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Modification of motor nerve terminal excitability by alkanols and volatile anaesthetics

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1 A method of local polarization-excitation was used to study changes in motor nerve terminal excitability produced by n-alkanols and volatile anaesthetics in mouse diaphragm preparations.

2 Ethanol and propanol caused an exaggeration of 'accommodation', i.e., the increase in excitation threshold produced by a conditioning depolarization. Butanol, hexanol and octanol had mixed effects, producing a rise in the minimum threshold (threshold after removal of resting accommodation) in addition to an increase in accommodation.

3 Volatile anaesthetics produced effects on excitability at concentrations comparable to minimum alveolar concentration. The action of enflurane was essentially only to increase accommodation while methoxyflurane produced an increase in threshold insensitive to conditioning polarization. Halothane and isoflurane produced intermediate effects.

4 Accommodation curves were little affected by Ba^{2+} or 4-aminopyridine and were consistent with accommodation being a reflection of inactivation of the Na^+ current system.

5 We conclude that volatile anaesthetics, at concentrations comparable to those producing anaesthesia, may substantially modify Na^+ channel gating and inactivation.

Introduction

Because it requires rather high concentrations of general anaesthetics to cause any substantial blockade of nerve conduction (Seeman, 1972), or to modify considerably the kinetics of the ion-gating systems underlying nerve impulse generation (e.g. Kendig *et al.*, 1979; Bean *et al.*, 1981; Haydon & Urban, 1983a), it has appeared unlikely that interference with the action potential generating system plays an important role in the production of the state of general anaesthesia by anaesthetics at clinical concentrations. Effects of anaesthetic agents are manifest at relatively low concentrations on crayfish stretch receptor firing (Roth, 1980) and on excitability of nerve terminals in the spinal cord (Morris, 1980), but at these sites it is possible that the drugs act by altering transmitter release or transmitter action at synapses, as is apparently the case in hippocampus (Richards & White, 1975), rather than by modifying cell excitability.

In the present experiments we have searched for changes in nerve terminal excitability at the mouse neuromuscular junction. At this synapse the local bathing medium can be rapidly modified and the nerve terminal easily stimulated via focal polarization (Hubbard & Schmidt, 1963; Cooke & Quastel, 1973). The use of a conditioning pre-pulse to modulate threshold (Quastel *et al.*, 1981) permits different mechanisms of excitability changes to be recognized. Thus, neuronal membrane excitability can be studied without the complications produced by the diversity of synaptic input present on neurones (and at nerve terminals) in the CNS.

Briefly, we have found that pentobarbitone and n-alkanols (at rather high concentrations) and volatile anaesthetics, at concentrations) very close to minimum alveolar concentration ('MAC'), cause substantial changes in nerve terminal excitability that may well be due to modification of Na-current gating.

Methods

All experiments were done on mouse diaphragm, *in vitro*. The technique employed for the mounting, superperfusion and focal polarization of nerve terminals has been described elsewhere (Cooke & Quastel, 1973). The bathing solution contained (mM): NaCl 125, NaH_2PO_4 1, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, $NaHCO_3$ 24, and glucose 11; it was bubbled with 95%

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O_2/5% CO_2 to give pH 7.4. Experiments were carried out at 26–28°C. For the addition of volatile anaesthetics the solution in a glass syringe (which constituted one of the reservoirs of the superperfusion system) was bubbled with gas which had been passed through a vaporizer calibrated to give the appropriate percentage saturation; the syringe was then closed with a gas-tight plunger. Concentrations of volatile anaesthetics employed were: enflurane, 1.5% (0.6 mM) and 3% (1.2 mM); halothane, 1% (0.4 mM) and 2% (0.8 mM); isoflurane, 3% (1.0 mM); methoxyflurane, 0.2% (0.5 mM). The concentrations in mM, at 27°C, were calculated using the water : gas partition ratios quoted in Steward *et al.* (1973), and the temperature coefficients in Allott *et al.* (1973). For comparison, values of 'MAC' for these agents in man (in O_2, at 37°C) are: enflurane, 1.68%; halothane, 0.75%; isoflurane, 1.15%; and methoxyflurane 0.16% (Saidman *et al.*, 1967; Gion & Saidman, 1971; Stevens *et al.*, 1975).

