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DIFFERENTIAL ACTIONS OF PENTOBARBITONE ON CALCIUM CURRENT COMPONENTS OF MOUSE SENSORY NEURONES IN CULTURE

BY ROBERT A. GROSS AND ROBERT L. MACDONALD

From the Department of Neurology, University of Michigan Medical Center, Ann Arbor, MI 48104-1687, U.S.A.

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SUMMARY

1. Using the single-electrode voltage clamp technique, three calcium current components were recorded at 35 °C from mouse dorsal root ganglion (DRG) neurones in culture. A transient low-threshold calcium current (T current) was recorded at clamp potentials (V_c) positive to −60 mV. Holding potentials (V_h) at or negative to −90 mV were required to fully remove inactivation. A large transient high-threshold calcium current component (N current) was recorded at V_c positive to −40 mV. V_h at or negative to −80 mV removed all steady-state inactivation. A slowly inactivating high-threshold calcium current component (L current) was recorded at V_c positive to −30 mV. Inactivation was removed by V_h at or negative to −60 mV. When currents were evoked at V_c positive to −20 mV from V_h negative to −60 mV, all three calcium current components were present.

2. Pentobarbitone (500 μM) had no effect on the isolated T current, but reduced the isolated L current 50–100% when evoked at V_c of −20 to 0 mV from V_h of −50 mV. Pentobarbitone had voltage-dependent effects on calcium currents containing all three calcium current components. Pentobarbitone produced small and equal reductions of the peak and late ($\geqslant$ 300 ms) calcium currents evoked at −20 to 0 mV from V_h at or negative to −80 mV, but at more positive V_h there was a greater reduction in the peak current. The rate of current inactivation was increased in the presence of pentobarbitone.

3. Current–voltage plots were constructed from currents recorded in the absence and presence of 500 μM-pentobarbitone. Pentobarbitone reduced the magnitude of the calcium current without affecting the voltage dependence of the current–voltage relation.

4. Calcium current traces were fitted with a multiexponential function to determine the amplitudes and inactivation time constants (τ_i) of the three calcium current components. Inactivation time constants decreased with more positive V_c for all three calcium current components. Pentobarbitone reduced only those τ_i corresponding to the N current.

5. Recovery from inactivation of the N current was determined using a two-pulse protocol. In control neurones, recovery from inactivation occurring at 0 mV was slower at $V_h = -65$ mV than at $V_h = -80$ mV. In the presence of pentobarbitone,

recovery from inactivation was faster, and occurred at a similar rate at both potentials.

6. Steady-state inactivation curves for the N current were derived from neurones in the absence and presence of pentobarbitone. The steady-state inactivation curve was shifted -10 to -15 mV by pentobarbitone at V_h positive to -80 mV.

7. Multiexponential curve fitting of barium current traces revealed that there was a greater proportion of the L current than in calcium currents, and that the τ_i were larger. In contrast, the proportion of the N current was less, and the τ_i were smaller. When barium currents were evoked from V_h of -80 mV, pentobarbitone reduced the magnitude of the L current only, and had no effect on the τ_i of any of the current components.

9. Pentobarbitone reduced the L current at V_h as negative as -90 mV, possibly by preventing channel activation. The reduction in the N current was due to enhancement of steady-state inactivation and reduction in the inactivation time constant.

INTRODUCTION

Barbiturates are widely used clinically and experimentally and have multiple synaptic and non-synaptic effects on the nervous sytem. Neurotransmitter release is inhibited by barbiturates (Brooks & Eccles, 1947; Kalant & Grose, 1967; Morgan & Bryant, 1977), an effect that may be due, in part, to a reduction of neuronal calcium influx. Support for this idea comes from studies showing that barbiturates reduce the duration of calcium-dependent action potentials (Heyer & Macdonald, 1982), reduce synaptosomal calcium influx (Blaustein & Ector, 1975; Leslie, Friedman, Wilcox & Elrod, 1980), and reduce voltage-dependent calcium currents of invertebrate (Nishi & Oyama, 1983; Ikemoto, Mitsuiye & Ishizuka, 1986) and vertebrate neurones (Werz & Macdonald, 1985; Gross & Macdonald, 1988*a*).

Recently, the co-existence of multiple voltage-dependent calcium current components in neurones has been reported. In addition to the 'classical' slowly inactivating (L) calcium current, which is activated at relatively positive membrane potentials (e.g. Hagiwara & Byerly, 1981; Hess, Fox, Lansman, Nilius, Nowycky & Tsien, 1986), a low-threshold transient (T) calcium current has been described in both invertebrate and vertebrate neurones (e.g. Llinás & Yarom, 1981; Carbone & Lux, 1984; Deitmer, 1984; Matteson & Armstrong, 1986; Carbone & Lux, 1987*a*, *b*). A third type of calcium current (N) has been observed in vertebrate sensory neurones (Nowycky, Fox & Tsien, 1985; Fox, Nowycky & Tsien, 1987*a*, *b*; Gross & Macdonald, 1987). In single-channel experiments, unitary barium currents of three different conductances have been recorded using voltage clamp protocols similar to those used to evoke the different calcium current components (Fox *et al.* 1987*b*).

In an earlier study we showed that the barbiturates phenobarbitone and pentobarbitone selectively reduced neuronal voltage-dependent calcium current components (Gross & Macdonald, 1988*a*). We therefore investigated the mechanisms by which pentobarbitone reduced N and L currents in cultured mouse dorsal root ganglion (DRG) neurones. We report that the reduction of the L current was most likely due to prevention of channel activation. In contrast, pentobarbitone reduced

the N current by enhancing steady-state inactivation of the channel, and by reducing its inactivation time constant, resulting in a more rapid inactivation of this calcium current component.

