing the preparation internally with a solution containing 20 mM EGTA, assuming that most intracellular free Ca^{2+} would be chelated. After the start of internal perfusion with the EGTA-solution, the fast component of the decay phase of I_{Ca} gradually slowed, but the slow one was not affected. The preparation was perfused with the solution for more than 30 min and pentobarbitone was then applied. Results revealed that, even with internal perfusion with the EGTA-solution for a long period of time, the accelerating effects on the decay phase of I_{Ca} persisted; the time course of I_{Ca} in the presence of pentobarbitone was almost identical to that without internal perfusion with EGTA (not illustrated).

Effects of various external Ca^{2+} concentrations on actions of pentobarbitone

In order to characterize the nature of the inhibitory action of pentobarbitone on MPA of I_{Ca} and its accelerating effects on the decay phase, effects of various concentrations of $[Ca^{2+}]_o$ on the actions of the agent were examined. The experimental procedures were essentially similar to the previous ones (Akaike *et al.*, 1981; Akaike, Ito, Nishi & Oyama, 1982). $[Ca^{2+}]_o$ was varied between 5 and 20 mM, and Lineweaver-Burk plots were used to clarify the inhibitory actions of pentobarbitone on MPA. Measurements were first performed in 5 mM $[Ca^{2+}]_o$, because of the difficulty in obtaining stable records of I_{Ca} in pre- and post-control periods; two satisfactory experiments were obtained from 8 preparations. Fig-

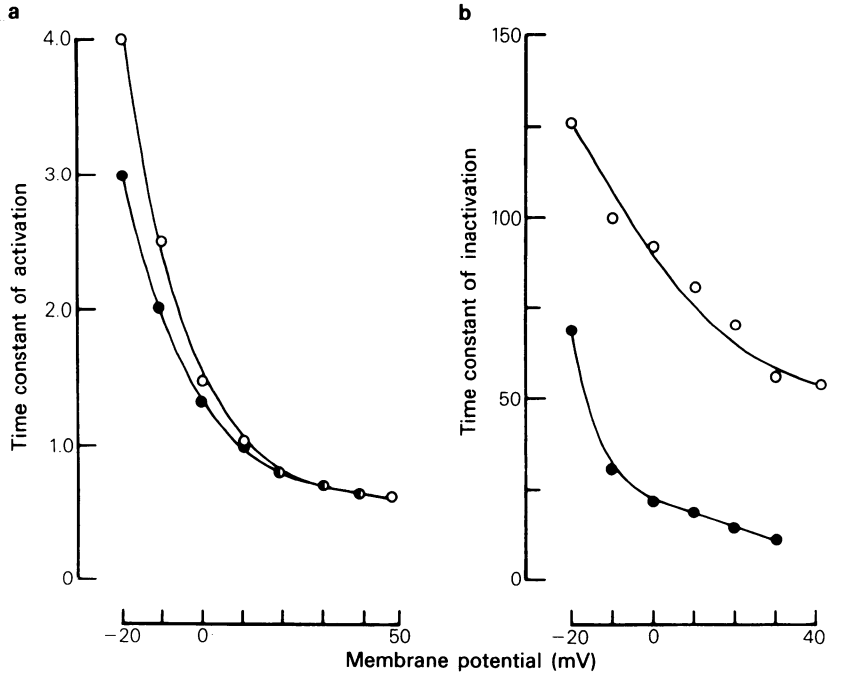

Figure 4 Effects of pentobarbitone on the time constants of activation (a) and the first component of inactivation (b). (○) Control; (●) pentobarbitone at a concentration of 3×10^{-4} M.