Figure 1 illustrates diagrammatically the method used for testing nerve terminal excitability. Stimuli were applied as square current pulses of fixed duration and variable strength, via micropipettes of about 20 μm tip diameter, filled with 3 M NaCl and sufficient agar to prevent oozing from the electrode tip. The firing of a presynaptic action potential was detected by the appearance of a large endplate potential (followed by an action potential) recorded intracellularly in the muscle fibre. With repeated excitation the movement of the impaled fibre always resulted in a drop of its membrane potential, and consequent failure of action potential generation i.e., the postsynaptic response became an 'all-or-nothing' endplate potential. In previous experiments it was found from strength-duration curves that the apparent time constant of nerve terminals was close to 1 ms (range 0.6–1.4 ms), in contrast to an apparent time constant of 0.1–0.2 ms for nerve stimulation. Except for experiments in which nerve terminal refractory period was measured, in which case test pulses of about 0.2 ms duration were used, test stimulating pulses (S_2) were of at least 4 ms duration, i.e., rheobasic, superimposed upon hyperpolarizing or depolarizing conditioning pulses (S_1) commencing 70 ms before the test pulse. Control experiments had shown that modulation of net threshold developed over a period of a few ms and 70 ms was more than sufficient for maximal modulation of threshold by a conditioning pulse. Thus, if transmembrane potential change is almost proportional to the applied current, the algebraic sum $S_1 + S_2$ will reflect the net displacement of transmembrane potential necessary to elicit an action potential.

Typically it required about ten minutes to obtain each curve of threshold vs conditioning polarization; several minutes were allowed for equilibration after each switch of the bathing solution. Results were rejected if data obtained after washing out the drug

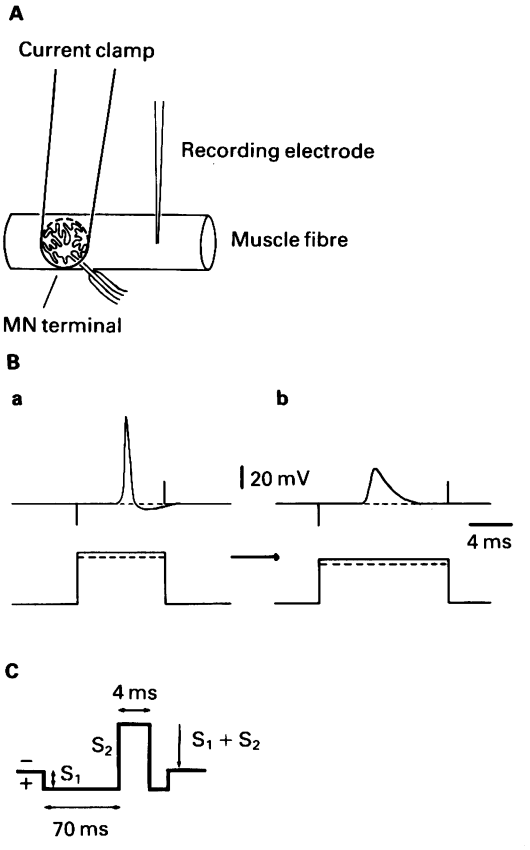

Figure 1 Diagram of the method used to test nerve terminal excitability. (A) A stimulating electrode about 20 μm in diameter was placed over a nerve terminal, its proximity being signalled by the effectiveness of steady depolarizing current to increase frequency of intracellularly recorded miniature endplate potentials. Subsequently, a depolarizing pulse of sufficient magnitude induced an action potential (and twitch) in the muscle fibre; a slightly smaller pulse (dotted lines in (B)) elicits no response. With time the resting potential of the muscle fibre falls and the response becomes a subthreshold endplate potential which is all-or-nothing with respect to stimulus intensity (B,b). The stimulus paradigm is shown in (C); a test pulse (S_2) is adjusted in amplitude until it is just threshold (eliciting responses to about one half of stimuli) at various values of the conditioning pulse (S_1).

were substantially different from the control; not infrequently, there was a drift of threshold, presumably due to movement of the polarizing electrode relative to the nerve terminal, upon which was superimposed the same alterations of threshold by drugs as found in experiments where there was no such drift.

