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Effects induced by the antiepileptic drug valproic acid upon the ionic currents recorded in rat neocortical neurons in cell culture*

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Summary. Rat neocortical neurons in culture were subjected to the whole cell mode of voltage clamping under experimental conditions designed to study Na^+, Ca^{2+} and K^+ currents in isolation. Following pharmacological blockade of most of the Ca^{2+} and K^+ channels, depolarizing commands which brought the membrane potential from −80 to +10 mV elicited an inward current. This current was sensitive to tetrodotoxin (TTX) and was therefore caused by the opening of voltage-dependent channels permeable to Na^+. Extracellular application of the antiepileptic drug valproic acid (VPA, 0.2–2mM) reduced in a dose-related, reversible way this Na^+ current. VPA also evoked an increase of the voltage-dependent inward current recorded in the presence of TTX and thus presumably carried by Ca^{2+}; this effect was seen in the presence of doses of VPA larger than 0.5 mM and was not reversible. Two types of outward K^+ currents evoked by depolarizing steps in the presence of Na^+ and Ca^{2+} channels blockers were not affected by VPA (up to 5 mM). Our data indicate that doses of VPA that are within the range present when it is used as an anticonvulsant, can influence inward currents generated by rat neocortical cells in culture. The reduction of the Na^+, inward current is in line with findings obtained in mouse neurons by using standard intracellular recording techniques. This effect might represent an important mechanism of action for VPA in neocortex.

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Introduction

Clinical studies conducted in the last two decades indicate that valproic acid (VPA) is effective in controlling generalized seizures including those associated with myoclonic epilepsy, absence and tonic-clonic (convulsive) attacks (Bruni and Wilder 1979). However, the actions exerted by VPA at the cellular level remain as yet poorly understood. Mechanisms which have been proposed for the anticonvulsant effects of VPA include the enhancement of inhibition mediated through the GABA receptor, the impairment of excitatory neurotransmission and several effects exerted directly upon intrinsic membrane currents (for review see Chapman et al. 1982; Capek and Esplin 1990).

The enhancement of GABAergic mechanisms induced by VPA has been reported by several investigators in both biochemical and electrophysiological studies (Godin et al. 1969; Iadarola and Gale 1979; Gent and Phillips 1980; Löscher 1981; Baldino and Geller 1981; Preisendörfer et al. 1988). However, these data have little relevance for understanding the therapeutical action of VPA since very high concentrations are necessary to modify GABA-mediated inhibition (cf. Perreault et al. 1989). A second hypothesis for the anticonvulsant action of VPA invokes a decreased efficacy of excitatory synaptic mechanisms. Following the demonstration that VPA can reduce transmitter release at the neuromuscular junc-

tion (Alkadhi and Banks 1984), in vitro-experiments in the CA1 subfield of the hippocampal slice have indicated that VPA can decrease both paired-pulse facilitation (Franceschetti et al. 1986) and the amplitude of post-tetanic potentiation (Griffith and Taylor 1988). A third hypothesis for the anticonvulsant action of VPA postulates an effect exerted by this antiepileptic drug on one or several intrinsic membrane currents. High doses of VPA appeared to increase K^+ outward currents in *Aplysia* nerve cells (Slater and Johnston 1977). More recently it has been shown that VPA can block Na^+ and K^+ ion channels (Van Dongen et al. 1987), regulate Na^+ and K^+ ion gating (Fohlmeister et al. 1984) and decrease high-frequency repetitive firing generated by mouse central neurons in cell culture (McLean and MacDonald 1986). As in the case of post-tetanic potentiation (Griffith and Taylor 1988), the effects observed in the latter study were obtained in the presence of therapeutic concentrations of VPA.

The action of VPA on intrinsic membrane properties is presumably relevant for its anticonvulsant effects since VPA can reduce the occurrence of epileptiform bursts generated by hippocampal neurons maintained in vitro and bathed in a medium containing low Ca^{2+} high Mg^{2+} (Agopyan et al. 1985; Franceschetti et al. 1986; Rose et al. 1986). This is an experimental model of in vitro epileptiform activity in which both inhibitory and excitatory synaptic potentials are to a large extent reduced or blocked (Haas and Jefferys 1984; Konnerth et al. 1986). The experiments reported in this paper were therefore designed to obtain further information on the effects induced by VPA upon inward and outward currents generated by cultured rat neocortical cells. A preliminary report of some of these findings has appeared (Zona et al. 1988).