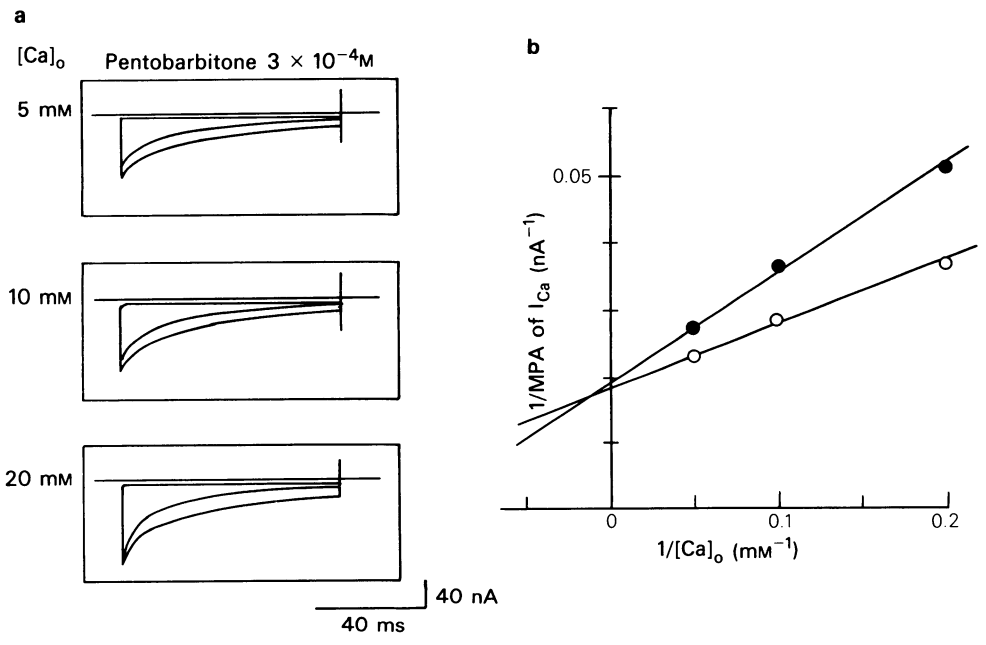

Figure 5 Effects of changes in extracellular calcium concentrations on I_{Ca} in the presence and absence of pentobarbitone (3×10^{-4} M). (a) Superimposed traces of I_{Ca} obtained before (lower trace) and 5 min after drug application (upper trace) at different external Ca^{2+} concentrations. (b) Lineweaver-Burk plots for pentobarbitone: (○) control; (●) pentobarbitone 10^{-4} M. Straight lines drawn by eye.

ure 5 shows records taken from an experiment in which the effects of 5, 10 and 20 mM $[Ca^{2+}]_o$ on the actions of pentobarbitone (3×10^{-4} M) were examined. Increases in $[Ca^{2+}]_o$ augmented I_{Ca} and shifted the potential to induce MPA to the positive direction. The depressant effects of the agent on MPA in 5 mM $[Ca^{2+}]_o$ were partially overcome in 20 mM $[Ca^{2+}]_o$. It is evident from this figure that the depressant effects of the agent on MPA are dependent on $[Ca^{2+}]_o$, but the accelerating effects on the decay phase of I_{Ca} are not. Lineweaver-Burk plots obtained from another experiment also demonstrate the inhibition of I_{Ca} by pentobarbitone, in which double reciprocal plots for MPA and $[Ca^{2+}]_o$ in the presence or absence of pentobarbitone intersected at different points on the ordinate scale. It is, therefore, reasonable to assume that pentobarbitone depresses MPA in a non-competitive manner in the snail neurone. Results are similar to those obtained with local anaesthetic agents (Akaike *et al.*, 1982).

