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DEVELOPMENT OF CALCIUM CURRENT SUBTYPES IN ISOLATED RAT HIPPOCAMPAL PYRAMIDAL CELLS

BY SCOTT M. THOMPSON* AND ROBERT K. S. WONG†

From the Department of Neurology, College of Physicians and Surgeons, Columbia University, 630 West 168th Street, New York, NY 10032, USA

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SUMMARY

1. Patch-clamp techniques were used to record from acutely dissociated rat hippocampal pyramidal cells of different postnatal ages to study the development, kinetics of activation and inactivation, and pharmacology of various components of whole-cell calcium current.

2. In both adult and immature pyramidal cells, the threshold of activation for Ca^{2+} current from the holding potential of −50 mV was about −35 mV. The current was non-inactivating near threshold, and slowly inactivating with stronger depolarizations.

3. In *adult* pyramidal cells, hyperpolarizing pre-pulses (−85 mV, 3 s) increased the peak amplitude of current, but had little effect on the amplitude of sustained current or on the threshold. In *immature* cells, hyperpolarizing pre-pulses (−85 mV, 3 s) revealed an additional component of Ca^{2+} current that had a threshold for activation around −60 mV, and inactivated rapidly and completely at potentials between −60 and −35 mV. This low-threshold Ca^{2+} current was found in all cells less than 12 days of age, and in no cells older than 29 days of age.

4. No difference was observed between the Ca^{2+} currents elicited from CA3 or CA1 pyramidal cells.

5. The time course of decay for inactivating Ca^{2+} currents in adult cells at −20 mV was well fit with a single exponential of roughly 120 ms time constant. In immature cells, the addition of a second, faster time constant (roughly 25 ms) was required to describe the decay of the inactivating current adequately. The persistent Ca^{2+} current elicited from the holding potential of −50 mV decayed with a time constant of roughly 750 ms. The rate of inactivation for all Ca^{2+} current components was faster with stronger depolarizations. Inactivating Ca^{2+} currents contributed a significantly larger percentage of the total Ca^{2+} current in immature than adult cells.

6. Steady-state inactivation of the low-threshold Ca^{2+} current in immature cells was described by the Boltzmann equation with half maximal inactivation at −76 mV and a slope factor of 6·8. Recovery from inactivation was exponential, with

* Present address and correspondence to: Brain Research Institute, University of Zürich, August Forel-Strasse 1, CH-8029 Zürich, Switzerland.

† Present address: Department of Pharmacology, SUNY Health Science Center at Brooklyn, 450 Clarkson Avenue, Brooklyn, NY 11203, USA.

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a time constant of 820 ms (at −85 mV). Steady-state inactivation of the high-threshold inactivating Ca^{2+} current in adult cells was described by the Boltzmann equation with half maximal inactivation at −78 mV and a slope factor of 9·9.

7. The low-threshold Ca^{2+} current in immature cells was blocked in a reversible and dose-dependent manner by amiloride (100–250 μM). Amiloride had no effect on high-threshold Ca^{2+} currents. Neither ethosuximide (350 μM) nor methsuximide (350 μM) had any effect on low-threshold Ca^{2+} current. ω-Conotoxin blocked both persistent and inactivating components of high-threshold Ca^{2+} current in adult cells.

8. We conclude that immature hippocampal pyramidal cells exhibit three macroscopically distinct Ca^{2+} currents: a low-threshold, rapidly inactivating T-type current; a high-threshold, inactivating N-type current; and a high-threshold, persistent L-type current. The kinetic and pharmacological properties of these current components are comparable to those described for other cell types. T-type low-threshold Ca^{2+} current, however, is not present in adult dissociated pyramidal cells.

INTRODUCTION

Influx of Ca^{2+} into neurons plays an important role in regulating cell excitability, neurotransmitter release, enzyme activity, and gene expression. Voltage-gated Ca^{2+} channels are thus critically involved in determining neuronal function. At least three types of voltage-dependent Ca^{2+} current may be distinguished in a variety of cell types on the basis of their thresholds, kinetics of activation and inactivation, single channel properties, and pharmacology. High-threshold Ca^{2+} currents are activated with relatively strong depolarizations from the resting membrane potential. In neurons, there are both rapidly and slowly inactivating components of high-threshold Ca^{2+} current. In cultured chick sensory neurons, these current components have been named N- and L-type Ca^{2+} current, and inactivate with time constants of roughly 100 ms and 1 s, respectively (e.g. Nowycky, Fox & Tsien, 1985; Fox, Nowycky & Tsien, 1987*a*; Plummer, Logothetis & Hess, 1989). In contrast, low-threshold T-type Ca^{2+} current is activated close to the resting membrane potential, and inactivates rapidly and completely with a time constant of roughly 20 ms (e.g. Carbone & Lux, 1984, 1987*a*, *b*; Fox *et al.* 1987*a*).

Hippocampal pyramidal cells produce action potentials, and bursts of action potentials, that contain pronounced Ca^{2+}-dependent components (e.g. Wong & Prince, 1978; Gähwiler & Brown, 1987). Although initial evidence in support of multiple Ca^{2+} currents in hippocampal cells was obtained with microelectrode recording (e.g. Halliwell, 1983), a full description of their kinetics and voltage-dependence requires the application of patch-clamp recording techniques. Whole-cell recording provides lower electrical resistance access to the cell interior and intracellular dialysis, allowing complete block of K^+ currents. Problems may arise in achieving adequate space- and voltage-clamp when recording currents from intact neurons *in situ* or in brain slices due to the extent and complexity of their dendritic arborization. Acute mechanical isolation of neurons following partial enzymatic digestion yields neurons with reduced dendritic trees which are well suited for patch-clamp recording of whole-cell currents (Kay & Wong, 1986). Previous experiments have shown that adult guinea-pig hippocampal CA1 pyramidal cells isolated with

this technique have exclusively high-threshold Ca^{2+} currents (Kay & Wong, 1987; Doerner, Pitler & Alger, 1988). In contrast, low-threshold Ca^{2+} current has been observed in cultured embryonic hippocampal neurons (Bley, Madison & Tsien, 1987; Doerner *et al.* 1988; Meyers & Barker, 1989) and neurons isolated from embryonic tissue (Yaari, Hamon & Lux, 1987). We wished to determine whether the previously observed absence of low-threshold Ca^{2+} current in dissociated adult hippocampal cells was a consequence of either the cell isolation procedure, or the use of CA1 and not CA3 cells, or rather reflected an on-going developmental process that is arrested or delayed in cultured embryonic cells (e.g. Gonoi & Hasegawa, 1988).

