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IONIC BASIS FOR THE ELECTRORESPONSIVENESS OF GUINEA-PIG VENTROMEDIAL HYPOTHALAMIC NEURONES *IN VITRO*

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SUMMARY

1. The difference between the ionic bases for electroresponsiveness in the three types of neurone in the ventromedial hypothalamic nucleus (v.m.h.) were studied in *in vitro* brain slice preparations of the guinea-pig.

2. The current–voltage relationships in all three types of neurone showed anomalous rectification.

3. Application of tetrodotoxin (TTX) abolished the fast action potentials in all three types of cell.

4. In type A cells, injections of outward current pulses did not evoke spikes when the cells were perfused with TTX alone, but the addition of tetraethylammonium chloride elicited broad spikes. In type B and C cells, broad spikes could be evoked with TTX alone. These results suggest the presence of a minimal high-threshold Ca^{2+} current in type A neurones, and a more prominent one in type B and C neurones.

5. In type B neurones, Ca^{2+} conductance blockage with Mn^{2+} or Co^{2+}, or replacement of Ca^{2+} by Mg^{2+}, abolished the low-threshold response (l.t.r.). Substitution with Ba^{2+} did not increase the duration of the l.t.r. significantly, suggesting that under normal conditions the falling phase of the response was caused by inactivation of Ca^{2+} conductance.

6. In type B and C neurones, the amplitude and duration of the after-hyperpolarization (a.h.p.) following direct activation by long outward current pulses were markedly reduced in Ca^{2+}-free solution. These findings indicate that a large component of this response was generated by the Ca^{2+}-dependent K^+ conductance increase. In type A cells the a.h.p. amplitude was originally small and was not affected by the above treatment, suggesting that the participation of this conductance was minimal in this type.

7. In type C neurones, the membrane potential following an inward current pulse showed a delayed return to the base line. This delay was produced by transient K^+ conductance, since it was reduced by 4-aminopyridine.

8. The frequency–current (*f–I*) relation of the first interval in type A and C cells was scarcely affected in Ca^{2+}-free solution, while the slope of the initial firing *f–I* curves in type B neurones which had the l.t.r. became flatter. Furthermore, the *f–I* curves of the third interval in type C cells became steeper in Ca^{2+}-free solution.

9. The data indicate that the distinct membrane characteristics related to the heterogeneity among cells in the v.m.h. can be attributed to their specific ionic mechanisms, with the type A neurones showing a minimal high-threshold Ca^{2+} current, the type B having l.t.r. and the type C having a transient K^+ (I_A) current.

INTRODUCTION

Heterogeneity among the neurones within the ventromedial hypothalamic nucleus (v.m.h.) has been reported in a previous paper (Minami, Oomura & Sugimori, 1986). The following three types of neurone were identified by their membrane properties. Type A neurones were characterized by a short membrane time constant, a small after-hyperpolarization (a.h.p.) with a short duration after direct activation by a long outward current pulse, and a steep slope of the frequency–current (*f–I*) curves of the first spike interval. Type B cells had a long time constant, a large a.h.p. with medium duration and a steep *f–I* slope for the first interval. Type C neurones had a long time constant, a large a.h.p. with a long time course and a flat *f–I* slope for first interval. Type C neurones alone exhibited glucoreceptor properties: depolarization with a decrease in K^+ conductance in response to external glucose application. Horseradish peroxidase (HRP) staining revealed three different types of cell in the v.m.h. based on dendritic arborization and soma size, and this classification correlated well with the membrane properties of the type A, B and C cells. Here, we describe extracellular ionic requirements and some pharmacological properties of the different membrane characteristics in these three types of cell. Particularly, the ionic mechanism of the low-threshold response (l.t.r.) in type B cells, and transient K^+ (I_A) conductance increase in type C neurones are shown in detail. Some of these results were included in a preliminary report (Minami, Oomura, Sugimori & Llinás, 1984).

METHODS

The methods were similar to those described previously (Minami *et al.* 1986). The control solution contained (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1·24; $CaCl_2$, 2·4; $MgSO_4$, 1·3; $NaHCO_3$, 26; glucose, 10 and was equilibrated with 95 % O_2 and 5 % CO_2 at pH 7·4. Tetraethylammonium chloride (TEA, Ishizu Osaka, Japan), tetrodotoxin (TTX, Sigma St. Louis, U.S.A.) and 4-aminopyridine (4-AP, Ishizu Osaka, Japan) were used in final concentrations of 5–10 mM, 3 μM and 1 mM respectively. Divalent cations Mn^{2+}, Co^{2+}, Mg^{2+} and Ba^{2+} were used as Cl^- salts and were dissolved in phosphate-free solutions which were buffered by Tris to maintain pH (Llinás & Sugimori, 1980*a*).

RESULTS

Anomalous rectification

The input membrane resistance of v.m.h. neurones was determined by injection of inward current pulses. As shown in Fig. 1, plots of current–voltage (*I–V*) relations had two components. Near the resting membrane potential, inward current pulses produced almost linear voltage displacement. As the amplitude of the inward current was increased, the membrane conductance indicated anomalous rectification. Electroresponsiveness of some specific ionic conductances was also revealed by this manoeuvre. Slow depolarizing potential at off responses in type B neurones indicated l.t.r.s which were one of the characteristics of this cell type (Fig. 1*Ba*) (Minami *et al.*

1986). Marked delayed return to the resting potential after inward current pulses was seen in type C neurones (Fig. 1*Ca*). This response was brought about by the I_A conductance increase which is described later.