Effects of changing extracellular pH on the inhibitory action of pentobarbitone

Since pentobarbitone is ionized in aqueous solution, the question arose as to whether the charged anionic form or the uncharged form is responsible for its action. We therefore examined the effects of changing external pH on the inhibitory actions of pentobarbitone. Since the pKa of pentobarbitone is 8.0, lowering pH would be expected to increase the ratio of uncharged form of the agent and *vice versa*, according to the Henderson-Hasselbach equation. Lowering the pH of the external solution bathing the preparation produces shifts in the current-voltage (I-V) relationships of I_{Ca} in the positive direction along the voltage axis (Oyama & Nishi, unpublished observations). In the present experiments, the potency of pentobarbitone at different external pH-values was assessed by comparing the MPA in each test solution containing a constant concentration of the agent (3×10^{-4} M). Figure 6a shows records obtained from the same cell. In the absence of the agent, an elevation of pH from 7.0 to 9.0 increased MPA by about 30–40%. However, MPA at pH 7.0 was markedly depressed by pentobarbitone, while MPA at pH 9.0 was not substantially affected. The mean inhibition by pentobarbitone of MPA at pH 7.0, 8.0 and 9.0 was 46%, 21% and 4% (5 experiments), respectively. Thus, pentobarbitone exhibited stronger inhibitory actions on I_{Ca} in the solution at low pH than in the solution at high pH, indicating that it may act on Ca^{2+} channels in the uncharged form to depress MPA and accelerate the decay of I_{Ca}.

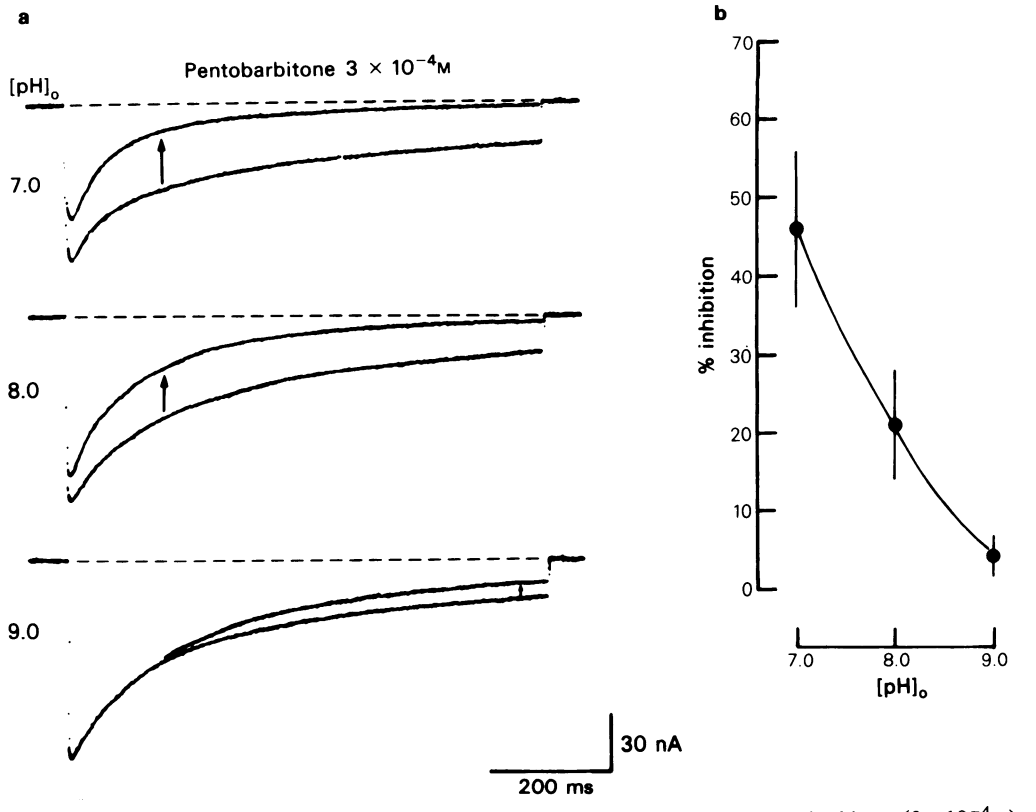

Figure 6 Effects of changes in extracellular pH on I_{Ca} in the presence and absence of pentobarbitone (3×10^{-4} M). (a) Superimposed traces of I_{Ca} before (lower trace) and 5 min after drug application (upper trace) at various pH. Arrows indicates changes in the time course of I_{Ca} before and after drug application. (b) Relative inhibition of MPA by pentobarbitone (3×10^{-4}M) in solutions of different pH. Each point indicates the average value of four experiments and vertical bars show s.e.mean.