Our results suggest that a low-threshold, rapidly inactivating Ca^{2+} current, similar to T-type Ca^{2+} current described in other cell types, can be recorded only in cells taken from rats at less than 2 weeks of age. Both inactivating N-type and persistent L-type high-threshold Ca^{2+} currents are apparent at all ages. Portions of these data have been presented to the Society for Neuroscience (Thompson & Wong, 1989).

METHODS

Isolation of cells

The method for acutely dissociating hippocampal neurons has been described in full elsewhere (Kay & Wong, 1986). In brief, Sprague–Dawley rats of various postnatal ages were anaesthetized with halothane, decapitated, and the hippocampus was dissected free. We will refer to cells taken from animals older than 29 days after birth as adult cells. In most cases adult rats were litter mates of rats used at immature ages. Transverse slices were cut at a thickness of 650 μm on a McIlwain tissue chopper. The slices were subdissected under a stereo microscope to isolate either the CA1 or CA3 region. These sections were then incubated in a HCO_3^--free, PIPES (piperazine-*N*-*N*′-bis(2-ethanesulphonic acid))-buffered saline containing 0·8 mg ml^{-1} trypsin (Sigma Type XI). The sections were gently circulated, continuously superfused with oxygen, and maintained at 32 °C for 1 h. After washing in fresh saline without trypsin for 1 h at room temperature, cells could be mechanically isolated as needed (up to 6 h) by gentle trituration through a graded series of three fire-polished pipettes in the recording chamber containing HEPES-buffered Dulbecco's modified Eagle's medium (GIBCO). After trituration, several minutes were allowed for the isolated cells to settle and to become attached to the glass bottom of the recording chamber before beginning perfusion with the recording saline. Viable cells appeared bright under phase contrast optics, and had one or more dendrites up to 80 μm in length.

Electrophysiological techniques

The extracellular recording solution was composed of (mM): Tris methylsulphate (from Tris base), 120; HEPES, 10; $MgCl_2$, 1; glucose, 25; and was titrated to pH = 7·4 with methanesulphonic acid. In addition, the recording solution contained 5 mM-CsCl, 15 mM-tetraethylammonium chloride, and 5 mM-4-aminopyridine to ensure pharmacological block of K^+ currents. The charge carrier through voltage-gated Ca^{2+} channels was Ba^{2+} (3–10 mM) to reduce problems with Ca^{2+}-dependent inactivation of Ca^{2+} channels (Eckert & Chad, 1984). We will nevertheless refer to our recorded currents as Ca^{2+} currents, as Ca^{2+} ions are undoubtedly the charge carrier *in situ*. The recording chamber was mounted on the stage of an inverted microscope, had a volume of 1·5 ml and was continuously perfused at a rate of 2 ml min^{-1}. Three minutes were allowed for equilibration when applying drugs. Experiments were performed at room temperature (22–24 °C).

The intracellular recording solution was composed of (mM): Tris methylsulphate (from Tris base), 130; HEPES, 10; tetracaesium bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′*N*′-tetraacetic acid (BAPTA), 10; and was titrated to pH = 7·3 with methanesulphonic acid. In addition, the intracellular solution contained 0·1 mM-leupeptin to inhibit Ca^{2+}-activated proteases, as well as 4 mM-$MgCl_2$ and 2 mM-TrisATP to retard run-down of Ca^{2+} currents. For pharmacological experiments, an 'ATP regenerating system' consisting of 20 mM-Tris creatine phosphate and 50 Units ml^{-1} creatine phosphokinase (bovine heart, Type III) was added to the patch pipettes, and Tris methylsulphate was isosmotically reduced (Forscher & Oxford, 1985). All chemicals were

obtained from Sigma, except BAPTA (Molecular Probes) and methanesulphonic acid (Aldrich). Ethosuximide was a gift of Parke-Davis Pharmaceutical Research.

Patch pipettes were pulled on a Kopf vertical puller in two stages from WPI borosilicate glass tubing (1·5 mm o.d.) to a tip diameter of 1–2 μm. They had a resistance of 1–3 MΩ when filled with intracellular solution. The steady-state series-resistance error would thus be expected to be less than 6 mV for the largest currents measured (about 1 nA). Indeed, use of an analog series-resistance compensation circuit had no noticeable effect on the membrane potential at which the peak current was observed. Tip potentials were compensated immediately before seal formation. No correction has been made in the results for current flow across the leak conductance, however, these cells have an extremely high input resistance (0·5–2 GΩ) (Kay & Wong, 1987). Uncorrected leak currents would be expected to result in slight underestimates of the true Ca^{2+} reversal potential and peak current amplitudes. Although these cells are electronically very compact (Kay & Wong, 1987), 'escape' of Ca^{2+} currents was occasionally observed. Cells were therefore excluded from study if the current obtained more than 50 % of its peak amplitude in a single 5 mV voltage step. The holding potential for all experiments was -50 mV, unless otherwise stated.

Whole-cell currents were measured using standard patch-clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) and a List L/M-EPC7 amplifier. Voltage commands were delivered to the amplifier, and membrane currents were digitized and stored on a hard disc, by an 8-bit analog/digital converter (Labmaster, Tecmar Inc.) under the control of an IBM-XT computer running 'pClamp' software (Axon Instruments). Ca^{2+} currents were low-pass filtered at 1 kHz with an 8-pole Bessel filter (Frequency Devices) and subsequently digitized at 0·67 kHz. Data are expressed as means $\pm$ standard deviation, unless otherwise stated.