It should be noted that with focal depolarization

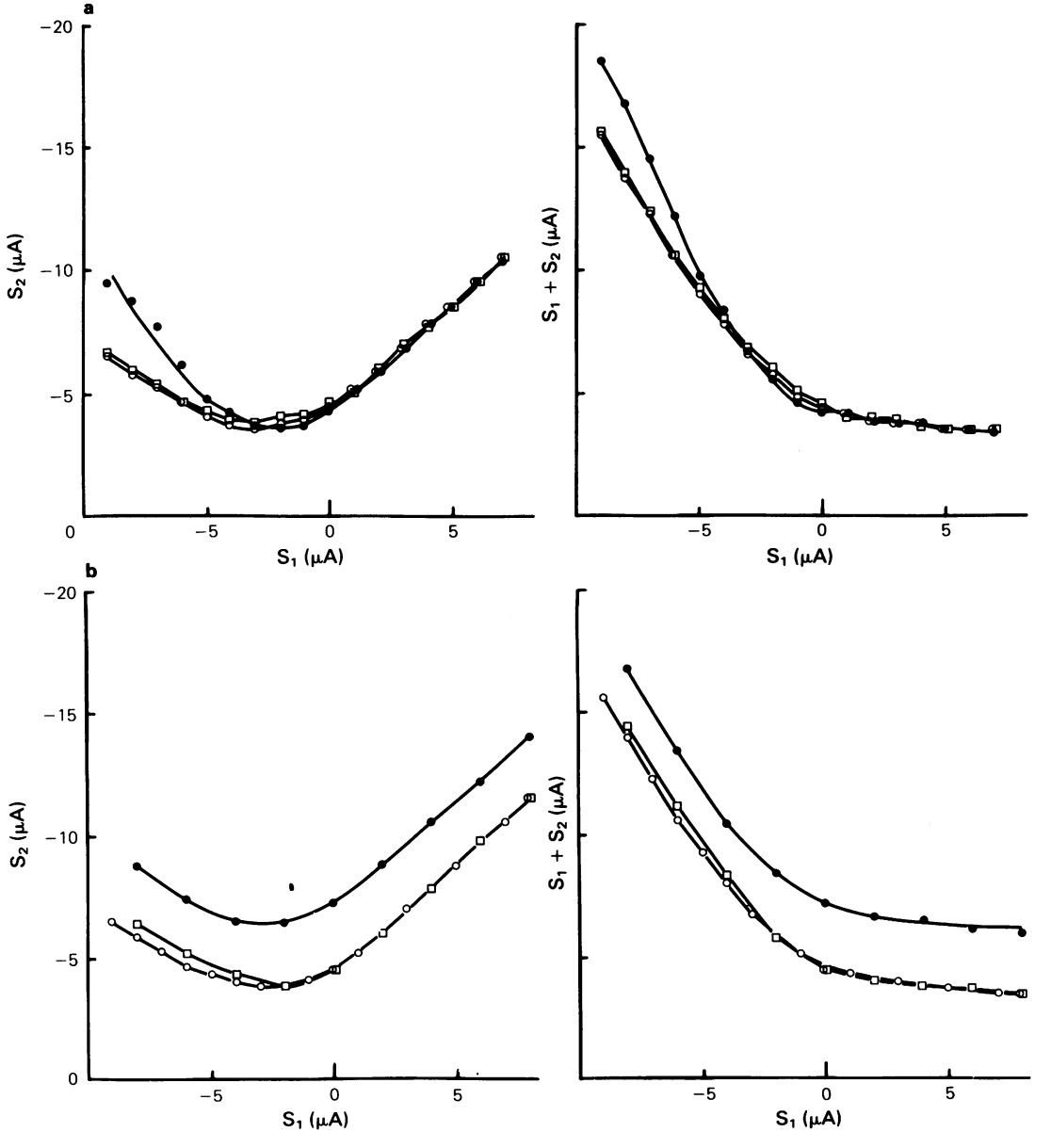

Figure 2 Modification by propanol 100 mM (a) and pentobarbitone 0.5 mM (b) of the curves of threshold (S_2) vs conditioning polarization (S_1). In each case the graphs on the left are of threshold S_2 vs S_1, while the graphs on the right show the same data plotted as net threshold ($S_1 + S_2$) vs S_1. Note increase of 'accommodation' by propanol, while pentobarbitone causes the same increase in threshold at all levels of conditioning polarization. In this and following figures (○) represent control data, filled symbols represent data obtained in the presence of the drug, and (□) represent data obtained after washout of the drug.

(local external negativity) the potential inside the nerve terminal and axon becomes more negative, i.e., at the first node there is hyperpolarization rather than depolarization. Thus, if the first node were the site of nerve impulse generation, threshold would be lower for hyperpolarizing pulses than for depolarizing pulses and action potential generation would occur as an off response. The reverse was always the case, and in view of the results of Brigant & Mallart (1982), showing action potential invasion of the proximal part of the nerve terminal, we conclude that excitation indeed occurs at the nerve terminal.