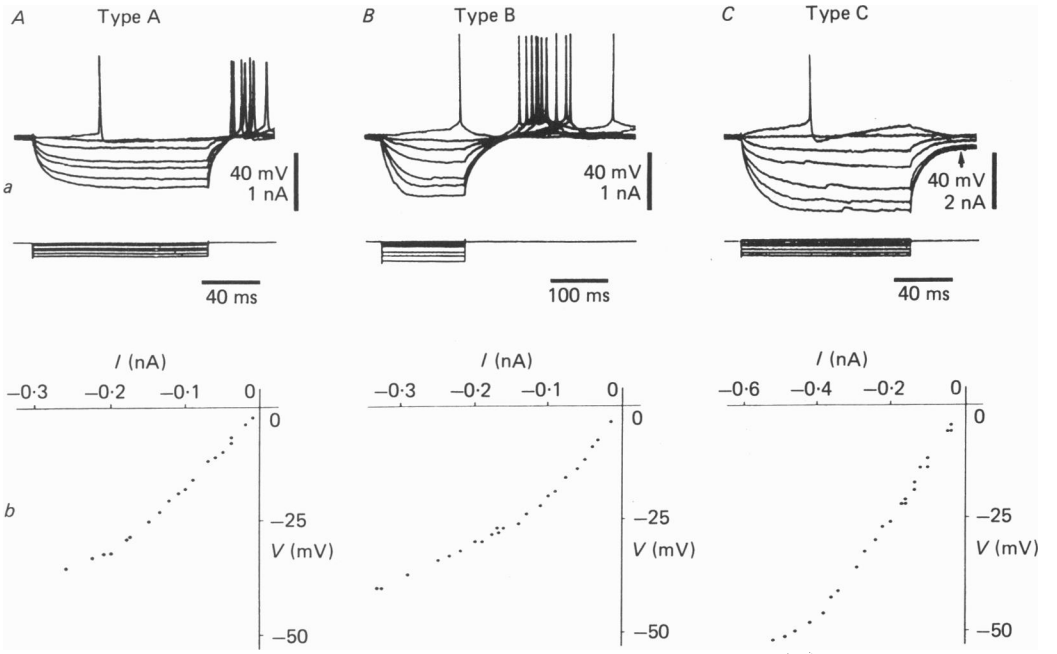

Fig. 1. Input membrane resistance of three different types of neurone within the v.m.h. *I–V* relations obtained by injection of inward square current pulses (130 ms duration). *A*, type A neurone; resting membrane potential (r.m.p.), −60 mV. *Aa*, membrane potentials produced by current steps. Off responses without l.t.r. *Ab*, *I–V* plot for *Aa*. *B*, type B neurone; r.m.p., −63 mV. *Ba*, membrane potential as in *Aa*. Off responses with l.t.r. showing slowly rising and falling depolarizing potential. *Bb*, *I–V* plot for *Ba*. *C*, type C neurone; r.m.p., −60 mV. *Ca*, membrane potential as in *Aa*. Arrow, delayed return to base line caused by I_A conductance. *Cb*, *I–V* plot for *Ca*. In *Ab*, *Bb* and *Cb*, *I–V* curves have concave upward shapes demonstrating anomalous rectification.

Ionic basis for generation of the action potentials

Na^+ action potential. Fast action potentials in all three types of neurones (Fig. 2*Aa*, *Ba* and *Ca*) were blocked by bath application of TTX (Fig. 2*Ab*, *Bb* and *Cb*). Therefore, these sharp spikes were assumed to be generated by increased Na^+ conductance.

High-threshold Ca^{2+} action potential. After TTX application, long, large outward current pulses could evoke action potentials with long duration and small amplitude in type B and C neurones (Fig. 2*Bb* and *Cb*). No such spikes were elicited in type A cells (Fig. 2*Ab*). The addition of TEA to the TTX-containing medium made it easier to evoke TTX-resistant action potentials in type B and C neurones; their spikes became larger and broader and were accompanied by enhanced a.h.p.s (Fig. 2*Bc* and *Cc*). Also in the type A cell, a large spike with long duration was observed in the presence of TEA and TTX (Fig. 2*Ac*). These action potentials disappeared in all three types of neurone in the presence of 5 mM-Mn^{2+} (Fig. 2*Ad*, *Bd* and *Cd*).