Effects of internal application of pentobarbitone on I_{Ca}

Internal application of pentobarbitone at concentrations lower than 10^{-5} M did not produce any appreciable changes in I_{Ca}. However, at higher concentrations, depression of I_{Ca} was observed. The agent at a concentration of 10^{-3} M applied internally, gradually reduced MPA, the effect increasing with longer perfusion times and 10 min after internal application MPA was reduced by 15–30% of control in 3 cases examined. Depression occurred over the entire voltage range and no shifts of the threshold and the voltage necessary to induce the MPA of I_{Ca} were observed. Pentobarbitone did not exert any accelerating actions on the decay phase of I_{Ca}, as observed in the case of the external application. On the contrary, the time course of decay of I_{Ca} was slightly slowed in the internal presence of pentobarbitone (Figure 7).

Effects of repetitive stimulation of I_{Ca}

The above results suggest that in the presence of pentobarbitone the accelerated decay phase of I_{Ca} evoked by a single depolarizing pulse would alter the response of the neuronal membrane to successively applied pulses. Therefore, we examined the effects of repetitive stimulation in the absence or presence of pentobarbitone. The MPA of I_{Ca} induced by depolarizing pulses of 10–20 ms duration was not affected when the interval of successive pulses was over 300–500 ms. However, MPA decreased with increase in the number of successive pulses, depending upon the interval, at intervals shorter than those mentioned. Figure 8b shows a frequency-dependent decrease of MPA in the control solution. The neurone was stimulated repetitively at an interval of 150 ms. The MPA induced by a 40th pulse was reduced to about 40% of the initial MPA. Pentobarbitone (10^{-4}M), which reduced the initial MPA by

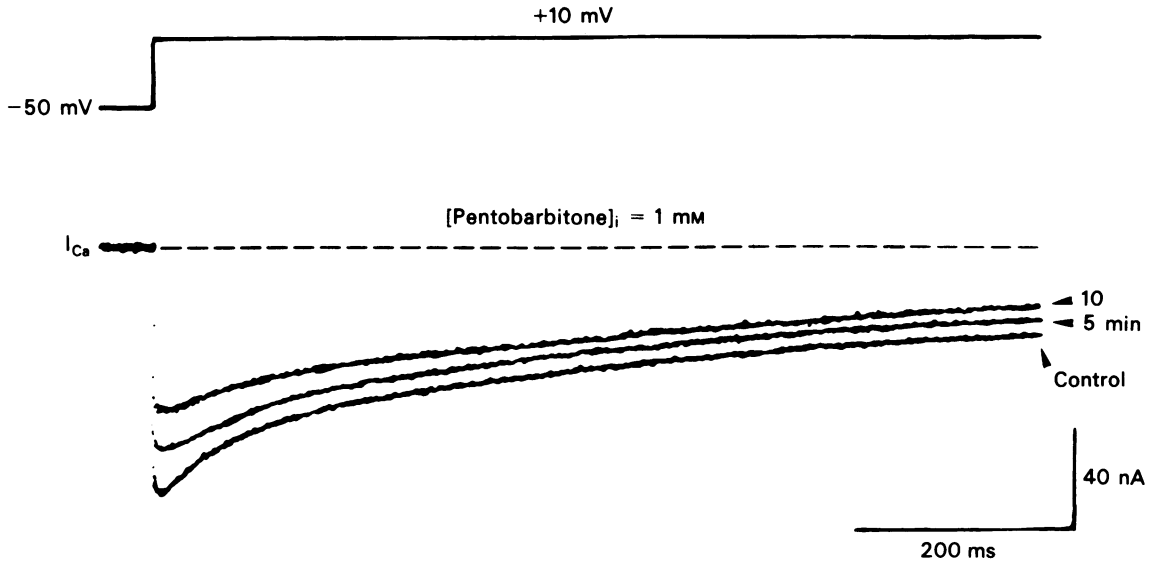

Figure 7 Effects of internal application of pentobarbitone on I_{Ca}: 5 min indicates 5 min after the start of internal perfusion with solution containing pentobarbitone (10^{-3} M); 10 indicates 10 min after internal application. I_{Ca} was elicited by a depolarizing pulse of +10 mV from the holding potential of −50 mV.

about 10% 5 min after the start of superfusion, markedly accelerated the decrease of the MPA of I_{Ca} induced by successive pulses (Figure 8c). Even at stimulus intervals which did not affect MPA during the control period, the agent depressed MPA induced by successive pulses. Thus, pentobarbitone induced a frequency-dependent depression of I_{Ca}.