RESULTS

Calcium currents in adult cells

Step depolarizations from a holding potential of -50 mV in solutions containing extracellular Ba^{2+} elicit pronounced inward currents. These currents are mediated by voltage-dependent Ca^{2+} channels because they are sensitive to the concentration of extracellular Ca^{2+} or Ba^{2+}, and abolished by 0·2 mM-Co^{2+}. In adult pyramidal cells, derived from either the CA3 or CA1 region, these currents had a mean threshold for activation of $-35{\cdot}8 \pm 5{\cdot}7$ mV ($n = 25$). The peak current amplitude was obtained at a mean membrane potential of $-3{\cdot}1 \pm 5{\cdot}0$ mV ($n = 8$). Data from a representative adult CA3 cell are illustrated in Fig. 1. Near threshold, the current showed little or no evidence of decay during maintained depolarizations. With stronger depolarizations there was a slow, monophasic decline in the amplitude of the current. The decay in current amplitude was never complete during maintained depolarizations of 600 ms in duration. The amplitude of the current at the end of the pulse was never less than 50 % of the peak amplitude. (Throughout this paper we will refer to this current elicited from the holding potential of -50 mV as 'persistent' Ca^{2+} current because it is never fully inactivated at this potential.)

Some Ca^{2+} currents exhibit complete voltage-dependent inactivation, and existing data suggest that several Ca^{2+} currents may be largely inactivated in experiments where the membrane potential is held at -50 mV (e.g. low-threshold Ca^{2+} current; Carbone & Lux, 1984). We therefore examined the effect of preceding the depolarizing test pulse with hyperpolarizing pre-pulses to a membrane potential of -85 or -90 mV for 3 s to remove voltage-dependent inactivation. In adult cells, hyperpolarizing pre-pulses increased the peak amplitude of the resultant Ca^{2+} currents up to 25 %, but had almost no effect on the amplitude of the Ca^{2+} current at the end of the pulse (Fig. 1*C* and *D*) or on the threshold for activation (Table 1).

These results suggest that the hyperpolarizing pre-pulse did indeed reveal a component of Ca^{2+} current that had been inactivated at the holding potential, but that this current component has the same threshold for activation as the persistent Ca^{2+} current.

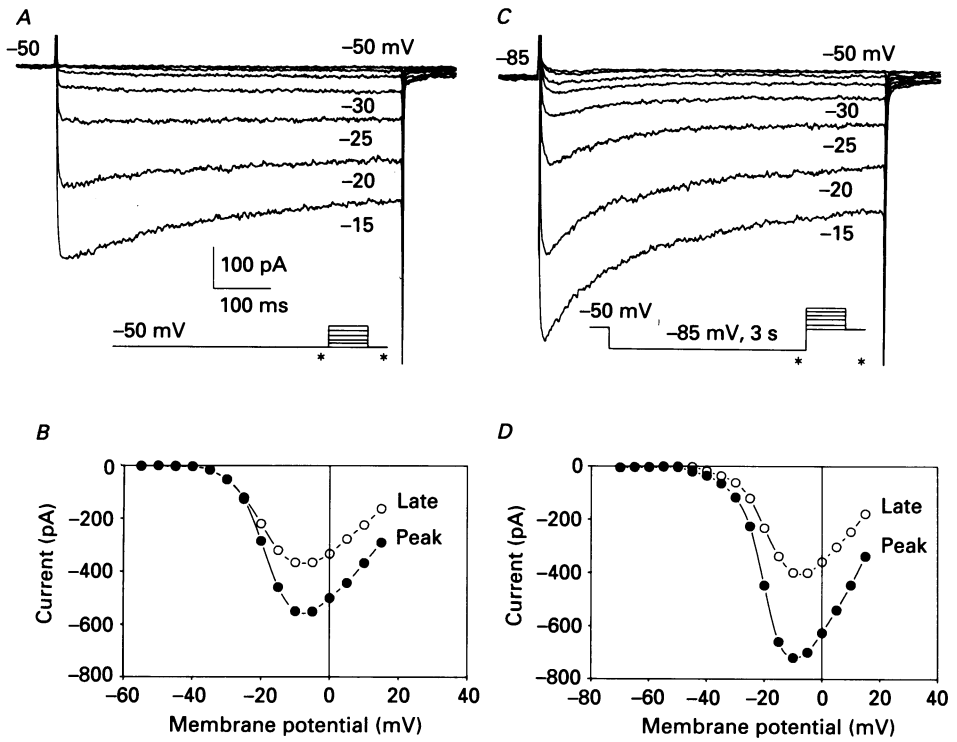

Fig. 1. Calcium currents in an adult CA3 cell. *A*, calcium currents elicited from the holding potential (−50 mV) with 600 ms step depolarizations every 5 mV between −50 and −15 mV. Note that the threshold for activation is −40 mV, and that currents are non-inactivating near threshold, and slowly and incompletely inactivating at more depolarized potentials. Throughout the text we refer to these currents as 'persistent' currents because they are never fully inactivated at −50 mV. The stimulation protocol is illustrated beneath in this and subsequent figures; only the currents between the two asterisks are shown. *B*, current–voltage plot for peak current (●), and current at the end of the pulse ('late', ○), from records in *A*. *C*, in the same cell, currents elicited with identical depolarizing commands but from a hyperpolarizing pre-pulse to −85 mV for 3 s. Note that peak current is enhanced compared to persistent currents in *A*, but that current at end of the pulse is relatively unaffected. *D*, current–voltage plot for peak current (●), and current at end of the pulse (○), from records in *C*. Note that the hyperpolarizing pre-pulse increased peak current amplitude, but did not affect activation threshold.

In most experiments, some 'run-down' of Ca^{2+} current was observed after 20–30 min, despite the addition of ATP to the intracellular solution. In adult neurons, all components of Ca^{2+} current appeared equally affected.

There were no detectable differences in the properties of Ca^{2+} currents obtained from adult CA1 and CA3 cells in terms of their threshold of activation (Table 1), peak current amplitudes, or inactivation properties.