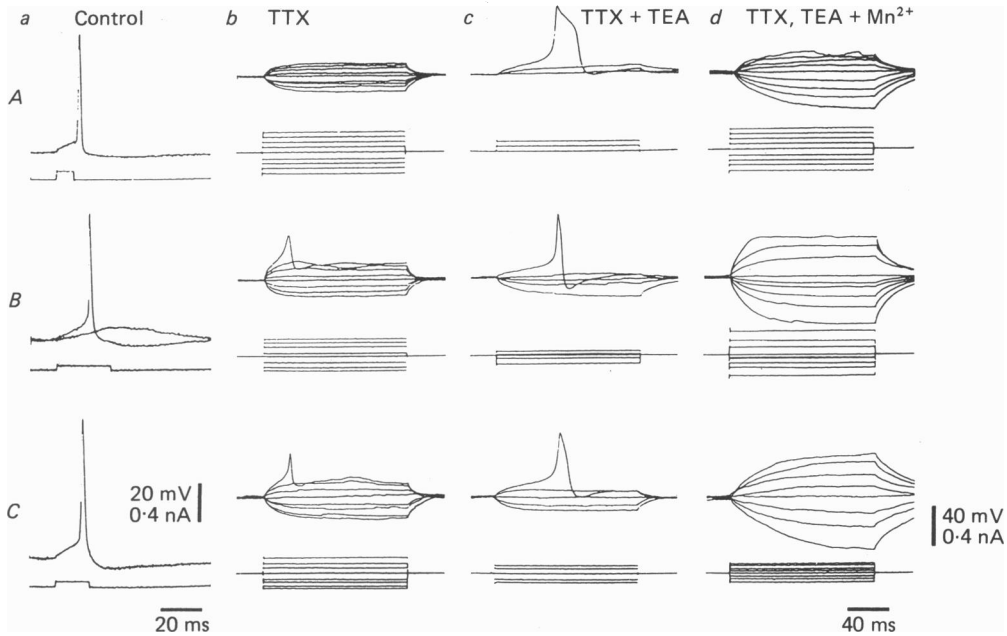

Fig. 2. Effects of TTX, TEA and Mn^{2+} on action potentials evoked by outward current pulses in three neurone types. *A*, *B* and *C* correspond to type A (resting membrane potential, r.m.p., −58 mV), B (r.m.p., −60 mV) and C (r.m.p., −61 mV) neurones respectively. *Aa*, *Ba* and *Ca*, single spike typically elicited in control solution. *Ab*, *Bb* and *Cb* responses to outward current pulses in 3 μM-TTX. *Ac*, *Bc* and *Cc*, responses in TTX plus 5 mM-TEA. *Ad*, *Bd*, and *Cd*, disappearance of TTX-resistant response, in 5 mM-Mn^{2+} plus TTX and TEA. Type A, no spike in TTX alone (*Ab*), but broad spikes elicited in TTX plus TEA (*Ac*). Types B and C, slow spikes even in TTX alone (*Bb*, *Cb*).

Ionic conductances generating low-threshold responses

In type B neurones, cells with resting potentials more negative than −65 mV can generate the l.t.r. (Minami *et al.* 1986). Application of an outward current pulse to a slightly hyperpolarized cell evoked a low amplitude, low threshold, slow rising and falling depolarizing potential with a fast high-frequency spike burst superimposed (Fig. 3*A*). A similar response was generated after termination of an inward current pulse (Fig. 3*B*). Addition of TTX to the bath, which abolished the fast Na^+ spikes, did not affect the l.t.r. or the high-threshold Ca^{2+} spikes elicited by injections of stronger currents (Fig. 3*C*). In the medium with TEA plus TTX, both potentials, especially the high-threshold spikes, became larger and broader (Fig. 3*D*). Addition of 5 mM-Mn^{2+} abolished the spikes (Fig. 3*E*).

Additional evidence for the presence of Ca^{2+} current in this l.t.r. was obtained by investigating the effects of Ca^{2+}-free solution. In the type B cell responses shown in Fig. 4, an outward current pulse from a slightly hyperpolarized level (−73 mV) generated a typical l.t.r. with a burst of fast spikes (Fig. 4*A*) as did rebound from an inward current pulse (Fig. 4*B*). Outward current pulses at the original resting membrane potential (−60 mV) produced slowly repetitive fast action potentials (Fig. 4*C*). Perfusion with Ca^{2+}-free solution (Ca^{2+} replaced by 12 mM-Mg^{2+}) during hyperpolarization reduced the l.t.r. but had no effects on the individual fast action