Discussion

Two main conclusions may be drawn. The first is that pentobarbitone can block MPA of I_{Ca} in a manner similar to lignocaine and propranolol, as reported previously (Akaike *et al.*, 1982). This conclusion is based on the following observations: (1) pentobar-

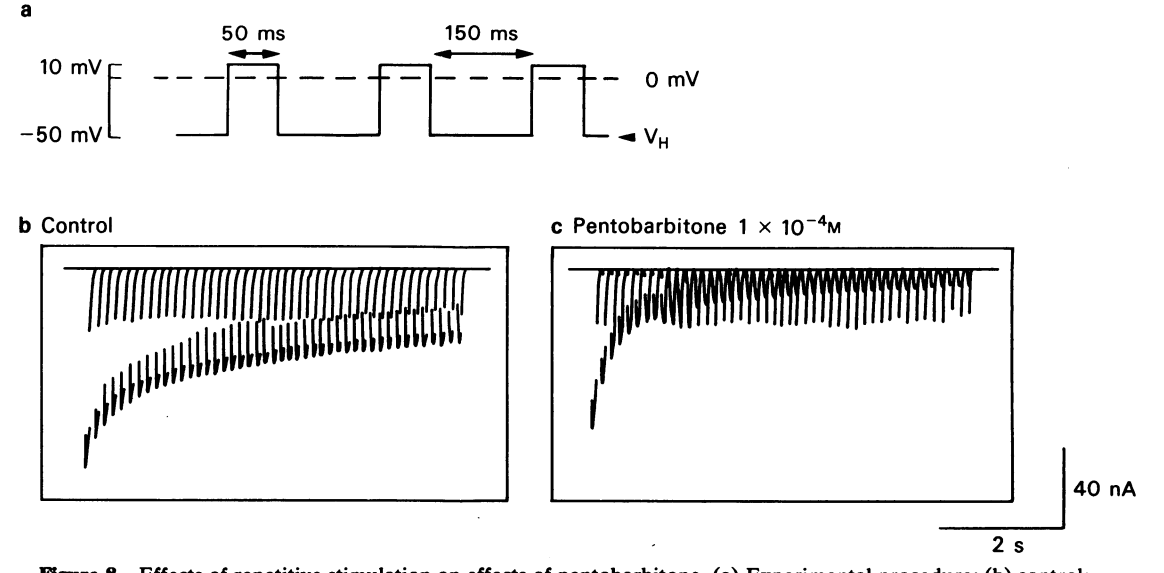

Figure 8 Effects of repetitive stimulation on effects of pentobarbitone. (a) Experimental procedure; (b) control; (c) in the presence of pentobarbitone (10^{-4} M). Current trace was obtained 5 min after the start of drug application.

bitone inhibited MPA of I_{Ca} dose-dependently over the entire voltage range of the I-V relationship without shifting the threshold and peak potentials of the I-V relationship; (2) increases in external Ca^{2+} concentration in the presence and absence of the agent gave Lineweaver-Burk plots which did not intersect on the ordinate scale; (3) internal application of the agent depressed the MPA of I_{Ca}.