METHODS

Cell culture. DRG and spinal cord neurones from 12- to 14-day fetal mice were grown in culture as previously described (Ransom, Neale, Henkart, Bullock & Nelson, 1977; Gross & Macdonald, 1987), with the following modifications. Initial medium was Eagle's minimal essential medium supplemented with 5% horse serum, 10% fetal calf serum (all from GIBCO, Grand Island, NY, U.S.A.), 5% Nu-Serum (Collaborative Research, Lexington, MA, U.S.A.) and 1·5 g $NaHCO_3$ and 5·5 g glucose/l. Nerve growth factor (NGF) was added to a final concentration of 500 ng/ml. Subsequent medium changes were at 3–4 day intervals, and the medium was supplemented with 5% horse serum, 5% Nu-Serum and 10 ng/ml NGF. Cultures were used for experiments after 4–12 weeks.

Intracellular recording and single-electrode voltage clamp. For voltage clamp experiments, recording medium (pH 7·3–7·4 at 35 °C) contained (in mM): choline chloride, 67·0; Tris base, 13·0; KCl, 5·3; $CaCl_2$, 2·0, or $BaCl_2$, 5·0; $MgCl_2$, 0·8; glucose, 5·6; tetraethylammonium chloride, 100. This eliminated sodium curents and virtually eliminated potassium currents.

Voltage clamp recordings were made as previously described (Gross & Macdonald, 1987). Briefly, recording micropipettes (20–30 MΩ) were filled with 3 M-CsCl to further suppress potassium currents. The single-electrode voltage clamp amplifier (Axoclamp 2, Axon Instruments, Burlingame, CA, U.S.A.) switched between voltage sampling and current injection at 6–12 kHz with a 70–30% duty cycle. The settling time of the voltage clamp was between 2 and 5 ms. The program pCLAMP (Axon Instruments) was used to generate voltage step commands and to digitize, store and analyse the voltage and current traces. Traces were sampled at 1·6–5·0 kHz, depending on the duration of the voltage command (512 samples per sweep).

Leak currents were determined by two different methods. Hyperpolarizing voltage commands of magnitudes equal to the depolarizing commands used to evoke the inward currents produced inward currents assumed to be equal and opposite in magnitude to leak currents. With large hyperpolarizing steps to clamp potentials (V_c) negative to −120 mV, however, a small inward current was evoked. This slowly activating (20–100 ms) current caused a slight overestimation of leak currents. Alternatively, leak currents were recorded during depolarizing commands equivalent to those used to evoke inward currents after block of calcium currents by cadmium. In either case, leak currents were subtracted digitally from the relevant inward currents to yield the calcium or barium currents.

Recordings were made from large DRG neurones (15–30 μm in diameter) that were nearly spherical and without large processes to minimize space clamp artifacts. If poor space clamp control occurred, those trials were not included in the analyses.

Drug preparation and application. Pentobarbitone (Sigma Chemical Co., St Louis, MO, U.S.A.), prepared fresh for each experiment, and $CdCl_2$ (100 μM) were delivered from micropipettes (tip diameters of 10–20 μm) positioned 10–60 μm from the cell body. Drugs were allowed to diffuse from the tip and/or were delivered by pressure ejection at 0·25–1·0 lbf/in² (1·68–6·7 kPa). Micropipettes containing diluent (recording medium) had no effect on evoked or holding currents.

Experimental paradigms and analysis of currents. The three calcium current components recorded in mouse DRG neurones were (see Fig. 1), given the differences in experimental conditions, quite similar to T-, N- and L-type currents previously recorded in chick DRG neurones (Nowycky *et al.* 1985; Fox *et al.* 1987*a*, *b*). Unlike the N and L currents, the T current was relatively resistant to the calcium channel blocker cadmium (Gross & Macdonald, 1987).

Although both L and T currents could be recorded in isolation, only the T current could be isolated consistently. At holding potentials (V_h) at or positive to −50 mV, the N current was largely inactivated, allowing the L current to be evoked at V_c positive to −30 mV. However, since slow inactivation of this current occurred at these V_h (over seconds), full recovery from drug effects was unusual.

From V_h at or negative to −80 mV, calcium currents evoked positive to −30 mV contained primarily the N and L current components, as well as the smaller T current component. The use

of long voltage step commands (300–500 ms) allowed substantial inactivation of the N current; the residual current at the end of the voltage command could then be used to estimate the magnitude of the L current component. The peak minus the late calcium current (i.e. at 300 or 500 ms) then served as an estimate of the N current component. In this way, the effect of drug treatment was assessed on each current component.

There were two sources of error introduced by this type of analysis. Estimation of the L current as the current remaining at 300 ms ignored the inactivation of this current during that time. The L current inactivated less than 20–30% during a 300 ms command at −30 to +10 mV, however. In neurones held at potentials negative to −60 mV, the peak calcium current also contained the T current. This rarely contributed more than 10–15% of the peak current, however. For most analyses, both sources of error could be ignored.

Another method for assessing drug effects on calcium and barium current components was used. Current traces were fitted using a multiexponential curve-fitting program (Asystant, Macmillan Software Co., New York, NY, U.S.A.). Digitized currents were truncated so that traces were fitted after the current peak and before the offset of the voltage step command. The program allowed an estimation of amplitude and inactivation time constants (τ_i) for each of the calcium current components. Leak-subtracted calcium currents were fitted according to the equation:

$$y(t) = A_1 \exp(-t/\tau_{i1}) + A_2 \exp(-t/\tau_{i2}) + A_3 \exp(-t/\tau_{i3}) + A_4,$$

where $A_{1\ldots3}$ represent the amplitudes of the current components, $\tau_{i1\ldots3}$ represent the corresponding inactivation time constants, and A_4 represents a residual value (usually an error in the estimation of leak currents). This method gave results similar to those described above with regard to the effect of pentobarbitone on the amplitude of the calcium or barium current components. The curve-fitting routine was used primarily to determine quantitatively the effect of drug treatment on the amplitudes and τ_i of the different current components. If current component amplitudes were too small to be accurately or precisely fitted (e.g. the L current in the presence of pentobarbitone), those data were excluded from the analyses.