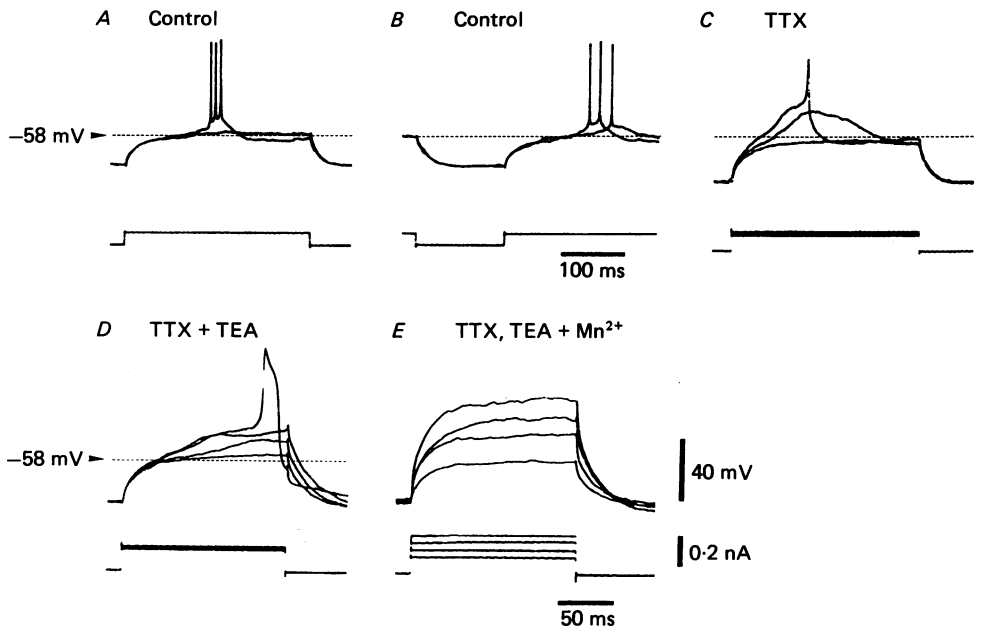

Fig. 3. Effects of TTX, TEA and Mn^{2+} on l.t.r. and high-threshold Ca^{2+} spikes in a type B neurone (resting membrane potential, r.m.p., −58 mV). *A*, fast spike burst with high frequency on slowly rising and falling, all-or-none l.t.r. produced by outward current during hyperpolarization (−77 mV). *B*, rebound post-anodal excitation with l.t.r. at original r.m.p. (−58 mV) (time scale, 100 ms). *C*, effect of TTX at hyperpolarized level (−87 mV). Disappearance of fast action potentials observed in *A* and *B*. Appearance of slow spike (high-threshold Ca^{2+} spike) on l.t.r. *D*, effect of TTX plus TEA. Arrow head, original r.m.p. (−58 mV). Both potentials, especially slow spikes, became larger and broader. *E*, abolition of both l.t.r. and slow action potential by 5 mM-Mn^{2+} addition. Time calibration for *A*, *C*, *D* and *E*: 50 ms.

potentials (Fig. 4*D*). Also, the rebound responses that followed an inward current pulse disappeared (Fig. 4*E*). The fast spikes produced by an outward current pulse at the original resting membrane potential level were not affected by removal of Ca^{2+} (Fig. 4*F*), but were blocked by TTX at hyperpolarized as well as resting membrane potentials (Fig. 4*G* and *H*). These results indicate that the l.t.r. in v.m.h. cells is generated by a voltage-dependent Ca^{2+} conductance increase.

Replacement of 0·5 mM-Ca^{2+} by Ba^{2+} increased the rate-of-rise and duration of the l.t.r. and increased the number of fast action potentials (Fig. 5*A* and *B*), however the prolonged l.t.r. did not last for many seconds as the Ca^{2+} plateau potential did in inferior olivary dendrites (Llinás, 1984). The train of fast spikes disappeared after the addition of TTX, while the l.t.r. and high-threshold spikes remained (Fig. 5*C*). Addition of 5 mM-Co^{2+} to the solution completely blocked these active reponses (Fig. 5*D*).

Ionic basis of after-hyperpolarization

Ca^{2+}-dependent K^+ conductance. The amplitude and half decay time of the a.h.p. that follows direct activation are important properties in differentiating cell types in the v.m.h. (Minami *et al.* 1986). To determine the contribution of the Ca^{2+}-