Methods

Experiments were performed on neocortical neurons removed from 14-day Wistar rat embryos and grown in dissociated cell culture for 2–3 weeks following methods that are standard in our laboratory (Dichter and Zona 1989). The whole cell patch-clamp technique was used to record the current from the cell soma. Patch-clamp electrodes were obtained from microhematocrit capillary tubes which were pulled with a BBCH micropipette puller. When filled with the appropriate solution (see below), the resistance of the electrodes was approximately 3 MΩ. Patch-clamp electrodes were connected to an EPC-7 patch-clamp amplifier which was controlled in turn by a computer-stimulation and data-acquisition system (PDP 11/23-based INDEC). The responses were recorded, digitized and stored on a hard disk for later analysis. Leak measurements (determined by application of small hyperpolarizing commands) were automatically subtracted from the response.

The bathing solution contained in mM: NaCl 120, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, glucose 20, HEPES 10 mM buffered to pH 7.3 with NaOH. Depending upon the type of experiment, 4-aminopyridine (4-AP, 2 mM), tetrodotoxin (TTX, 5 μM) and Cd^{2+} (0.2 mM) were also added to the extracellular medium. The solution used for filling the electrodes employed for measuring Ca^{2+} or Na^+ currents contained in mM: CsCl 130, $CaCl_2$ 0.24, EGTA 5, glucose 10, HEPES 10, $MgCl_2$ 1, ATP 2 and the pH was adjusted to 7.3 with CsOH. For monitoring K^+ currents, the electrode solution contained in mM: KCl 120, EGTA 5, $CaCl_2$ 0.24, glucose 30, 10 mM HEPES buffered to pH 7.3 with KOH. The experiments were performed at room temperature 21–24° C.

VPA was freshly prepared for each experiment. It was dissolved by adding 1 M NaOH to achieve water solubility and was then diluted in a solution that had the same composition as the bathing medium. The values of the VPA concentration to be used in our experiments were indicated by pharmacokinetics studies performed in humans (Gugler et al. 1977) and rats (Albertson et al. 1981). We used concentrations ranging between 0.2 and 5 mM, where the highest value represents a toxic concentration of VPA while the therapeutical range is between 0.2 and 1 mM (cf. McLean and MacDonald 1986).

The solutions containing known concentrations of VPA were ejected by pressure from blunt micropipettes with tips broken back to 10–15 μm diameter. During the period of solution ejection (approx. 60–90 s), the pipettes were situated close to the cell membrane, but were otherwise kept away from the cell under study to prevent effects due to the diffusion of the solution.

Results

Inward Na^+ and Ca^{2+} currents

In a first series of experiments, Ca^{2+} and K^+ currents were pharmacologically blocked by extracellular application of Cd^{2+} and 4-AP as well as by addition of Cs^+ and TEA in the recording electrode. In this type of experimental condition, depolarizing pulses from a holding potential of −80 mV elicited a voltage-dependent, inward current that was sensitive to TTX (not illustrated)

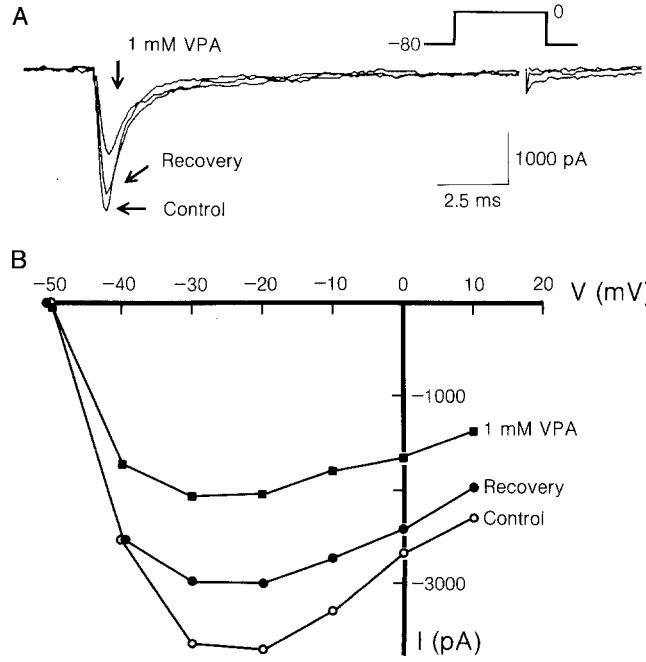

Fig. 1A, B. Effects induced by VPA upon the voltage-dependent inward current generated by a cultured rat neocortical cell following blockade of most of the Ca^{2+} and K^+ channels. In **A**, the response to a single command which brought the membrane potential from −80 to 0 mV is shown in control, during and after application of 1 mM VPA. In this and following figures the inset shown in the top right portion of the raw data panel represents the pulse protocol. In **B**, changes induced by 1 mM VPA upon the I–V plot of this inward Na^+ current