Results

Figure 2 shows typical curves of threshold vs conditioning polarization, and the modification of this relation by propanol and pentobarbitone. Notably, with propanol there was no change in minimum net threshold ($S_1 + S_2$), obtained when a hyperpolarizing conditioning pulse preceded the test pulse (i.e. at positive values of S_1). However, propanol caused an increase in thresholds following depolarization prepulses (S_1). The most straightforward interpretation of this observation is an increase in apparent 'accomodation', which might arise either from an enhancement of Na-current or an increase in K-current. Identical results were obtained with ethanol (not illustrated); even with concentrations of ethanol of 0.5 M and greater, which caused block of axonal conduction and a large increase of threshold in the absence of conditioning polarization, a sufficiently large conditioning hyperpolarization completely removed any effect on threshold. In contrast, a general increase in threshold, essentially unaltered by conditioning polarization, was found with 0.5 mM pentobarbitone (Fig 2b). This concentration of pentobarbitone is much greater than that found to depress Ca^{2+}-dependent action potentials (Heyer & MacDonald, 1982). With butanol, hexanol and octanol an increase in 'accommodation' was also visible (Figure 3), but threshold was also raised throughout the hyperpolarizing range of S_1.

Figures 4 and 5 show the effects on excitability of four volatile anaesthetics, methoxyflurane (0.2%), halothane (1% and 2%), isoflurane (3%) and enflurane (1.5% and 3%). It is evident that there was substantial alteration of nerve terminal excitability by these agents at concentrations close to those used clinically in man. With methoxyflurane the increase in threshold was uniform throughout the range of S_1, while with enflurane the increase in threshold was removed by conditioning hyperpolarization; isoflurane and halothane showed intermediate behaviour.

In principle, the increase in 'accommodation' produced by enflurane might reflect an increase in inactivation of g_{Na}, or an increase in membrane conductance to K^+ (or Cl^-). The experiment was therefore repeated in the presence of added 2 mM Ba^{2+} (Figure 5b) which should be sufficient to block calcium-activated g_K (Hagiwara *et al.*, 1978; Hotson & Prince, 1980), and in the presence of 1 mM 4-aminopyridine (4-AP) to block delayed rectification (Pelhate & Pichon, 1974; Yeh *et al.*, 1976). With 2 mM Ba^{2+} there was a small change in the apparent accommodation, but no significant alteration of the effect of enflurane (Figure 5b). In the presence of 4-AP the actions of enflurane, methoxyflurane, or halothane were apparently unaltered (Figures 4c and 5c).

Figure 6 shows the effect of enflurane (3%) on the refractory period of the nerve terminal. At one junction, 3% enflurane increased the absolute refractory period from 2.4 ms to 3.5 ms and at another from 1.6 ms to 2.6 ms. At the latter junction the addition of 1 mM 4-AP increased the control refractory period to 5 ms and 3% enflurane caused an additional increase to over 8 ms. The increased refractory period produced by 4-AP alone is presumably due to blockade of delayed rectification leading to a prolongation of the presynaptic action potential, which is terminated mainly by sodium inactivation under these conditions (c.f. Chiu *et al.*, 1979; Hodgkin & Huxley, 1952a,b). The further prolongation of the refractory period induced by enflurane, in the presence of 4-AP, is most easily explained in terms of an increase by enflurane of the inactivation of g_{Na} produced by the action potential.

Discussion

The present results indicate that the inhalation anaesthetics have an appreciable effect on nerve terminal excitability at concentrations close to those employed in clinical anaesthesia. In evaluating these data one must keep in mind that the change in transmembrane potential produced by an extracellular polarizing current pulse depends not only on the distance from the terminal of the polarizing electrode, but also on the resistance of the nerve terminal membrane. For a uniformly polarized terminal, the membrane polarization will be proportional to $R_t/(R_t + R_a)$ where R_t is the resistance of the presynaptic terminal membrane and R_a is the resistance of the current pathway(s) to ground via the interior of the axon and nodes of Ranvier (see Cooke & Quastel, 1973); the time constant will be $C_t/(R_t^{-1} + R_a^{-1})$. Briefly, one can predict the following modifications of the 'accommodation curve': (a) a reduction of R_t (or movement of the polarizing electrode away from the nerve terminal) will cause a flattening of the curve of threshold (S_2) vs conditioning polarization (S_1), with a rise of S_2 at $S_1 = 0$; (b) an intrinsic hyperpolarization or de-