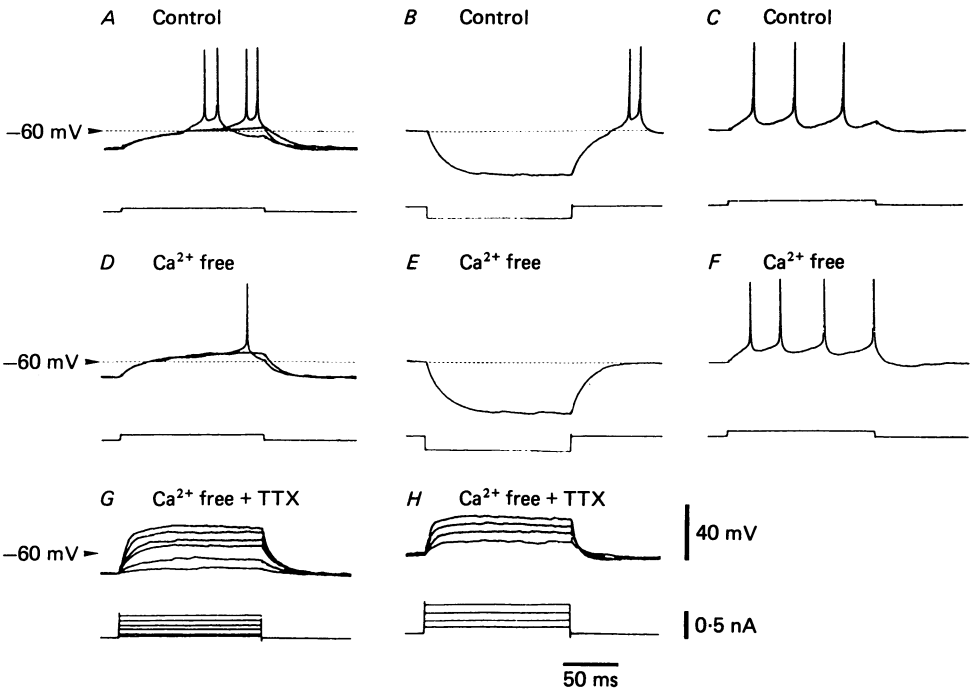

Fig. 4. Effects of Ca^{2+}-free solution on l.t.r. of a type B neurone. Arrowhead, resting membrane potential (r.m.p., −60 mV). *A*, *B* and *C*, control responses. *A*, burst of fast action potentials on slow all-or-none l.t.r. evoked by direct outward current pulse from hyperpolarized level (−73 mV). *B*, off response of l.t.r. at r.m.p. (−60 mV). *C*, fast action potentials with lower frequency from original r.m.p. *D*, *E* and *F*, responses 15 min after perfusion with Ca^{2+}-free solution (Ca^{2+} replaced by 12 mM-Mg^{2+}). *D*, attenuation of l.t.r. *E*, disappearance of off response. *D* and *F*, no influence on fast spikes. *G* and *H*, disappearance of fast action potentials in Ca^{2+}-free solution plus TTX at hyperpolarized and resting levels.

dependent K^+ conductance increase, the effects of Ca^{2+}-free solutions on the a.h.p.s. were studied. The amplitude of the a.h.p. of type A neurones, which was small at normal Ca^{2+} concentration (Fig. 6*Aa*), did not change significantly after 15 min perfusion in Ca^{2+}-free solution (Fig. 6*Ab*). However, in type B and C neurones, the amplitude and half decay time of a.h.p. were markedly decreased in Ca^{2+}-free solution (Fig. 6*Ba*, *Bb*, *Ca* and *Cb*).

Transient K^+ (I_A) conductance. A characteristic membrane property of the type C cells is the slow return to original resting membrane potential level after an inward current pulse. 30 min perfusion with 1 mM-4-AP, which blocks the I_A conductance (Thompson, 1977; Kenyon & Gibbons, 1979; Gustafsson, Galvan, Grafe & Wigström, 1982), accelerated the return to base line (Fig. 7*A* and *B*).

Effects of Ca^{2+}-free solution on f–I relations

To study the participation of Ca^{2+} in firing behaviour, the effects of Ca^{2+}-free solution (replaced by 12 mM-Mg^{2+}) on *f–I* relations and repetitive firing were observed. In type A neurones, perfusion with Ca^{2+}-free solution for 15 min produced

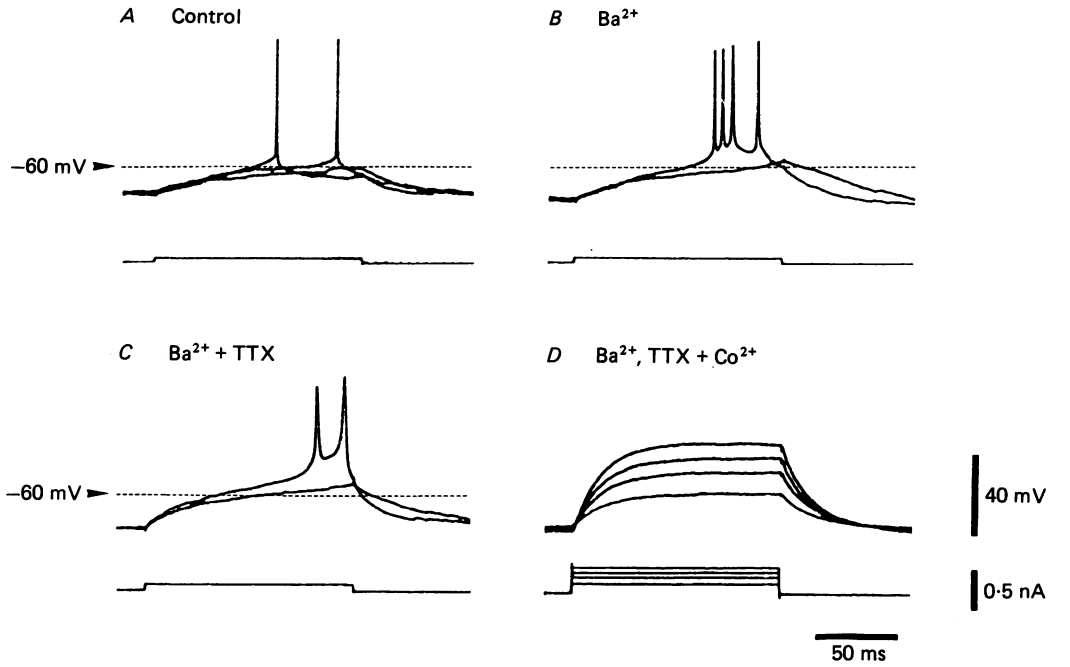

Fig. 5. Effects of Ba^{2+} on l.t.r. of a type B neurone. *A*, control. Hyperpolarized level (−72 mV). Arrowhead, resting membrane potential (r.m.p., −60 mV). *B*, replacement of 0·5 mM-Ca^{2+} by 0·5 mM-Ba^{2+}. Increased amplitude of l.t.r. and fast spike burst. *C*, further addition of TTX. Disappearance of fast action potentials. High-threshold spikes and l.t.r. remained. *D*, disappearance of l.t.r. and high-threshold action potential in 5 mM-Co^{2+}.