RESULTS

The reduction of calcium current by pentobarbitone was due to a selective effect on calcium current components

In all neurones tested, pentobarbitone reversibly reduced calcium currents. The lowest concentration that produced a reproducible effect was 50 μM, but 500 μM, which produced a maximal effect, was used for the experiments reported here. The isolated T current, however, was unaffected by pentobarbitone in twelve out of twelve neurones (Fig. 1*A*). The voltage range of steady-state inactivation of this current was −90 to −50 mV. When evoked at −60 to −50 mV from V_h of −90 to −100 mV, inactivation was usually complete within 50 ms, but in many neurones incomplete inactivation was seen.

The L current was recorded at V_c positive to −30 mV (Fig. 1*B*). The voltage range of steady-state inactivation was −60 to −20 mV. When evoked at −20 to 0 mV from −50 to −40 mV, less than 30% of the L current inactivated during a 300 ms voltage command. This current component was reduced 50–100% in the presence of 500 μM-pentobarbitone in all cells tested. In one series of experiments, the mean reduction in the L current was 72 ± 10·2% (S.E.M., $n = 7$ neurones).

The N current was evoked at V_c positive to −40 mV. The voltage range of steady-state inactivation for this current component was −80 to −30 mV. From V_h of −80 mV, calcium currents evoked at −20 to 0 mV contained the N and L currents, as well as the smaller (partially inactivated) T current (Fig. 1*C*). The N current inactivated completely between 300 and 800 ms.

The effect of pentobarbitone on combined N and L currents was voltage dependent. When calcium currents were evoked at V_c positive to -30 mV from a holding potential at or negative to -80 mV (Fig. 1*C a*), pentobarbitone produced a small reduction in the peak calcium current; as previously reported (Werz & Macdonald, 1985; Gross & Macdonald, 1988*a*), the current inactivated at a faster

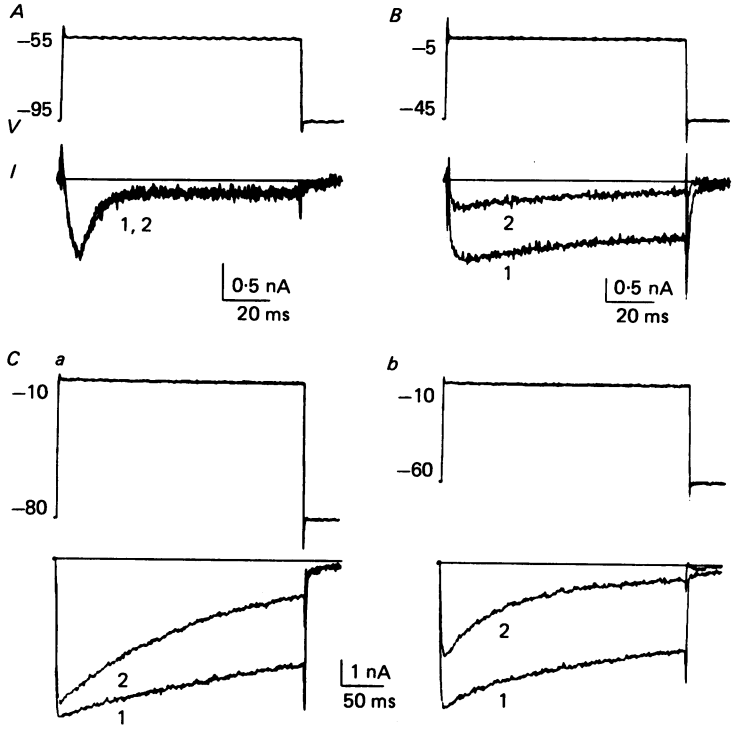

Fig. 1. Pentobarbitone selectively reduced calcium current components in DRG neurones. In this figure, digitized voltage traces are shown above the current traces, and in subsequent figures illustrative voltage traces are shown; in all cases, they show the holding potentials (V_h) and voltage steps (mV). Digitized leak-subtracted currents are shown; inward currents are downward. The currents were recorded in the absence (trace 1) and presence (trace 2) of 500 μM-pentobarbitone in two different neurones (*A*, *B* and *C*). *A*, the T current was unaffected by pentobarbitone. *B*, the L current was reduced by pentobarbitone. Note that in the control trace (trace 1), there is a residual N current at this holding potential. *C*, pentobarbitone hastened current inactivation and reduced the peak current more at relatively positive V_h. Calcium currents were evoked from $V_h = -80$ mV (*Ca*) or $V_h = -60$ mV (*Cb*).

rate. The reduction in the peak and late calcium current amplitudes was similar in those neurones in which the N current had largely inactivated by the end of the voltage command (see Figs 4*A a* and 6*A*). In contrast, the reduction in the peak calcium current was greater when currents were evoked at V_h positive to -80 mV (Fig. 1*C b*). Again, the calcium current inactivated at a faster rate.

Pentobarbitone had selective effects on the different calcium current components of DRG neurones. The T current was unaffected, while the L current was reduced by

pentobarbitone. We concluded that the reduction of the L current accounted for the reduction in the peak and late calcium currents evoked from very negative V_h. The faster inactivation of the calcium current and the greater reduction of the peak current at relatively positive V_h were due, therefore, to effects on the N current. The next series of experiments was designed to test this hypothesis, and to determine the mechanism by which pentobarbitone affected calcium currents.