The second major conclusion is that pentobarbitone, acting directly on the soma membrane, exerts accelerating effects on the decay phase of I_{Ca}. These are completely different from those of other organic and inorganic Ca^{2+} antagonists and the local anaesthetics so far examined in our laboratory. This conclusion stems from the observations that pentobarbitone did not augment I_{NS} and that, in the presence of internal EGTA which would be expected to chelate intracellular free Ca^{2+}, pentobarbitone still accelerated the decay of I_{Ca}. Thus, the accelerating effects of the pentobarbitone were not due to secondary effects mediated through other mechanisms. Implicit in the second conclusion is that the accelerating actions of the agent on the decay of I_{Ca} is due to open channel block. The actions of the agent on I_{Ca} in many ways simulate those of pancuronium on sodium current (I_{Na}) in the giant axon of the squid (Yeh & Armstrong, 1978) and quinacrine on open endplate channels (Adams & Feltz, 1980); they can be largely explained by analogy with the actions of these agents in inactivating the Na^+ channel. Once the Ca^{2+} channel is activated by a voltage-sensitive gate, Ca ions as well as pentobarbitone molecules in an uncharged form enter the channels. Pentobarbitone will then bind at receptor sites in common with Ca^{2+} in the pore of the channel and block the translocation of Ca^{2+} from the external phase to the cell interior. However, it can only equilibrate with its binding site if the channel is activated, and equilibration requires an appreciable fraction of a millisecond. Therefore, the channel allows passage of Ca^{2+} transiently. This would explain why the activation process of I_{Ca} was not much affected by pentobarbitone. However, the agent gradually binds to the site blocking the open-state of Ca^{2+} channels. Similar ideas in relation to the endplate current have been developed by Adams (1976), suggesting barbiturates block open channels. However, further characterization of the effects of pentobarbitone on the inactivation process of I_{Ca} in the snail neurone are necessary (cf. studies on pancuronium and I_{Na} by Yeh & Narahashi, 1977) before drawing a final conclusion. Nevertheless, the present experiments are the first to demonstrate that pentobarbitone exerts accelerating effects on the decay phase of I_{Ca} in the snail neurone. This pharmacological action of pentobarbitone is different from its known effects on the excitable membrane.

The present results regarding the potency of pentobarbitone at various pH values lend strong support to the notion that the active form of the agent is the uncharged one, as in the case of the endplate (Adams, 1976). However, there is one aspect of the data that requires further experimental examination. In Figure 6a the MPA of I_{Ca} at pH 9.0 was larger (about 150%) than that obtained at pH 7.0 in the absence or pentobarbitone, while pentobarbitone was more potent at low pH. One can argue that this is simply due to the greater driving force inducing I_{Ca} which would overcome the inhibitory action of pentobarbitone, just as observed on increasing $[Ca^{2+}]_o$. However, this is unlikely since in the I-V relationships for I_{Ca} the inhibitory effects of the agent were almost the same over a certain range of depolarizing voltages. At a concentration of 10^{-4}M, peak amplitude of I_{Ca} at +20 mV (150% relative to that induced at −5 mV) was reduced by about 23% while peak amplitude of I_{Ca} elicited at −5 mV was depressed by about 25% (Figure 2). Therefore, it is reasonable to assume the potency of pentobarbitone increases at a pH lower than its pKa where the ratio of the uncharged to the charged form is elevated. However, the possibility that changes in pH may modify characteristics of the Ca^{2+} channel cannot be ruled out.

As to the site of action of pentobarbitone in inhibiting MPA and accelerating the decay of I_{Ca}, the present results do not allow the same conclusion as in the case of pancuronium on I_{Na} (Yeh & Armstrong, 1978). Internal application of pentobarbitone depressed MPA of I_{Ca}, but exerted practically no effect on the decay of I_{Ca}. This was unexpected. The present results indicate that a pentobarbitone molecule in the uncharged form may act on the outer end of the Ca^{2+} channel and thus block the open state of the channel. The pentobarbitone molecule might bind to a site with a hydrophobic component within the pore of the Ca^{2+} channel so reducing MPA of I_{Ca}. Even if a certain amount of pentobarbitone passed from the internal phase of the cell to the surface of the membrane, the agent would be washed away with the constant flow of external perfusate, and hence could not act on the outer end of the pore of the Ca^{2+} channel.

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