no remarkable change in the *f–I* relation for the first interval (Fig. 8*A*). In type B neurones, perfusion with Ca^{2+}-free solution markedly reduced the initial firing rate at around 0·2 nA, and the steep rise of the *f–I* curve became somewhat flat at the same current intensity (Fig. 8*B*, *E* and *G*). These results suggest that the initial firing rate in this type of neurone is controlled mainly by the l.t.r. which is related to Ca^{2+} current. In type C neurones, Ca^{2+}-free solution had little or no effect on the *f–I* curves for the first interval (Fig. 8*C*), but the frequency in later intervals, for example the third interval (interval between third and fourth spikes), increased (Fig. 8*D*). The build-up of the a.h.p. with successive action potentials is relatively slow, so its effects on discharge frequency are greater at later interspike intervals. Thus Ca^{2+}-free solution has little effect on the first interval, but substantially shortens the later intervals of the train (Madison & Nicoll, 1984).

DISCUSSION

In the present study we examined the ionic bases for the different membrane properties observed in three distinct types of cell in the v.m.h. In addition to the traditional Na^+ and K^+ conductance changes, some distinct ionic conductance changes, e.g. the l.t.r. or the I_A conductance, form the bases of specific membrane properties, and underlie the heterogeneity among neurones of the v.m.h.

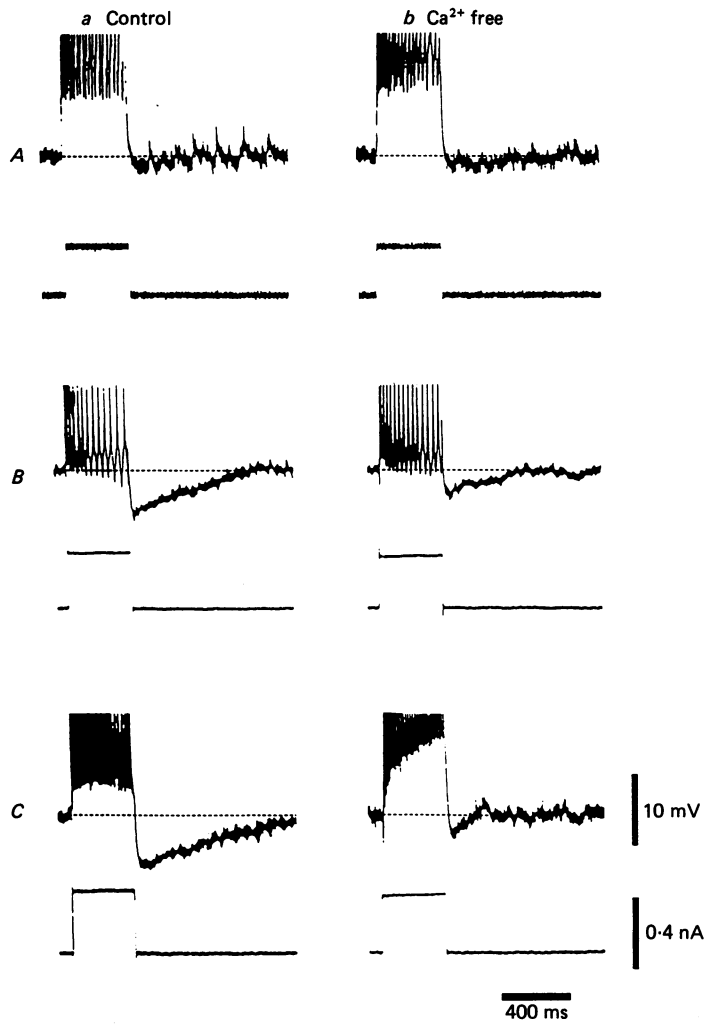

Fig. 6. Effect of Ca^{2+}-free solution on a.h.p. in three neurone types. *a*, control responses; *b*, 15 min after perfusion with Ca^{2+}-free solution (replaced by 12 mM-Mg^{2+}). *A*, type A cell (resting membrane potential, r.m.p., −63 mV); a.h.p. amplitude, small in control solution (*Aa*) and only slight change in Ca^{2+}-free solution (*Ab*). *B*, type B neurone, (r.m.p., −60 mV); large a.h.p. (*Ba*); marked attenuation of a.h.p. amplitude (6·3 mV to 3·3 mV; *Bb*). *C*, type C cell (r.m.p., −62 mV); large a.h.p. with long time course (*Ca*); marked attenuations of a.h.p. amplitude (7·0 mV to 2·6 mV) and half decay time (415 ms to 148 ms; *Cb*).

Action potentials

The fast action potentials with rapid rate-of-rise and short duration, found in all three types of cell, could be Na^+ spikes since these fast spikes were blocked by TTX (Fig. 2*Ab*, *Bb* and *Cb*) but persisted in Ca^{2+} conductance-blocked solutions (Fig. 6*Ab*, *Bb* and *Cb* and Fig. 8).