Calcium currents in immature cells

In immature pyramidal cells, Ca^{2+} currents elicited from the holding potential of -50 mV had a mean threshold for activation of $-34{\cdot}5 \pm 9{\cdot}1$ mV ($n = 31$), and the peak current amplitude was obtained at a mean membrane potential of $-5{\cdot}3 \pm 7{\cdot}9$ mV ($n = 14$). These values are not significantly different from those

TABLE 1. Activation thresholds for Ca^{2+} currents

	Threshold (mV)	
	Holding potential (-50 mV)	Pre-pulse to -85 mV
Adult		
CA3	$-33{\cdot}8 \pm 6{\cdot}0$ (8)	$-35{\cdot}6 \pm 3{\cdot}0$ (8)
CA1	$-36{\cdot}7 \pm 6{\cdot}2$ (7)	$-37{\cdot}1 \pm 6{\cdot}5$ (7)
Immature		
Peak	$-34{\cdot}5 \pm 9{\cdot}1$ (31)	$-57{\cdot}4 \pm 4{\cdot}9$* (37)
End	$-34{\cdot}7 \pm 8{\cdot}3$ (31)	$-41{\cdot}7 \pm 5{\cdot}6$ (37)

The threshold of Ca^{2+} current subtypes, and the effects of hyperpolarizing pre-pulses to -85 or -90 mV for 3 s, at various ages are shown. Values expressed as means $\pm$ standard deviation, with the number of cells in parentheses. The threshold for the peak current and for the current at the end of the pulse are given for immature cells (< 2 weeks of age), and for peak current in adult cells (> 28 days old). The threshold for peak current in adult cells was not different from the threshold at the end of the pulse, because the current is non-inactivating near threshold. *Indicates significantly more negative than either the threshold in adult cells with pre-pulse, immature cells without pre-pulse, or at the end of the pulse in immature cells; $P < 0{\cdot}001$ as assessed with Mann–Whitney U test.

measured in adult pyramidal cells (Table 1). Data from a representative immature CA3 cell, obtained from an animal 6 days after birth, are illustrated in Fig. 2. As in adult cells, this persistent current exhibited little or no inactivation close to threshold, and inactivated slowly and incompletely at more depolarized levels.

Calcium currents that were preceded by 3 s hyperpolarizing pre-pulses to -85 mV were qualitatively different from both currents elicited without the pre-pulse, and currents elicited under identical conditions in adult cells (Fig. 2*C* and *D*). In immature cells, the threshold for activation of Ca^{2+} currents was more negative when preceded by a hyperpolarizing pre-pulse. The mean threshold was $-57{\cdot}4 \pm 4{\cdot}9$ mV, significantly more negative than both the threshold of the persistent current and the current in adult cells (Table 1). Close to threshold (i.e. -60 to -35 mV), this current inactivated rapidly and completely during the depolarizing pulse. With depolarizations more positive than -35 mV, there was an initial rapid decay of the current, followed by a slower, incomplete decay (see below). The threshold of the incompletely inactivating Ca^{2+} current, as indicated by the amplitude of the current at the end of the pulse, was not significantly different from the threshold of the persistent current (Table 1).

These data suggest that the hyperpolarizing pre-pulse revealed a component of Ca^{2+} current in immature pyramidal cells that was not observed in adult neurons. This current has a more negative threshold of activation, and inactivates more rapidly, than any component of Ca^{2+} current observed in adult pyramidal cells. This

current was observed in all CA1 ($n = 24$) and CA3 ($n = 27$) cells 12 days of age or younger. There was no appreciable difference in the activation and inactivation of Ca^{2+} currents between immature CA3 or CA1 cells. In cells in which there was appreciable 'run-down' of high threshold Ca^{2+} currents, the low threshold Ca^{2+} current was apparently unaffected.

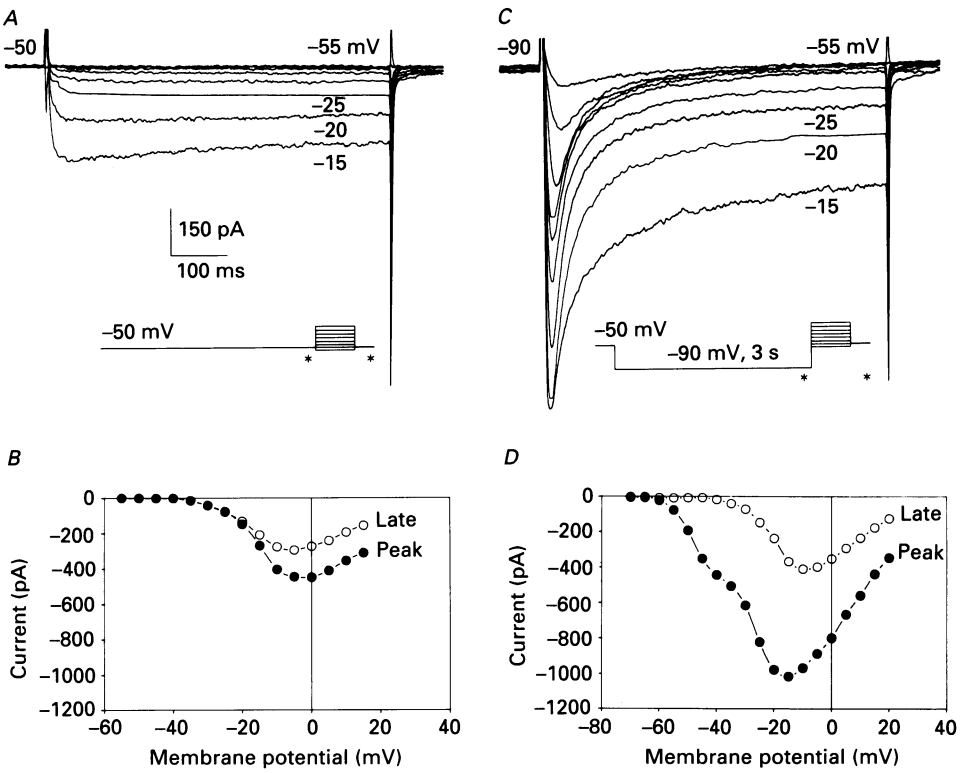

Fig. 2. Calcium currents in a 6-day-old CA3 cell. *A*, persistent calcium currents elicited from the holding potential (−50 mV) with step depolarizations between −55 and −15 mV. Note that, like adult persistent currents shown in Fig. 1, the threshold for activation is −40 mV, and that currents are non-inactivating near threshold, and slowly and incompletely inactivating at more depolarized potentials. *B*, current–voltage plot for peak current (●), and current at end of the pulse ('late', ○), from records in *A*. *C*, in same cell, currents elicited with identical depolarizing commands but from a hyperpolarizing pre-pulse to −90 mV for 3 s. Note that not only is peak current enhanced, but there is also current activated at −55 mV which decays rapidly and completely at potentials between −55 and −40 mV. *D*, current–voltage plot for the peak current (●), and current at end of the pulse (○), from the records in *C*. Note that the hyperpolarizing pre-pulse both increased peak current amplitude, and shifted the activation threshold for peak current to −60 mV without affecting the threshold for current at end of the pulse.