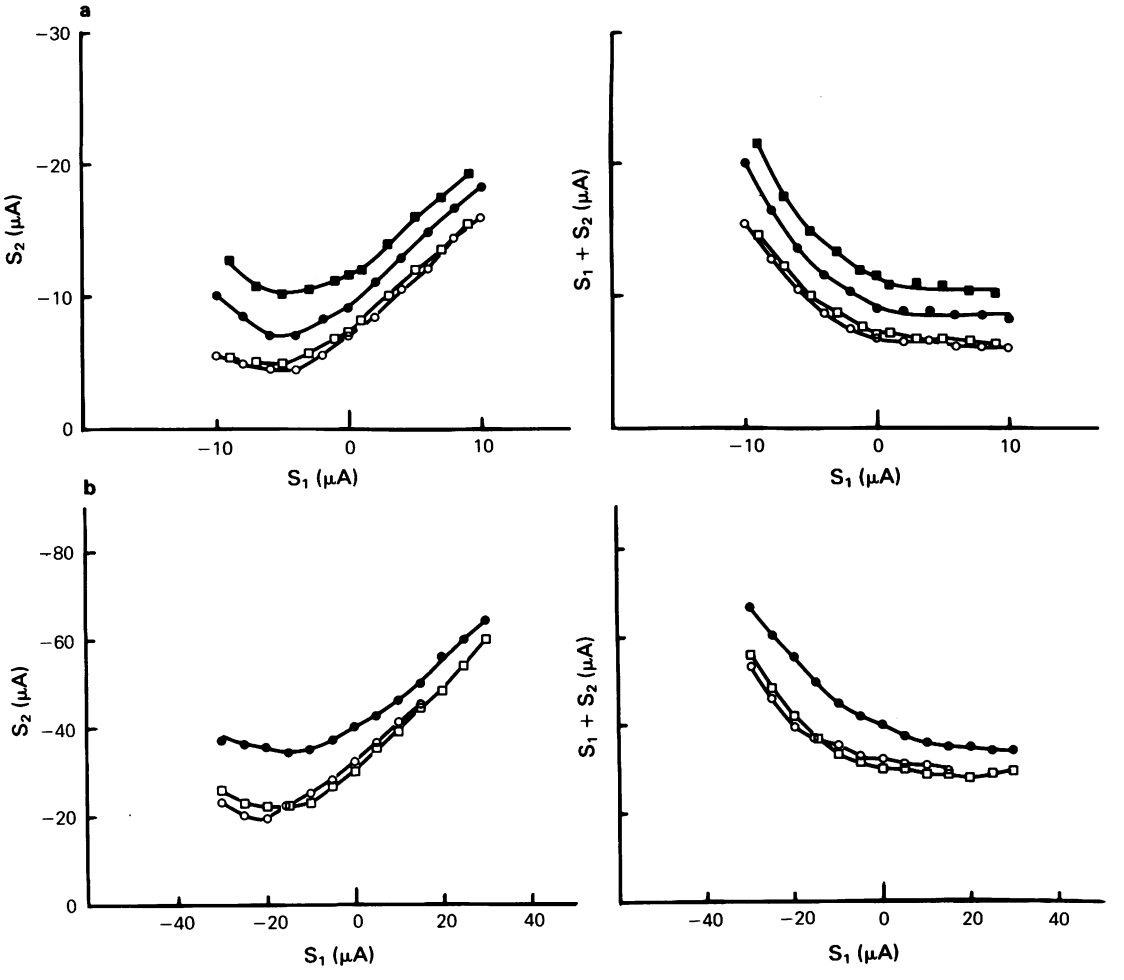

Figure 3 Effects of (a) butanol 20 mM (●) and hexanol 1 mM (■) and (b) octanol 0.1 mM (●) on nerve terminal excitability. Data were plotted in the same way as in Figure 2.

polarization of the nerve terminal will cause a parallel shift, to the right or left respectively, of the curve of S_2 vs S_1; (c) a reduction in maximum voltage-sensitive g_{Na} will cause an increase in S_2 at all values of S_1, and (d) a shift of the curve of g_{Na} inactivation vs membrane potential, in the hyperpolarizing direction, will appear as an increase in 'accommodation'; i.e., an alteration of the curve of threshold vs S_1 in the same way as observed with propanol (Figure 2) and enflurane (Figure 4). However, any depolarization-sensitive increase of membrane conductance would be manifest in the same way, The finding that accommodation was little, if at all, altered in the presence of 2 mM Ba^{2+} or 1 mM 4-AP strongly suggests that accommodation owes little to delayed rectification or to Ca-dependent g_K. Moreover, the ability of enflurane to prolong the refractory period, already enhanced in the presence of 4-AP, suggests that the membrane process responsible for accommodation is brought into play during a single normal action potential. It therefore seems reasonable to conclude, tentatively, that accommodation represents inactivation of the sodium current system (Hodgkin & Huxley, 1952a), and at relatively low levels of depolarization this process is enhanced by propanol, enflurane, and, to varying extents, by other alkanols and volatile anaesthetics. A shift in the hyperpolarizing direction of the inactivation curve for g_{Na} has been demonstrated with alkanols and volatile anaesthetics in crayfish and squid giant axon (Oxford & Swenson, 1979; Haydon & Urban, 1983a,b), and