The slow spikes with high threshold of type B and C neurones, but not those of type A, could be evoked in TTX alone. A broad spike in type A neurones was elicited

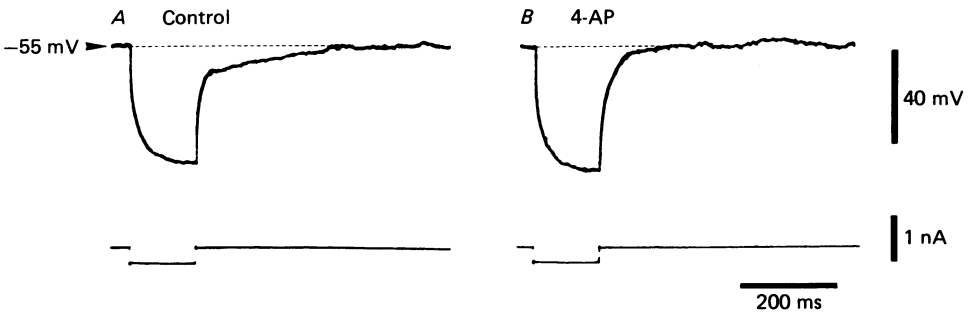

Fig. 7. Effect of 4-AP on delayed return to base line after inward current pulse in type C neurone. *A*, control. Depolarized membrane potential at −55 mV. *B*, after 30 min 1 mM-4-AP perfusion. Marked reduction of delayed return to base line.

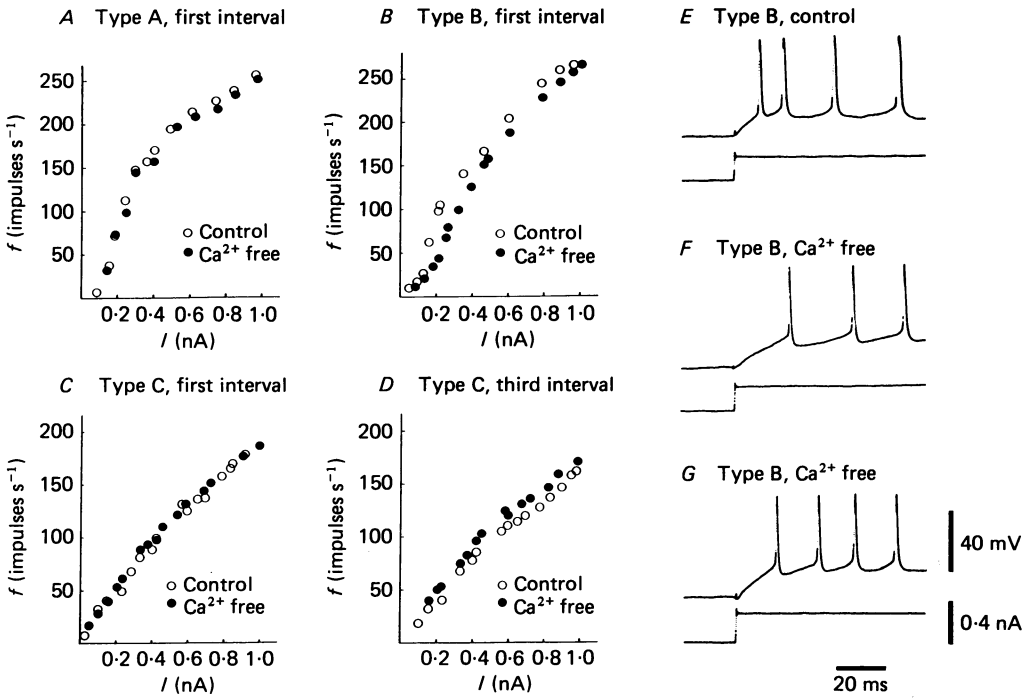

Fig. 8. Effects of Ca^{2+}-free solution on *f–I* relations. *A*, type A cell. 15 min after perfusion of Ca^{2+}-free solution (replaced by 12 mM-Mg^{2+}), no remarkable change in *f–I* relation for first spike interval (indicated as frequency *vs.* applied outward current pulses at various values of stimulus intensity). *B*, type B neurone. After perfusion with Ca^{2+}-free solution, marked decrease in *f–I* slope of first interval at about 0·2 nA. *C* and *D*, type C cells. After perfusion with Ca^{2+}-free solution, no change in *f–I* relation for first interval (*C*); increase in *f–I* relation of third interval (*D*). *E*, actual responses of type B cell to outward current of 0·2–0·3 nA in control solution. *F* and *G*, same as in *E* but in Ca^{2+}-free solution at different outward current intensities.

only after the addition of TEA. These TTX–TEA resistant action potentials could be high-threshold Ca^{2+} spikes since they were abolished by Mn^{2+} or Co^{2+}. The permeability responsible for high-threshold Ca^{2+} spikes is greater in the dendrites than in other neurone membrane areas (Llinás & Sugimori, 1980*b*; Jahnsen & Llinás,

1984). The above findings suggest that the high-threshold Ca^{2+} spike component would be minimal in type A neurones because this type, corresponding to bipolar types, possesses fewer dendrites than the others (Minami *et al.* 1986).

The l.t.r.