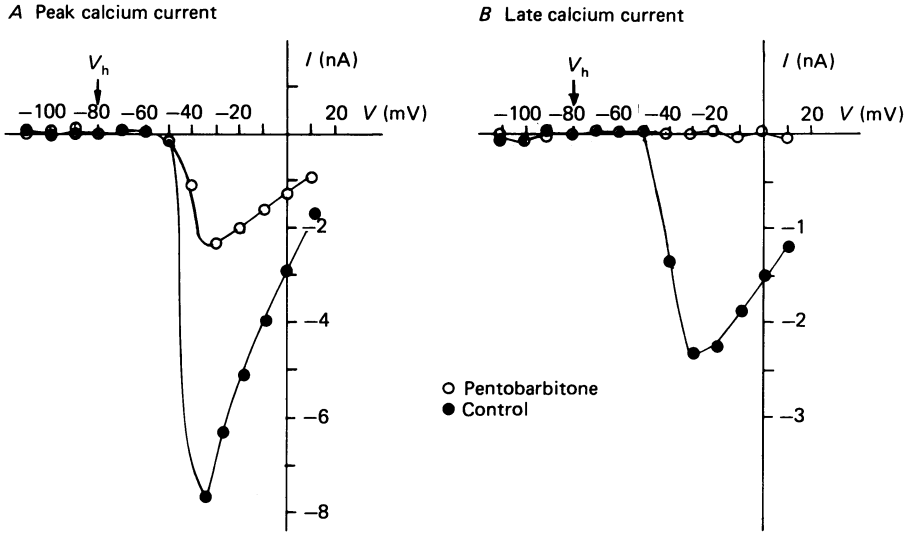

Fig. 2. Current–voltage relation of calcium currents in the presence of pentobarbitone. Plots were derived from cadmium leak-subtracted currents in a single neurone. V_h = −80 mV, with 300 ms voltage commands in 10 mV increments from −110 to 10 mV. Measurements of peak calcium current (*A*; at ~ 10 ms) and late calcium current (*B*; at 300 ms) were taken from currents in which the N current had largely inactivated by the end of the voltage command. There was no change in the voltage dependence of the calcium current–voltage relation in the presence of pentobarbitone (500 μM, ○).

Pentobarbitone did not affect the voltage dependence of the calcium current–voltage relation

To determine if the reduction of calcium current by pentobarbitone was due to a change in the voltage dependence of activation of the current, calcium current–voltage plots were constructed from calcium current traces obtained in the absence and presence of 500 μM-pentobarbitone (Fig. 2). From a holding potential of −80 mV, 300 ms hyperpolarizing and depolarizing commands were applied in 10 mV increments from −110 to +10 mV. Because of limited cell viability, voltage commands were applied every 5 s. With this frequency of stimulation, however, the calcium currents did not fully recover from inactivation between voltage steps (see below). The result was an accumulation of inactivation during that part of the trial which consisted of the depolarizing voltage commands.

Current–voltage plots were constructed from currents in which the N current had largely inactivated by the end of the 300 ms voltage command. Both peak calcium

current–voltage plots ($\sim$ 10 ms; Fig. 2*A*) and late calcium current–voltage plots (300 ms; Fig. 2*B*) were derived after cadmium leak subtraction. The peak and late calcium currents were maximally activated at -40 to -30 mV, and at -30 to -20 mV, respectively. Pentobarbitone reduced the peak current and abolished the late current without affecting the voltage dependence of the calcium current–voltage relation. The reduction in the late calcium current, consisting primarily of the L current, was consistent with the effect on this current component previously noted (Fig. 1*B*). The effect of pentobarbitone on the peak calcium current, however, was larger than usually seen with single voltage commands from a holding potential of -80 mV. Since pentobarbitone did not affect the time or voltage dependence of current activation, the exaggerated reduction of the peak calcium current seen in the current–voltage plots could be explained by enhanced accumulation of inactivation in the presence of pentobarbitone. We therefore tested the effect of pentobarbitone on the inactivation of the calcium current components, especially that of the N current.

Pentobarbitone reduced the inactivation time constant of the N current

To test whether pentobarbitone affected the time dependence of inactivation of any of the calcium current components, currents were evoked every 20 s at -30, -10 and $+10$ mV from a holding potential of -80 mV, in the absence and presence of 500 μM-pentobarbitone (Fig. 3*A*). In control conditions (Fig. 3*A a*), at -30 mV, the calcium current usually inactivated to a near-plateau by the end of the 300 ms voltage command; at -10 mV, the peak current was reduced but the late current was similar to that at -30 mV. At $+10$ mV both the peak and late currents were reduced from their values at -10 mV. Pentobarbitone (Fig. 3*A b*) reduced the peak and late currents to a similar extent, and hastened current inactivation (compare with Fig. 1*C a*, *b*).

These current traces were fitted using a multiexponential curve-fitting program, and inactivation time constants (τ_i) were estimated for each of the three calcium current components (Fig. 3*B*). For each component, τ_i decreased with increasingly positive V_c. Pentobarbitone had no effect on either the short τ_i, presumably those of the T current (Fig. 3*B a*), or the long τ_i of the L current (Fig. 3*B c*). In contrast, pentobarbitone reduced the intermediate τ_i of the N current at both -30 and -10 mV (Fig. 3*B b*). At $+10$ mV, the τ_i of the N current was similar in the absence and presence of pentobarbitone. In the presence of pentobarbitone, the τ_i of the N current were reduced to similar values at all V_c; at more positive V_c, therefore, the effect of pentobarbitone appeared to lessen.

Pentobarbitone had no effect on the amplitude of either the T or the N currents (as determined by curve fitting) when evoked at -30, -10 or $+10$ mV from a holding potential of -80 mV. There was, in contrast, a reduction of 50–80% in the (curve-fitted) magnitude of the L current in the presence of pentobarbitone. For example, in five neurones in which the L current could be precisely fitted, the L current was reduced to $46 \pm 0{\cdot}06$% (S.E.M.) of control. In the same neurones, the N current was $106 \pm 0{\cdot}10$% of control.

Two-pulse protocols were used to test whether pentobarbitone affected the time dependence of recovery from inactivation of the N current. The recovery from