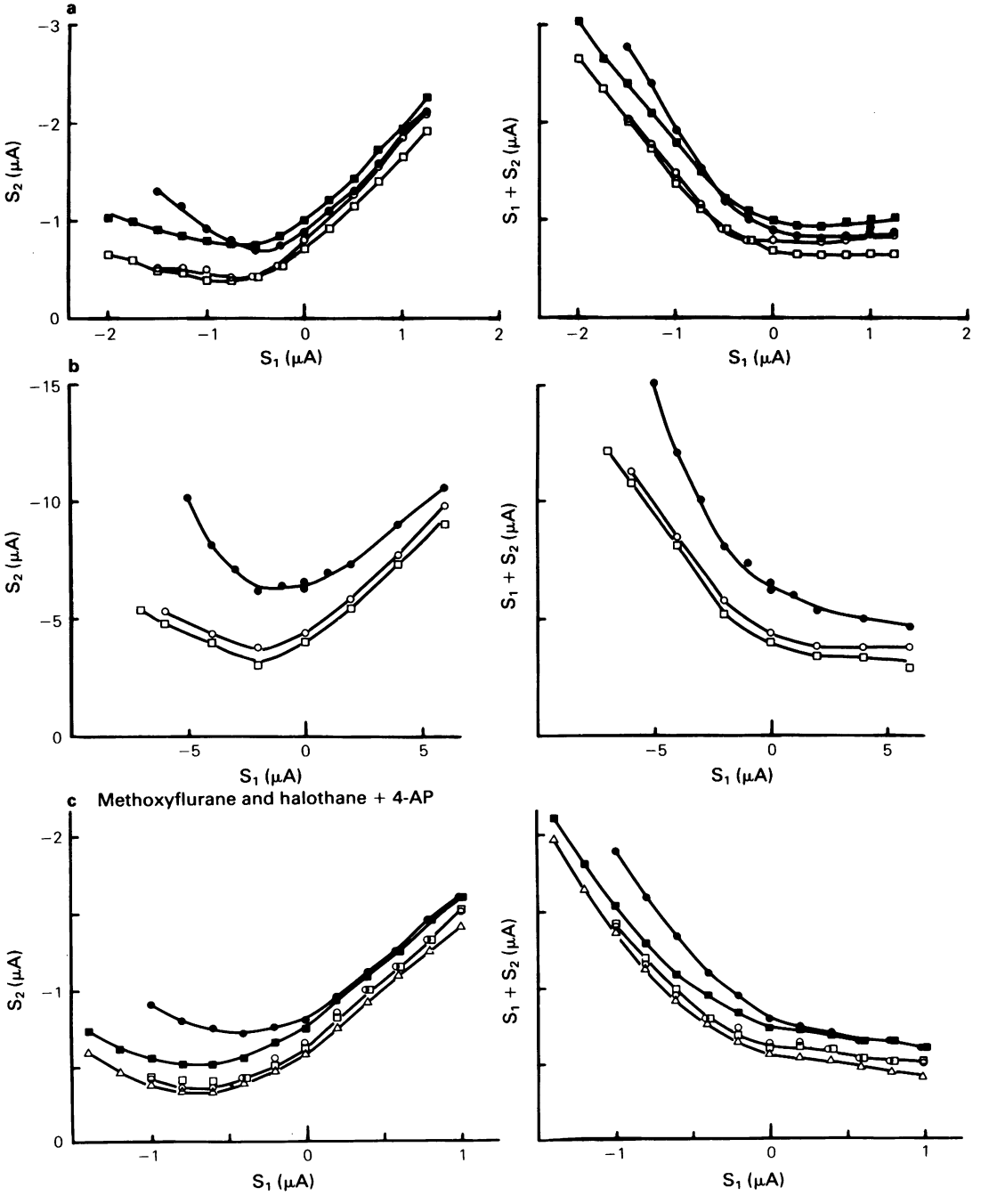

Figure 4 Modification of nerve terminal excitability by (a) methoxyflurane 0.2% (■) and isoflurane 3% (●), and (b) halothane 2% (●) in the presence (c) and absence (a and b) of 4-aminopyridine (4-AP) 1 mM. Data were plotted in the same way as in Figure 2.