The time course of this developmental loss of low-threshold Ca^{2+} current can be seen in Fig. 3. The low-threshold current, operationally defined as having a threshold more negative than −45 mV and complete inactivation close to threshold, was readily observed in all cells less than 12 days of age, and never observed in any cell older than 29 days. At intermediate ages, the probability of observing low-threshold

current progressively declined. At intermediate ages, cells with and without low-threshold current were occasionally obtained from individual rats. Although current densities were not directly determined, there was a progressive increase in the amplitude of the persistent Ca^{2+} current during development. This increase could

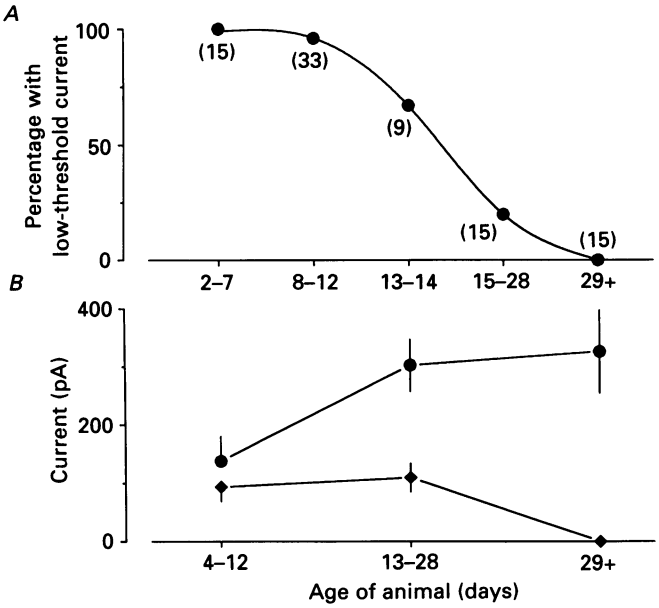

Fig. 3. Summary of time course of Ca^{2+} current development in both CA1 and CA3 cells. *A*, percentage of cells found to have low-threshold Ca^{2+} current is plotted for several different age groups. Number of cells tested given in parentheses. All cells less than 12 days old were found to have low-threshold current, while cells older than 29 days never had low-threshold current. Low-threshold current was defined as activating at potentials more negative than −45 mV, and inactivating completely near threshold. *B*, maximum persistent current (●) and maximum low-threshold Ca^{2+} current (◆) for several different age groups. Persistent current was elicited from holding potential of −50 mV. Low-threshold current was measured just subthreshold to activation of high-threshold currents; only cells that possessed low-threshold current between ages 13 and 28 were included. Data are from cells in saline containing 10 mM-Ba^{2+}, and given as means ± standard error. $n = 12$, 8 and 11 cells for the persistent current; $n = 15$ and 4 for the low-threshold current. Note that although persistent current continues to increase, low-threshold current is not significantly larger in the oldest cells in which it was observed.

reflect both increases in channel density and/or increases in cell area. Unlike high-threshold Ca^{2+} current however, the amplitude of the low-threshold current was not significantly different in the oldest cells in which it could be observed (Fig. 3*B*).

Properties of inactivating Ca^{2+} currents

As described above, removal of voltage-dependent inactivation, by preceding a depolarizing test pulse with a hyperpolarizing pre-pulse, reveals Ca^{2+} current components in both adult and immature pyramidal cells which are not observed following depolarizations from the holding potential of −50 mV. In order to examine these inactivating currents more clearly, it is helpful to isolate them from the

persistent component of Ca^{2+} current. This can be done by digitally subtracting the currents elicited from the holding potential, which contain persistent Ca^{2+} current, from those elicited from the hyperpolarizing pre-pulse, which contain both inactivating and persistent Ca^{2+} currents. Any inactivation of the persistent Ca^{2+}