and was therefore carried by Na^+ ions. During steps that brought the membrane potential to +10 mV, this inward Na^+ current rose within 0.7–0.8 ms to a peak of approximately 3–3.3 pA and then decayed to zero with a time constant of 0.8 ms (Fig. 1A).

In any of the neurons studied ($n > 20$), the application of VPA (0.2–2 mM) diminished reversibly the Na^+ inward current (Fig. 1A). As shown in the I–V plot of Fig. 1B, the decrease induced by VPA was observed on the responses to any of the voltage commands to which the cell under study was subjected. The reduction of the Na^+ inward current seen during application of VPA was dose-dependent with a threshold dose of 0.3 mM. The histogram shown in Fig. 2 was obtained by calculating the percentage reduction of the peak of the Na^+ inward current induced in different trials by concentrations of VPA which ranged between 0.2 and 2 mM. In order to avoid differences related to the experimental protocol used, these values were obtained in the different experiments by analyzing the changes of VPA upon the inward response which was elicited by bringing the membrane potential from −80 mV to a holding potential of 0 mV. It is clear from this histogram that the percentage reduction induced upon the Na^+ inward current reached a plateau at 1 mM concentration. This maximal effect consisted of a decrease, not of a blockade of the Na^+ inward current.

In a second series of experiments, inward currents were elicited by depolarizing pulses following blockade of both Na^+ currents (which was caused by addition of TTX to the extracellular solution) and K^+ outward current (i.e., 4–AP in the extracellular solution and Cs^+ and TEA in the intracellular electrode). Therefore these inward currents were presumably carried by Ca^{2+} ions. As reported by Dichter and Zona (1989), stepping the membrane potential from −80 to 0 mV evoked an inward response which reached a peak within 8–10 ms and decayed slowly with a time constant of several hundred ms.

Surprisingly, application of VPA (0.5–2 mM) in this type of experimental condition evoked an increase of the

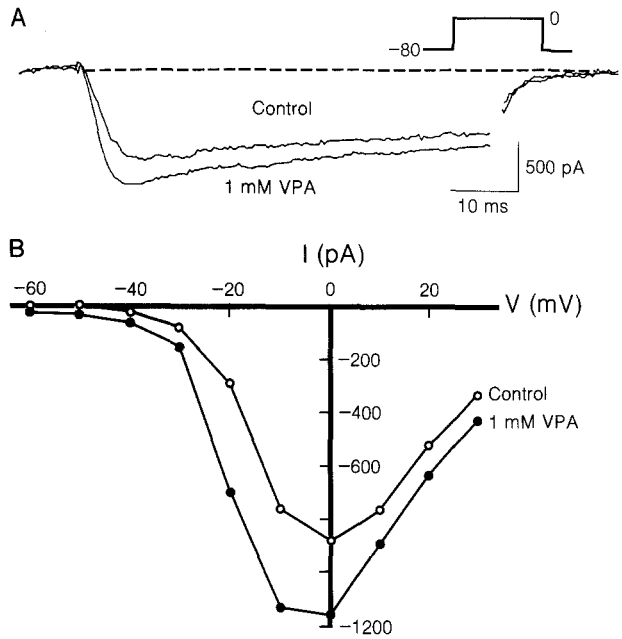

Fig. 3A, B. Effects induced by VPA upon the voltage-dependent inward current generated by a cultured rat neocortical cell following blockade of Na^+ and K^+ currents. In **A**, the response to a single command which brought the membrane potential from −80 to 0 mV is shown in control and during application of 1 mM VPA. Note the increase of the current evoked by VPA; this effect is detected both at the peak (i.e., 6–8 ms after the command's onset) and during the late part of the response. In **B**, changes induced by 1 mM VPA upon the I–V plot computed for this inward, Ca^{2+} current