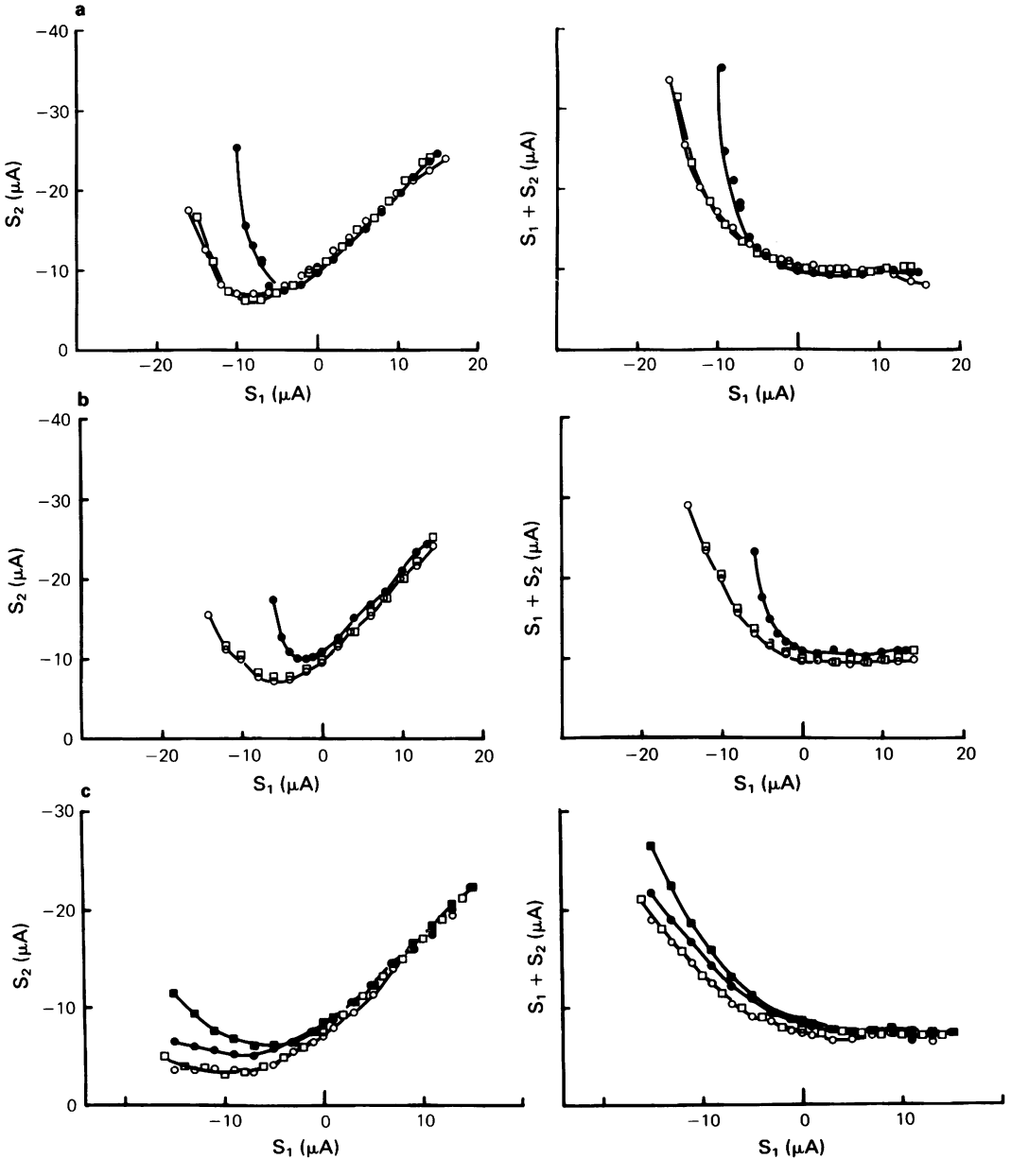

Figure 5 Effect of enflurane 3% (●, a and b; ■, c) and 1.5% (●, c), on nerve terminal excitability in the absence (a) and presence of (b) Ba^{2+} (2 mM) or (c) 4-aminopyridine 1 mM. (a) and (b) Results from the same junction. Data were plotted in the same way as in Figure 2.