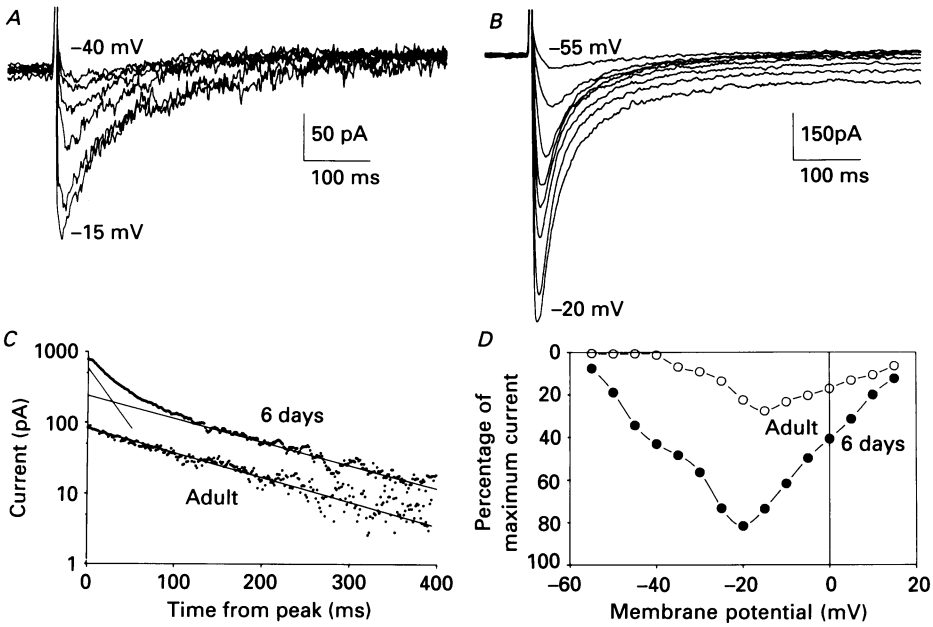

Fig. 4. Isolation of inactivating Ca^{2+} current. Inactivating currents were obtained by subtracting persistent currents from currents elicited from a hyperpolarizing pre-pulse. *A*, inactivating Ca^{2+} currents at potentials between −40 and −15 mV from adult cell shown in Fig. 1. Note relatively slow rate of inactivation. *B*, inactivating Ca^{2+} currents at potentials between −55 and −20 mV from 6-day-old cell shown in Fig. 2. Note complete and relatively fast rate of inactivation close to threshold, and appearance of second component at more depolarized potentials. *C*, inactivating Ca^{2+} currents at −20 mV plotted on a logarithmic scale as positive values. Note that the adult current can be well fitted with a single exponential (time constant 125 ms) while the immature cell is described by the addition of two exponentials, a fast one (27 ms) and a slower one comparable to the decay time constant of the adult current (118 ms). *D*, the relative contribution of inactivating Ca^{2+} currents to the total Ca^{2+} current is shown by plotting the amplitude of the isolated inactivating Ca^{2+} current as a percentage of the maximum Ca^{2+} current obtained from that cell. Note that inactivating current comprises about 80% of maximal Ca^{2+} current in the 6-day-old cell (●) but only 25% of maximal current in the adult cell (○).

current (see below) will also be eliminated by this technique. Isolated inactivating currents for the exemplar cells in Figs 1 and 2 are shown in Fig. 4. As expected from the data described above, the threshold for activation of the inactivating current in adult cells was roughly −35 mV (Fig. 4*A*), while the threshold for the immature cells was roughly −60 mV (Fig. 4*B*).

The time course of inactivation was clearly different for the two age groups as well. For adult neurons, the time course of the decay of the inactivating Ca^{2+} current could be well described with a single exponential at all potentials positive to threshold (Fig.

4*C*). The rate of decay was progressively increased at more depolarized membrane potentials (Fig. 5). In contrast, in immature cells the decay of the inactivating Ca^{2+} current could only be fitted with a single exponential at membrane potentials between −60 and −35 mV, i.e. subthreshold to activation of the high-threshold

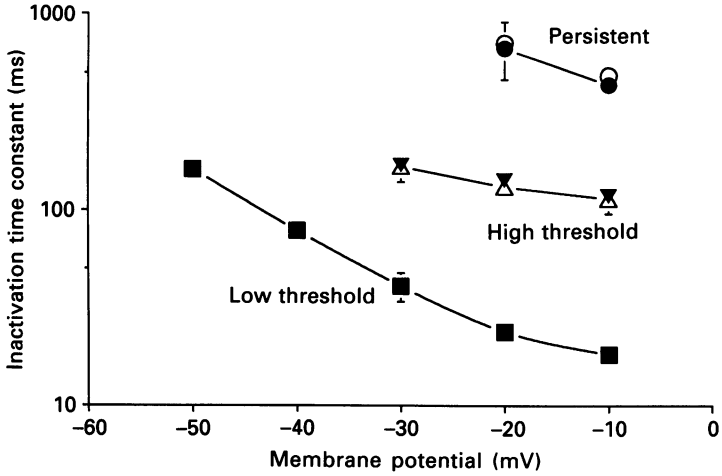

Fig. 5. Inactivation time constants for Ca^{2+} current subtypes in adult (open symbols) and immature (filled symbols) pyramidal cells. The rate of inactivation for low-threshold (squares) and high-threshold (triangles) inactivating Ca^{2+} current were determined after subtraction of persistent currents. The rate of inactivation for persistent currents (circles) was determined with pulses from the holding potential of −50 mV. Values displayed as means ± standard error; n = 8–12 cells at each potential.

current. At more depolarized potentials, the inclusion of a second, slower exponential was required to adequately describe the data (Fig. 4*C*). These exponentials differed from each other by about an order of magnitude, and both time constants became smaller as the membrane potential was depolarized (Fig. 5). The slower rate of inactivation in the immature cells was identical to the rate of inactivation of Ca^{2+} current in adult cells (Fig. 5).

The relative contribution of these currents that are inactivated at −50 mV to the total calcium current in the cell was much larger for immature cells than for adult cells. Normalized to maximum calcium current, inactivating calcium current contributed up to 75% of the maximal current in immature neurons, but up to 25% of the maximal current in adult cells (Fig. 4*D*).

Some inactivation was observed in the persistent calcium current elicited from the holding potential in both adult and immature neurons at potentials more positive than −30 mV (Figs 1*A* and 2*A*). The rate of decay of these currents was well fitted with a single exponential having a mean time constant of many hundred milliseconds (Fig. 5). The rate of decay of these currents was identical in adult and immature neurons.

The kinetic properties of the low-threshold Ca^{2+} current may be examined in isolation in immature cells at potentials more negative than −35 mV, i.e. subthreshold to activation of high-threshold Ca^{2+} currents. Activation of the low-

threshold Ca^{2+} current was relatively slow close to threshold (about 50 ms to peak) and became progressively accelerated at more depolarized potentials (Fig. 4*B*), as in other cell types (e.g. Coulter, Huguenard & Prince, 1989*b*; Droogmans & Nilius, 1989).