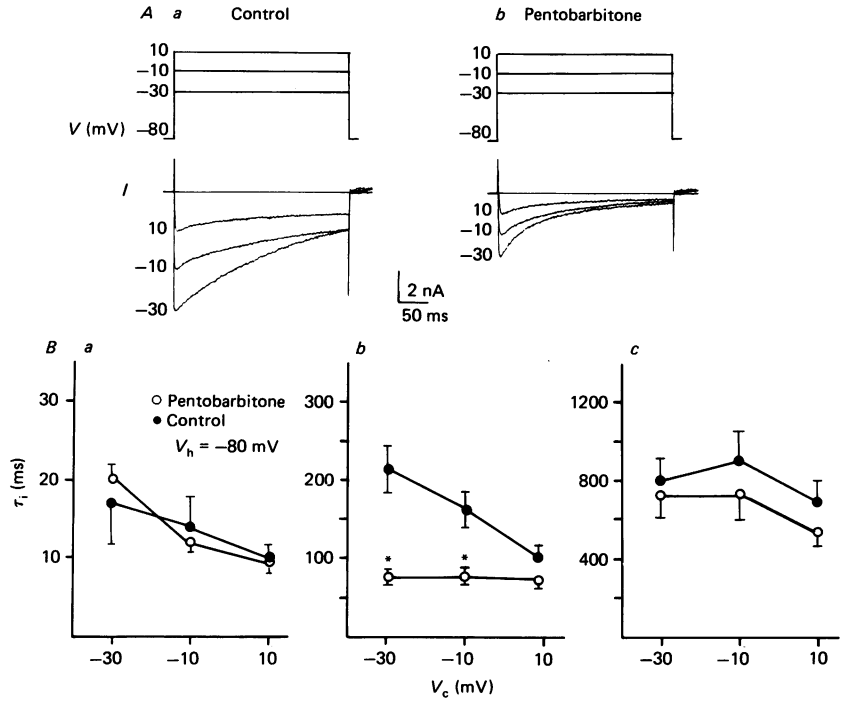

Fig. 3. Pentobarbitone reduced calcium currents and hastened inactivation of the N current. *A*, currents were evoked for 300 ms at $V_c = -30$, -10 and $+10$ mV from $V_h = -80$ mV. Control currents (*A a*) were evoked every 20 s, and then 500 μM-pentobarbitone was applied by diffusion for 30 s, after which the voltage commands were repeated (*A b*). Digitized leak-subtracted current traces from a single representative neurone are shown. Pentobarbitone reduced both peak and late calcium currents at each test potential. While the apparent rate of calcium current inactivation was increased at each potential, this effect seemed less pronounced at more positive potentials. *B*, calcium current traces similar to those in *A* were fitted according to a multiexponential function (see Methods) to determine the inactivation time constants (τ_i) of the three calcium current components. Data were obtained from five to seven neurones, and the mean τ_i values (±S.E.M.) are plotted as a function of the clamp potentials (V_c) for the T (*B a*), N (*B b*) and L (*B c*) currents, both in the absence (●) and presence (○) of 500 μM-pentobarbitone. The τ_i decreased with more depolarized V_c; pentobarbitone reduced the τ_i only for the N current (*B b*). Error bars are sometimes shown in one direction only for clarity of illustration. Asterisks represent a difference from control values by Student's paired two-tailed *t* test at the $P < 0.05$ level.

inactivation produced at $V_c = 0$ mV was determined at V_h of -80 and -65 mV (Fig. 4*A a* and *b*, respectively). The first of the paired pulses was 300 ms in duration ('conditioning' step); after a variable recovery period (10–15000 ms), a 50 ms voltage step was used to assess the extent of recovery ('test' step). Pairs of currents were recorded in individual neurones in the absence and presence of 500 μM-pentobarbitone.

The current remaining at 300 ms in the conditioning steps (I_{300}) was subtracted from the peak current value of the test step (I_{p2}) to yield the net recovery from inactivation. Subtraction of I_{300} from the peak current value of the conditioning step

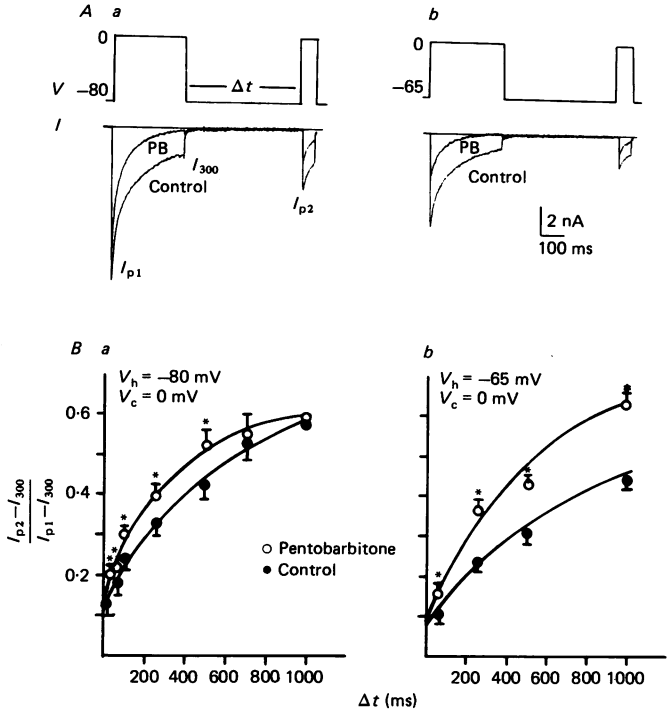

Fig. 4. Pentobarbitone hastened recovery from inactivation of the N current. *A*, digitized leak-subtracted currents from a single neurone are shown. A 300 ms conditioning voltage step to 0 mV was followed at varying intervals by a 50 ms test voltage step to 0 mV. $V_h = -80$ mV (*A a*) or -65 mV (*A b*). After control currents were recorded, pentobarbitone (500 μM) was applied by diffusion for 30 s, and the voltage commands repeated (PB). Pentobarbitone reduced the peak calcium current more at $V_h = -65$ mV (*A b*) than at -80 mV (*A a*). The peak (I_{p1} and I_{p2}) and late (I_{300}) calcium current values were used to calculate the degree of recovery from inactivation at various time intervals (Δt). *B*, the recovery from inactivation occurring at $V_c = 0$ mV at $V_h = -80$ mV (*B a*) or -65 mV (*B b*) was calculated (see text) from calcium currents in the absence (●) or presence (○) of 500 μM-pentobarbitone. The fractional recovery from inactivation was plotted as a function of the time interval (Δt) between the conditioning and test voltage steps. Pentobarbitone caused a more rapid recovery from inactivation at $V_h = -65$ mV (*B b*) than at $V_h = -80$ mV (*B a*). Values are the mean ± S.E.M. ($n = 6$–10 neurones, except for the 1000 ms point in *B a* ($n = 2$)). Error bars are shown in one direction only. The asterisk represents a difference from control using Student's paired two-tailed t test at the $P < 0{\cdot}05$ level.