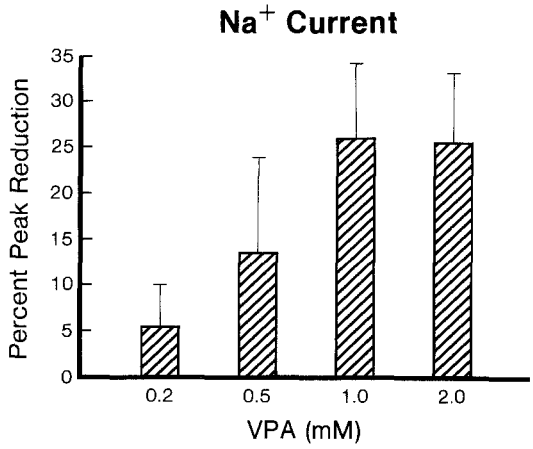

Fig. 2. Histogram of the percent reduction (ordinate) induced by different concentrations of VPA (abscissa) upon the Na^+ current. To avoid inconsistency in evaluating the responses observed in different experiments, the amplitude of the current was measured at the time of the peak before and during VPA application and this measurement was performed in all the cases by stepping the membrane potential from −80 mV to a holding potential of 0 mV. Bars indicate the standard deviation. The numbers of experiments performed by using the different doses of VPA were: 3, 4, 7 and 5 for 0.2, 0.5, 1 and 2 mM respectively

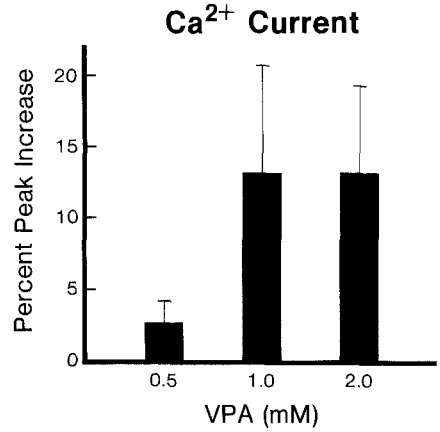

Fig. 4. Histogram of the percent increase (ordinate) induced by different concentrations of VPA (abscissa) upon the Ca^{2+} current. As in the case of the experiments performed for analyzing the Na^+ current, the amplitude of the Ca^{2+} current was measured at the time of the peak before and during VPA application and this measurement was performed in all the cases by stepping the membrane potential from −80 mV to a holding potential of 0 mV. Bars indicate the standard deviation. The numbers of experiments performed using the different doses of VPA were: 4, 7 and 5 for 0.5, 1 and 2 mM respectively

Ca^{2+} inward currents in over 15 cells (Fig. 3). This effect, which appeared to have a concentration threshold of 0.5 mM, did not however recover to the control values following cessation of drug application. As in the case of Na^+ inward currents, the increase in Ca^{2+} inward currents induced by VPA appeared to be dependent upon the dose used. This is shown in Fig. 4, which represents a summary of the percentage increases induced by different concentrations of VPA upon the Ca^{2+} inward currents.

K^+ outward currents

In the presence of TTX and Cd^{2+} in the extracellular solution, depolarizing pulses applied from a holding potential of −80 mV elicited a time- and voltage-dependent outward response that rose rapidly to a peak and then decayed to a steady level (Fig. 5A). In agreement with a previous report from our laboratory (Zona et al. 1988), this outward response was caused by at least two different K^+ currents, namely a fast, transient current which was half-inactivated at holding potentials more positive than −50 mV and a late persistent or slowly activating current which was unaffected by bringing the holding potential to depolarized levels.

As illustrated in Fig. 5, both components of the outward responses induced by depolarizing command pulses were not modified during application of VPA as achieved by ejecting a 1 mM solution from a nearby pipette ($n=10$ cells). A similar lack of effect upon the outward K^+ currents was also observed when the concentration of VPA was raised to 5 mM ($n=2$ cells). In Fig. 5B one can appreciate that the I/V curves obtained by measuring the peaks of the outward currents in control and during application of VPA closely overlap.