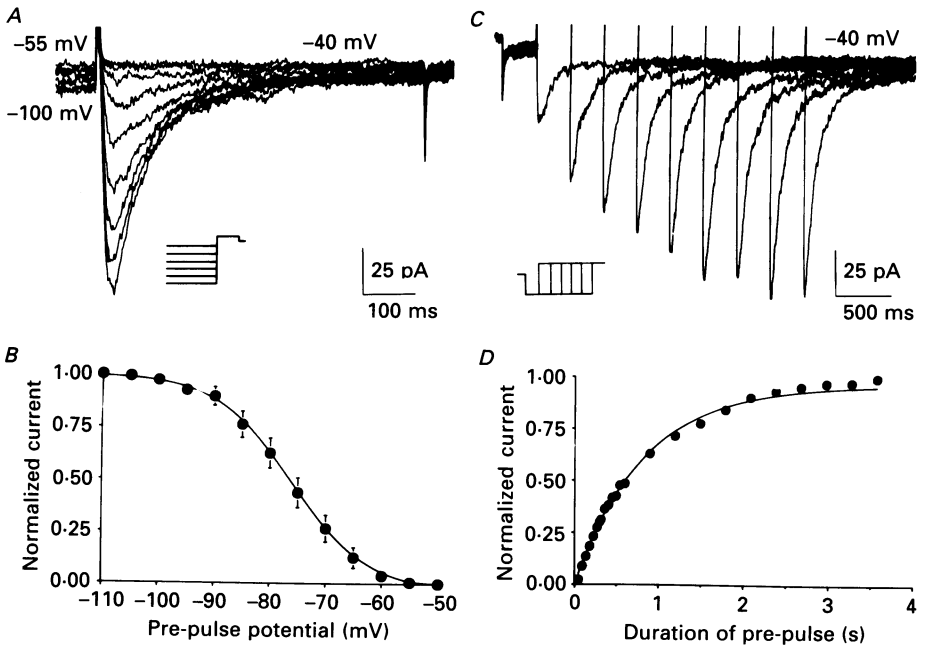

Fig. 6. Steady-state inactivation of low-threshold Ca^{2+} current in immature neurons (< 14 days old). *A*, low-threshold current in an 11-day-old CA3 cell elicited at a test potential of −40 mV following hyperpolarizing pre-pulses for 3 s to potentials between −55 and −100 mV. *B*, plot of the amplitude of low-threshold Ca^{2+} current as a function of pre-pulse potential, normalized to the maximum amplitude. Data from 8 cells have been pooled and expressed as means±standard error. The line is described by the Boltzmann equation with half-maximal amplitude at −76 mV and a slope factor of 6·8. *C*, time course of removal of steady-state inactivation for low-threshold current elicited at a test potential of −40 mV following hyperpolarizing pre-pulses to −85 mV of increasing duration in a CA1 cell. *D*, plot of the amplitude of low-threshold current as a function of the duration of pre-pulse, normalized to maximum amplitude. Data from 6 cells have been pooled. The line is described by a single exponential of time constant 820 ms.

The voltage-dependence of inactivation of the low-threshold Ca^{2+} current was examined by holding the membrane potential at −50 mV and preceding the test depolarization (to −40 mV) with hyperpolarizing pre-pulses to various potentials between −50 and −110 mV. As shown in Fig. 6*A*, steady-state inactivation at the holding potential was removed by such hyperpolarizing pre-pulses. The results could be well described with a Boltzmann equation having a half-maximal inactivation at −76 mV and a slope factor of 6·8 (Fig. 6*B*). The time-to-peak of the Ca^{2+} current remained constant, indicating the adequacy of the voltage control (Fig. 6*A*). Because the threshold for activation of the low-threshold Ca^{2+} current is more positive than

−60 mV, and the current is fully inactivated at membrane potentials more positive than −60 mV, there would appear to be little-or-no tonically activated low-threshold current at the resting membrane potential. Hyperpolarization to −100 mV is necessary for full removal of inactivation. The time course of the removal of

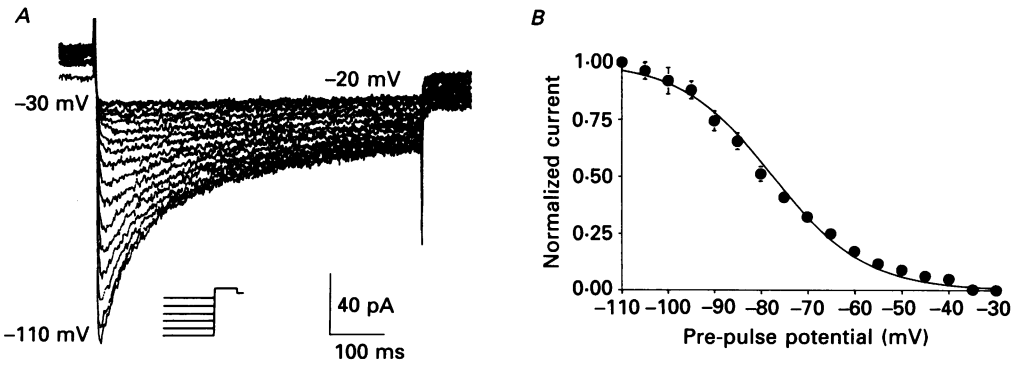

Fig. 7. Steady-state inactivation of high-threshold inactivating current in adult neurons. *A*, persistent and inactivating Ca^{2+} currents elicited at a test potential of −20 mV following hyperpolarizing pre-pulses for 3 s to potentials between −30 mV and −110 mV to remove steady-state inactivation resulting from the holding potential of −30 mV in a CA1 cell. *B*, plot of the amplitude of inactivating Ca^{2+} current as a function of pre-pulse potential, normalized to amplitude at −110 mV. Inactivating Ca^{2+} current was measured as the difference between peak current and current at end of pulse. Data from 7 cells have been pooled and expressed as means ± standard error. The line is described by the Boltzmann equation with half-maximal amplitude at −78 mV and a slope factor of 9.9.

inactivation for the low-threshold Ca^{2+} current was examined by varying the duration of the hyperpolarizing pre-pulse to −85 mV (Fig. 6*C*). The results could be well described as a single exponential process, with a mean time constant of 820 ms (Fig. 6*D*).