The l.t.r. in type B neurones was similar to that reported in inferior olivary (Llinás & Yarom, 1981) and thalamic neurones (Jahnsen & Llinás, 1984). Following membrane hyperpolarization, direct or synaptic stimulation may evoke the l.t.r., on which the fast high-frequency spike burst rides. The l.t.r. was Ca^{2+} dependent since it was blocked by the usual Ca^{2+} blockers or in Ca^{2+}-free solution, but not by TTX. This potential was still evident when Ba^{2+} was substituted for Ca^{2+}. Moreover, the duration of the l.t.r. was not greatly prolonged in the presence of Ba^{2+}, suggesting that its termination may have been due to voltage-dependent inactivation and not to the Ca^{2+}-dependent K^+ conductance change (Jahnsen & Llinás, 1984) since this conductance is not activated by Ba^{2+} (Eckert & Lux, 1976). This self-limited inactivation is a critical point in differentiating the l.t.r. generated in the cell soma from the dendritic Ca^{2+} plateau potentials (Jahnsen & Llinás, 1984). In inferior olivary dendrites, replacement of Ca^{2+} by Ba^{2+} produced prolonged plateau potentials (for many seconds) because the dendritic Ca^{2+} channel possesses very little inactivation (Llinás, 1984).

The a.h.p.

The a.h.p. in v.m.h. neurones was generated by distinctive ionic mechanisms. TEA increased the duration of the spikes in all three types of cell, suggesting the increase of voltage-dependent K^+ conductance in these neurones. In B and C type neurones, Ca^{2+}-free solution markedly reduced the amplitude of the a.h.p. (Fig. 6), indicating involvement of the Ca^{2+}-dependent K^+ conductance increase observed in other mammalian neurones (Alger & Nicoll, 1980; Hotson & Prince, 1980; Morita, North & Tokimasa, 1982; Harada & Takahashi, 1983). However, the a.h.p. in the type A cell was minimal to begin with, and was virtually unaffected in Ca^{2+}-free solution. These findings suggested minimal contribution of Ca^{2+}-dependent K^+ conductance increase in the type A cell due to the small contribution of high-threshold Ca^{2+} spikes generated in dendrites (Llinás & Sugimori, 1980*b*; Jahnsen & Llinás, 1984). This is consistent with our morphological evidence of fewer dendrites in this cell type.

In addition to these two conductance changes, the a.h.p. in type C cells was further enhanced, especially in duration, by I_A current. As in other neurones (Hagiwara, Kusano & Saito, 1961; Connor & Stevens, 1971*a*; Gustafsson *et al.* 1982; Jahnsen & Llinás, 1984), this conductance change was inactive at depolarized membrane potential levels, and was de-inactivated at the hyperpolarized level beyond the resting potential, and was then activated if a sufficient depolarization occurred from this new level. Once activated, this conductance prevented an immediate return of the membrane potential to the resting level (Minami *et al.* 1986). This conductance was markedly reduced by perfusion with 4-AP which can block the I_A current. This I_A current may contribute to the flat *f–I* slope of the first interval in type C neurones (Connor & Stevens, 1971*b*).

In the present experiments, the factors affecting generation of the a.h.p.s. were thought to be: voltage-dependent K^+ and minimal Ca^{2+}-dependent K^+ conductance

for the type A neurones; voltage-dependent K^+ and Ca^{2+}-dependent K^+ conductance for the type B neurones; and voltage-dependent K^+, Ca^{2+}-dependent K^+ and I_A conductance for the type C neurones.

Repetitive firing mechanisms

In type B neurones, Ca^{2+}-free solution decreased the initial firing rate in the *f–I* plots because the Ca^{2+}-free solution reduced the fast spike burst generated by the l.t.r. This suggests that the sharp inflexion of the *f–I* curves can be attributed to the l.t.r. as in thalamic neurones (Jahnsen & Llinás, 1984). The increase in firing rate at the third interval in type C cells was caused by blockade of the Ca^{2+}-dependent K^+ conductance, indicating that this conductance suppressively regulates repetitive firing of the cell in later intervals (Calvin & Schwindt, 1972; Baldissera & Gustafsson, 1974*a*, *b*; Madison & Nicoll, 1984). I_A current was also thought to be involved in regulation of the firing of type C neurones.

As mentioned, many kinds of ionic conductance participate in controlling the repetitive firing of v.m.h. neurones. Specifically, the six different ionic conductances summarized in Table 1 were observed in the three different types of cell in our present study. Each of the three cell types in the v.m.h. appears to have its own distinctive ionic mechanism for response to complicated information input.

TABLE 1. Six different ionic conductances in v.m.h. neurones

	Classification of cells		
	Type A	Type B	Type C
Action potential	Na^+ fast spike	Na^+ fast spike	Na^+ fast spike
	High-threshold Ca^{2+} spike (minimal)	High-threshold Ca^{2+} spike	High-threshold Ca^{2+} spike
		Low-threshold Ca^{2+} response	
K^+ current	Voltage-dependent K^+ conductance	Voltage-dependent K^+ conductance	Voltage-dependent K^+ conductance
	Ca^{2+}-dependent K^+ conductance (minimal)	Ca^{2+}-dependent K^+ conductance	Ca^{2+}-dependent K^+ conductance
			Transient K^+ (I_A) conductance

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