(I_{p1}) yielded the net inactivated current. The ratio $(I_{p2}-I_{300})/(I_{p1}-I_{300})$ thus represented the proportion of the current recovered from inactivation. Since the N current constituted the largest component of inactivating current, this calculation estimated the recovery from inactivation of the N current. A slight overestimate resulted, however, because this method did not take into account the contribution of the T current to the peak currents, nor the inactivation of the L current.

In control neurones, at $V_h = -80$ mV (Fig. 4*B a*), the N current recovery from inactivation was about 20% at 50 ms and about 60% at 1000 ms. We found that full

recovery from inactivation required 15 s at this V_h (data not shown). Pentobarbitone enhanced the rate of recovery from inactivation up to 500 ms, but not at longer intervals. At $V_h = -65$ mV, recovery from inactivation in control neurones was slower than at $V_h = -80$ mV (Fig. 4*B b*); recovery was about 20% at 200 ms, and only 40% at 1000 ms. At this holding potential, pentobarbitone speeded recovery from inactivation at intervals of up to 1000 ms; longer intervals were not tested. In the presence of pentobarbitone the rate of recovery was similar at both V_h.

Taken together, these experiments showed that pentobarbitone selectively reduced the τ_i of the N current at depolarized potentials and speeded its recovery from inactivation.

Pentobarbitone enhanced steady-state inactivation of the N current

To determine the effect of pentobarbitone on the steady-state inactivation of calcium currents, currents were evoked every 30 s with 300 ms voltage commands to 0 mV from V_h ranging from -90 to -20 mV. In some neurones, complete sets of calcium currents were obtained in the absence and presence of pentobarbitone (500 μM). In other neurones, either control or pentobarbitone currents were recorded.

In control neurones, V_h of -90 to -70 mV yielded similar calcium currents (Fig. 5*A a*). Positive to -70 mV there was a diminution in peak and late currents, and positive to -30 mV, little current remained (for illustrative purposes, only traces from V_h of -90 to -50 mV are shown). In the presence of pentobarbitone, there was a small reduction in the peak and late calcium currents at $V_h = -90$ and -80 mV, but at more positive V_h the reduction in peak calcium currents was greater.

From these data we constructed steady-state inactivation curves for the N current. Subtraction of the late calcium current (I_{300}) from the peak calcium current (I_p) yielded an estimate of the N current. Division of this value at any given potential by the maximum peak N current (i.e. at V_h of -90 or -80 mV) yielded the proportion of non-inactivated calcium current remaining at any given potential (Fig. 5*B*). In control neurones, the N current showed steady-state inactivation positive to -80 mV, inactivation being complete at -30 to -20 mV. Pentobarbitone produced a greater degree of inactivation at all V_h positive to -80 mV, i.e. a shift of -10 to -15 mV in the steady-state inactivation curve.

We also constructed steady-state inactivation curves using the N current amplitudes obtained from curve fitting of these current traces. These curves were similar to those described above (inset, Fig. 5*B*); the V_h at which the N current was inactivated 50% were within 3 mV.

Pentobarbitone did not appear to increase the rate of inactivation of the L current (visual inspection of Fig. 1*B*; Fig. 3*B c*). It was possible, however, that pentobarbitone may have increased the steady-state inactivation of this current component. We therefore constructed steady-state inactivation curves using either the late calcium current magnitudes to approximate the L current or using L current amplitudes obtained from curve fitting. Steady-state inactivation curves for control neurones showed inactivation beginning at V_h at or positive to -60 mV. In the presence of pentobarbitone, however, reliable steady-state inactivation curves could not be constructed because the L current magnitudes were so small that small variations in amplitude resulted in large variations in the normalized curves.