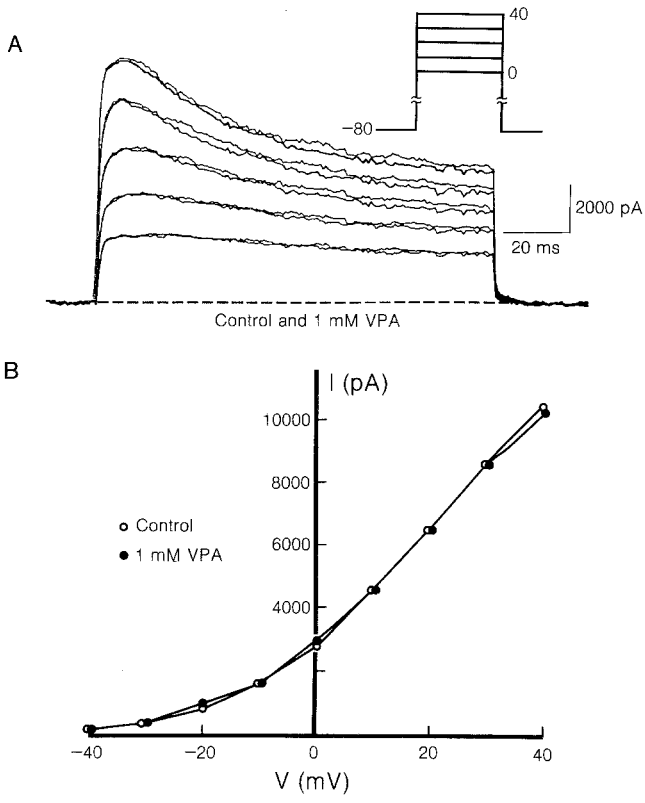

Fig. 5A, B Effects induced by 1 mM VPA upon the outward responses elicited by depolarizing steps in a neocortical cell following blockade of Na^+ and Ca^{2+} currents. Note the lack of change during application of VPA in both the raw data (**A**) and in the I–V plot (**B**) that was obtained from the same neuron

Discussion

These experiments demonstrate that the antiepileptic drug VPA induces at least two direct effects on the membrane properties of rat neocortical cells in culture. These consist of: (i) a reversible decrease of the fast, transient Na^+ inward current and (ii) a non-reversible increase of the Ca^{2+} inward current. In addition, the present experiments also show that at least in this type of preparation VPA does not modify K^+ outward currents.

The reduction exerted by VPA upon the Na^+ current generated by neocortical cells is in line with the findings obtained by McLean and MacDonald (1986) in mouse central neurons in cell culture. In their study, concentrations of VPA close to the clinically useful therapeutic range limited sustained repetitive firing of action potentials and reduced the maximal rate of rise of the Na^+ action potential. Interestingly, this type of action was not accompanied by a decreased ability of the cells treated with VPA to generate single action potentials. That in our experiments even supratherapeutic doses of VPA (i.e. >1 mM) could only reduce but never block the inward Na^+ current indicates that this antiepileptic drug interferes with the ability of the neurons to undergo prolonged and sustained firing of fast Na^+ action potentials. This type of neuronal activity typically occurs during epileptiform discharges (for review, see Prince and Connors 1986). The effect of VPA upon the voltage-dependent Na^+ current generated by rat neocortical neurons is also in line with the demonstration that VPA reduces a Na^+ current recorded in the nodal membrane of peripheral nerve fibers of *Xenopus levis* (Van Dongen et al. 1986).

The increase in Ca^{2+} currents observed during VPA application is a finding that does not offer a ready explanation for a possible mechanism of action of this antiepileptic drug. It is well known that Ca^{2+} currents play an important role in epileptogenesis (for review, see Heinemann and Hamon 1986). Therefore it is not easy to envisage an action through which the increase of a Ca^{2+} inward current might account for the anticonvulsant action of VPA. However, though not reversible, this effect was consistently observed in all experiments performed and displayed a dose-related response. Both these features would suggest that the changes induced by VPA upon the Ca^{2+} currents are not an artefact. A possible mechanism through which the VPA-induced increase in Ca^{2+} might affect neuronal excitability is that of modulating the efficacy of Ca^{2+}-dependent K^+ conductances (Lancaster and Adams 1986). However, at least in the CA1 subfield of the rat hippocampal slice, VPA failed to

modify the long-lasting afterhyperpolarization that follows repetitive firing induced by depolarizing current pulses (Perreault et al. 1989).

The failure of VPA to modify at least two different K^+ outward currents deserves some attention since a previous report by Slater and Johnston (1977) had indicated that high doses of VPA could hyperpolarize *Aplysia* nerve cells through a mechanism mediated by an increase in the membrane conductance to K^+. This lack of effect was observed even when using supratherapeutic concentrations of VPA (i.e. 5 mM), thus indicating that the findings obtained in the molluscan brain do not apply to mammalian neocortical neurons. This conclusion is in line with findings obtained in the rat hippocampus in which VPA did not affect repolarizing conductances such as the downstroke or time of repolarization of the action potential or the mechanism of adaptation (Perreault et al. 1989).

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