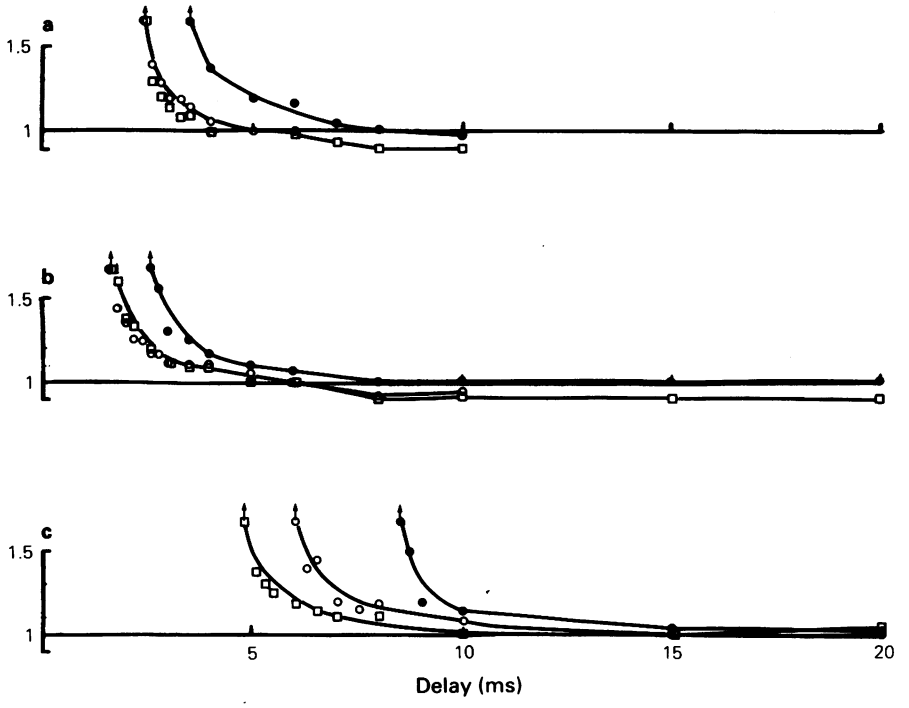

Figure 6 Prolongation of the refractory period of nerve terminals by enflurane. Each graph depicts the threshold, relative to control, vs time after a supramaximal stimulation. (○, □) Control and re-control points (●) points obtained in the presence of 3% enflurane. (b) and (c) Results obtained from the same endplate, the latter (c) being obtained in the presence of 1 mM 4-aminopyridine throughout.

(with diethylether) at the frog node of Ranvier (Kendig *et al.*, 1979). Nevertheless, we cannot exclude the possibility that the observed accommodation, enhanced by enflurane and propanol, instead represents activation of a g_K that is insensitive to Ba^{2+} or 4-aminopyridine, or even a voltage-sensitive g_{Cl}.

In other experiments, we have found that with raised K^+ there does indeed occur a parallel shift of the S_2 vs S_1 curve to the left, as predicted for an intrinsic depolarization, and in the presence of 15 mM K^+ (and 2 mM Ca^{2+}) minimum net threshold ($S_1 + S_2$) is close to 0. Thus it would appear that when accommodation is removed by conditioning hyperpolarization, absolute rheobasic threshold is about -60 mV. A rough estimate of the amount of shift in the accommodation curve can therefore be derived if we assume that the resting membrane potential of the terminal in 5 mM K^+ is about -90 mV. The flat portion of the curve of net threshold ($S_1 + S_2$) vs S_1 thus reflects a depolarization of about 30 mV. In the example in Figure 5a this was produced by a current of 10 μA. The shift in the curve produced by 3% enflurane, about 3 μA on the S_1 axis, then represents a shift of about 9 mV, in the hyperpolarizing direction, of accommodation vs membrane potential. Such a shift could be expected to have a significant effect on the function of axons and nerve terminals only under conditions where transmission is marginal because of depolarization (perhaps during presynaptic inhibition), or at points of axon branching (Hatt & Smith, 1976; Grossman *et al.*, 1979; Grossman & Kendig, 1982). However, the same shift at the sites of neuronal initiation of action potentials might have a substantial effect on cell firing in response to excitatory synaptic input.

A variety of mechanisms could underlie the overall increase in threshold found with most of the agents tested, the most obvious being reduced maximum g_{Na}, which has been observed with alkanols and volatile anaesthetics in both invertebrate and vertebrate axons under voltage-clamp conditions (e.g., Kendig *et al.*, 1979; Oxford & Swenson, 1979; Haydon & Urban, 1983a,b). By and large our data for the potency of alkanols on the mouse nerve terminal are consistent with those on squid axon (Haydon & Urban, 1983a), since we found substantial effects on threshold at about one third of the concentrations that reduce peak sodium currents by 50% in the voltage-clamped squid axon. However, Haydon & Urban (1983b) found a

rather small reduction in peak sodium current in squid axon with concentrations of methoxyflurane and halothane six and ten times larger than used here, indicating either that the mouse nerve terminal is more sensitive, or that other mechanisms underlie the change in excitability that we observed with these agents. Notably, the overall rise in threshold is consistent with a combination of an increase in accommodation and a hyperpolarization, perhaps secondary to an increase in intracellular Ca^{2+} and activation of Ca-dependent g_K (Krnjevic, 1974; Morris, 1980; Carlen *et al.*, 1983). Either depression of peak g_{Na} or increase in accommodation might well be sufficient to cause an inhibition of neuronal action potential generation at concentrations of these anaesthetics used clinically.

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