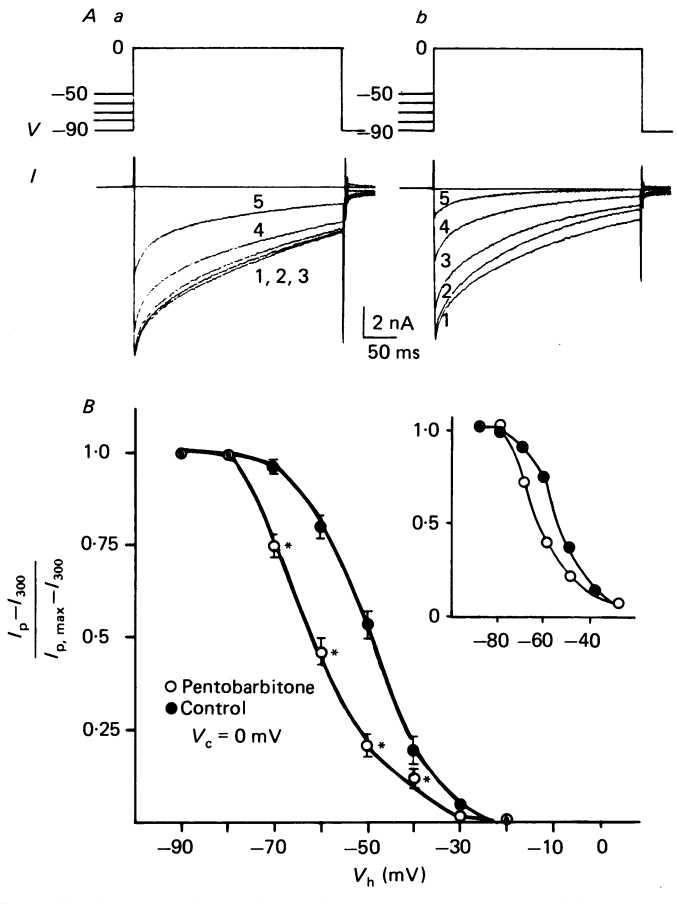

Fig. 5. Pentobarbitone enhanced steady-state inactivation of the N current at potentials positive to −80 mV. *A*, neurones were initially held at $V_h = -90$ or −80 mV and currents were evoked for 300 ms at $V_c = 0$ mV. The holding potential was reduced by 10 mV after the voltage command, which was repeated after 30 s. The range of V_h tested was −90 to −20 mV. The digitized leak-subtracted current traces shown here are from a single neurone held at potentials between −90 and −50 mV (current traces 1–5) in the absence (*A a*) and presence (*A b*) of 500 μM-pentobarbitone. Pentobarbitone increased steady-state inactivation at all V_h at or positive to −70 mV. *B*, steady-state inactivation curves for the N current in the absence (●) or presence (○) of 500 μM-pentobarbitone. The inset shows similar curves using the amplitudes of the N current derived from curve fitting ($n = 5$ neurones). The plots show the probability of *not* being inactivated as a function of V_h. The $V_c = 0$ mV. Pentobarbitone increased the steady-state inactivation at V_h at or positive to −70 mV, producing a −10 to −15 mV shift in the steady-state inactivation curve. Values are the means ± S.E.M. of three to thirteen neurones. Error bars are not shown if they fall within the symbol. Asterisks represent a difference from control using Student's paired two-tailed *t* test at the $P < 0{\cdot}05$ level.

Nevertheless, some reduction of calcium current was present at V_h of -90 or -80 mV, which was due to an effect on the L current (see above).

Pentobarbitone selectively reduced barium current components in DRG neurones

Barium can substitute for calcium as the charge carrier for voltage-dependent calcium channels, but is thought to lessen or eliminate calcium-dependent inactivation of calcium currents (e.g. Eckert & Chad, 1984). If pentobarbitone

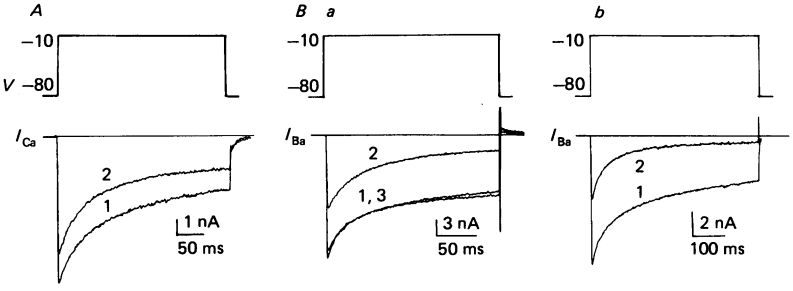

Fig. 6. Pentobarbitone reduced barium current in DRG neurones. Digitized leak-subtracted calcium (I_{Ca}) or barium currents (I_{Ba}) from different neurones are shown. Barium (5 mM; *B*) substituted for calcium (2 mM; *A*) in the recording medium, yielding currents of similar peak magnitude. Currents were recorded before (trace 1), during (trace 2) and after (trace 3) application of 500 μM-pentobarbitone. A representative calcium current (*A*) is compared with barium currents during 300 ms (*B a*) or 500 ms (*B b*) voltage commands. The effect of pentobarbitone was fully reversible after 1–2 min when the shorter commands were used; with longer commands, recovery (not shown) was between 75 and 90% of control.

increased inactivation of the N current by affecting, at least in part, calcium-dependent inactivation, then the effect of pentobarbitone on barium currents might differ from those on calcium currents. We therefore substituted barium (5 mM) for calcium in the recording medium for some experiments; this concentration of barium produced peak currents of similar magnitude to those obtained in 2 mM-calcium. The rate of decay of the barium currents suggested that they inactivated less than calcium currents (Fig. 6).

Pentobarbitone reduced the peak barium current by $45 \pm 5{\cdot}1\%$ (S.E.M., $n = 7$ neurones) when evoked at -10 mV from a holding potential of -80 mV. This was a greater proportional reduction than seen with comparable calcium currents (compare Fig. 6*A* with Fig. 6*B*). The reduction in the late barium current was $63 \pm 1{\cdot}0\%$ ($n = 4$) at 300 ms and $86 \pm 1{\cdot}9\%$ ($n = 3$) at 500 ms. In contrast to the faster inactivation of calcium currents at these V_c, there was no apparent increase in the rate of barium current inactivation in the presence of pentobarbitone.

The barium current traces were also analysed using mutiexponential curve-fitting. Like calcium currents,they had three components; these had distinct τ_i of $17{\cdot}3 \pm 1{\cdot}9$, $80{\cdot}6 \pm 4{\cdot}0$ and $1249{\cdot}8 \pm 169{\cdot}2$ ms (as compared to τ_i of $14{\cdot}1 \pm 5{\cdot}3$, $162{\cdot}6 \pm 26{\cdot}5$ and $896{\cdot}2 \pm 166{\cdot}0$ ms, respectively, for calcium currents). Pentobarbitone had no significant effect on any of the three τ_i of barium currents ($21{\cdot}4 \pm 2{\cdot}7$ ms; $74{\cdot}1 \pm 5{\cdot}7$ ms; $1193{\cdot}7 \pm 111{\cdot}6$ ms ($n = 7$)).

The component with the short τ_i (the T current) was a smaller proportion of the total current in barium than in calcium, and was not reliably estimated in most neurones. The amplitudes of the intermediate and long τ_i barium current components (N and L currents, respectively) could be determined in all neurones, however. The N current was the major calcium current component, but the L current was the predominant component of barium currents. There was no effect of pentobarbitone on the magnitude of the N current, whereas the L current was reduced 60–80 % (data not shown). These results were similar to those obtained from curve fitting of calcium currents at this holding potential.

DISCUSSION

Pentobarbitone selectively reduced calcium currents in mouse DRG neurones

Three calcium current components were recorded in mouse DRG neurones. They were distinguishable by their different activation and inactivation voltage ranges, inactivation time constants and different sensitivity to the calcium channel blockers cadmium and nifedipine (Gross & Macdonald, 1988*a*), findings consistent with others' results (Hess, Lansman & Tsien, 1984; Fox *et al.* 1987*a*, *b*).

Pentobarbitone selectively reduced calcium currents in mouse DRG neurones. There was no effect of pentobarbitone on the T current, but both the N and L currents were reduced, and the rate of inactivation of the N current was increased. This could account for the effect of the barbiturates on calcium currents noted previously (Werz & Macdonald, 1985). These data could also explain the findings of Nishi & Oyama (1983), who noted that pentobarbitone reduced the magnitude and hastened inactivation of invertebrate calcium currents.

The effect of pentobarbitone on the N and L currents possibly occurred by different mechanisms. The results presented suggest that the reduction in the latter may have been due to prevention of channel activation. Pentobarbitone reduced the L current without a shift in the voltage dependence of the late calcium current–voltage relation; there was no change in the L current inactivation time constants; and there was a similar percentage reduction in this current component evoked from V_h of -80 or -50 mV. The experiments with barium currents support this view. Pentobarbitone reduced the L current (a larger proportion of total current in barium) to a similar extent in barium and in calcium, with no effect on the inactivation time constants in barium. Our data do not, however, entirely eliminate the possibility of an effect of pentobarbitone on the steady-state inactivation process of the L current. If this were the case, however, the shift in the steady-state inactivation curve would have to be greater than -20 mV.

In contrast, the reduction of the N current by pentobarbitone was produced by an enhancement in steady-state inactivation at V_h positive to -80 mV and a reduction in the τ_i at all but the most depolarized V_c tested ($+10$ mV). Pentobarbitone also hastened recovery from inactivation of the N current. This effect, seen at relatively negative membrane potentials, suggests that pentobarbitone altered the N channel so as to enhance both entry into the inactivated state from the open state, and departure from the inactivated state to the closed state. Because pentobarbitone increased steady-state inactivation, however, it probably enhanced the transition

from the closed state to the inactivated state more than it facilitated the reverse transition.

Selective effects of pentobarbitone were also noted on barium current components, although pentobarbitone did not reduce the τ_i of the N current. It is of interest that the τ_i of this current component was smaller in barium than in calcium; if, at a given clamp potential, voltage-dependent inactivation is similar in barium and calcium, then barium may be more effective at producing inactivation of the N current. In this regard it is tempting to speculate that pentobarbitone may reduce the τ_i of the N current by increasing the efficacy of calcium-dependent inactivation.

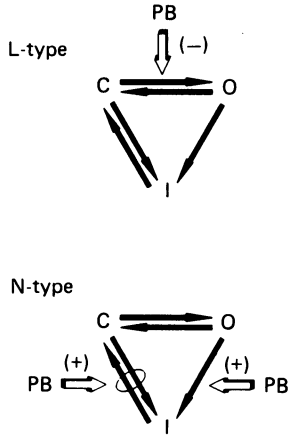

Fig. 7. A schematic illustration showing the proposed action of pentobarbitone (PB) on the L and N calcium channels. The arrows represent transitions from one state to another (C, closed state; O, open state; I, inactivated state), and the effect of pentobarbitone on those transitions, i.e. an inhibition (−) or an enhancement (+). See text for details.

If the N and L currents represent different states of the same channel, then pentobarbitone would affect the two states by different mechanisms. If, on the other hand, these current components are subserved by two different calcium channels, then pentobarbitone would affect the two channels differentially. At present, we favour the latter hypothesis (Fig. 7). Further experiments using single-channel analysis will be necessary, however, to clarify the mechanisms of action of pentobarbitone on the N and L currents.

Pentobarbitone and the alteration of neuronal function

The co-existence of multiple calcium channel types in neurones may mean that they have distinct physiological roles, an idea supported by the finding that these channels may not be uniformly distributed within neurones (Fox *et al.* 1987*b*; Miller, 1987). If this were the case, then the selective reduction of calcium current components by pentobarbitone could result in specific alterations in calcium-dependent neuronal processes such as neurotransmitter release. In fact, recent experiments suggest that the N and L currents may differentially modify neurotransmission. For example, the N calcium channel appeared to be the primary regulator of neurotransmitter release from sympathetic ganglion neurones in culture

(Perney, Hirning, Leeman & Miller, 1986; Hirning, Fox, McCleskey, Olivera, Thayer, Miller & Tsien, 1988); the L channel may play a similar role in substance P-releasing DRG neurones (Perney *et al.* 1986).

The selective reduction of calcium currents by pentobarbitone may underlie its ability (and that of other barbiturates) to inhibit neurotransmission. Near the resting membrane potential, the predominant effect of pentobarbitone was to reduce peak calcium current by preventing L current activation and enhancing N current steady-state inactivation. Calcium influx during an action potential might be further reduced due to faster inactivation of the N current. This might result in a suppression of neurotransmission, and may account for some of the clinical effects of the barbiturates, particularly in their use as anaesthetic agents. The reduction of calcium influx could also alter the regulation of potassium conductance (Meech, 1978), or the activation of calcium-dependent second messenger system components such as protein kinase C or calmodulin (Nishizuka, 1984; Berridge & Irvine, 1984; Johnson & Mills, 1986).

Finally, the reduction of N and L currents by pentobarbitone could modify the actions of those neurotransmitters that regulate voltage-dependent calcium channels, such as noradrenaline, adenosine and dynorphin A (Dunlap & Fischbach, 1981; Galvan & Adams, 1982; Forscher & Oxford, 1985; Dolphin, Forda & Scott, 1986; Macdonald, Skerritt & Werz, 1986; Macdonald & Werz, 1986; Gross & Macdonald, 1987). Similarly, the regulation of calcium channels by protein kinases A or C (DeRiemer, Strong, Albert, Greengard & Kaczmarek, 1985; Levitan, 1985; Rane & Dunlap, 1986; Strong, Fox, Tsien & Kaczmarek, 1987; Werz & Macdonald, 1987*a*, *b*; Gross & Macdonald, 1988*b*) could be affected by pentobarbitone. Thus, the marked reduction of the predominant voltage-dependent calcium current components by pentobarbitone and other barbiturates could be expected to have multiple direct and indirect effects on neuronal function.

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