The kinetic properties of the high-threshold, inactivating Ca^{2+} current may be distinguished from those of the low-threshold Ca^{2+} current by studying adult rat pyramidal cells where the low-threshold current is absent. Unfortunately, it is not possible to separate so readily the persistent and inactivating high-threshold currents because their activation voltages are comparable. However, because the persistent Ca^{2+} current does not appreciably inactivate during a 600 ms pulse at −20 mV (Figs 1 and 2), a reasonable measure of the high-threshold inactivating current may be obtained from the difference between the peak amplitude of the current and the amplitude at the end of the pulse. Ca^{2+} currents were steady-state inactivated by holding the membrane potential constant at −30 mV and hyperpolarizing pre-pulses of 3 s duration were made to potentials between −30 and −110 mV before stepping to a test potential of −20 mV. The results were well described with a Boltzmann equation having a half-maximal inactivation at −78 mV and a slope factor of 9·9 (Fig. 7). Considerable increases in the amplitude of the current at the end of the pulse were also observed (Fig. 7*A*), suggesting that at least part of the persistent Ca^{2+} current also undergoes considerable inactivation within this range of potentials.

Pharmacology of calcium currents

The kinetic properties of the calcium currents we have described above closely resemble those described in a variety of other cell types. We therefore wished to compare their pharmacological properties with those described for other cell types,

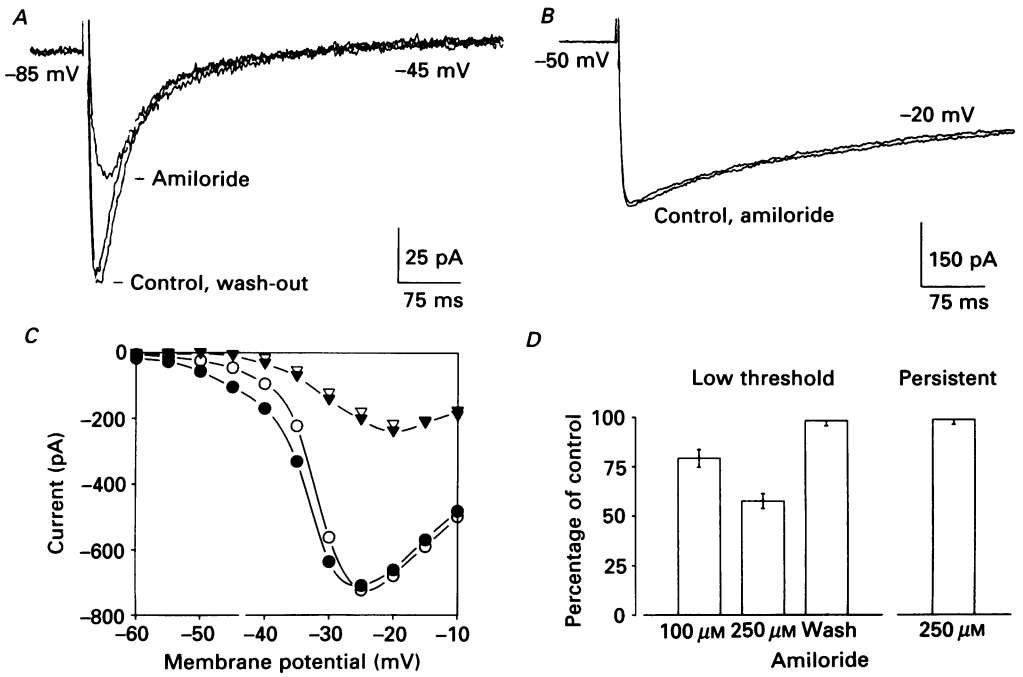

Fig. 8. Effects of amiloride on Ca^{2+} currents. *A*, low-threshold Ca^{2+} currents elicited at −45 mV following a hyperpolarizing pre-pulse to −85 mV in a 9-day-old CA1 cell. Amiloride (250 μM) reduced the amplitude by about 50%, and the effect was reversed upon wash-out of amiloride. *B*, amiloride (250 μM) had no effect on persistent Ca^{2+} currents elicited in the same cell after depolarization to −20 mV from the holding potential. *C*, current–voltage relationship for Ca^{2+} currents elicited from hyperpolarizing pre-pulse in a 14-day-old CA1 cell. Peak current amplitude (circles) and the amplitude of current at end of the pulse (triangles) are plotted before (solid symbols) and after application of 250 μM-amiloride (open symbols). Note that amiloride only blocked low-threshold current, without affecting high-threshold components. *D*, the effects of amiloride on low-threshold current were dose-dependent and fully reversible. No effect was seen on persistent current (includes both adult and immature cells). Data represent the means ± standard error for 4–9 cells.

as well. For pharmacological experiments, we found it necessary to reduce further the amount of run-down of Ca^{2+} currents than that seen with ATP-containing pipettes alone. We therefore included the 'ATP-regenerating system' described in the methods section which markedly enhanced the stability of Ca^{2+} current (Forscher & Oxford, 1985).

Amiloride, at concentrations lower than 500 μM, has been shown to block low-threshold Ca^{2+} current, without significantly affecting high-threshold Ca^{2+} currents, in chick neurons (Tang, Presser & Morad, 1988; McCobb, Best & Beam, 1989). When

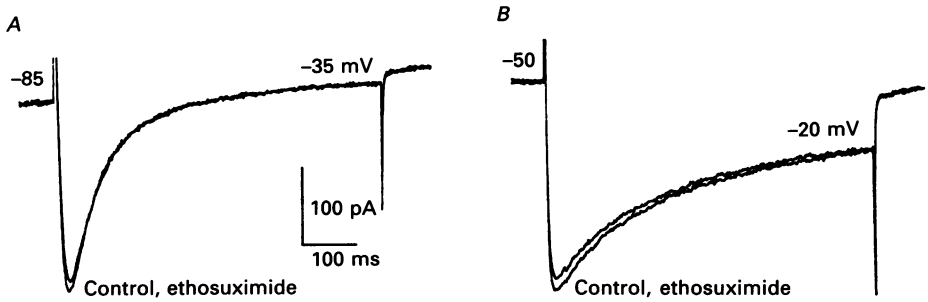

Fig. 9. Effects of ethosuximide on Ca^{2+} currents. *A*, low-threshold Ca^{2+} currents were elicited in a 14-day-old CA3 neuron at a test potential of −35 mV following a 3 s pre-pulse to −85 mV. Ethosuximide (350 μM) had no significant effect. *B*, high-threshold Ca^{2+} currents in the same cell, elicited with depolarization from −50 to −20 mV, were similarly unaffected.

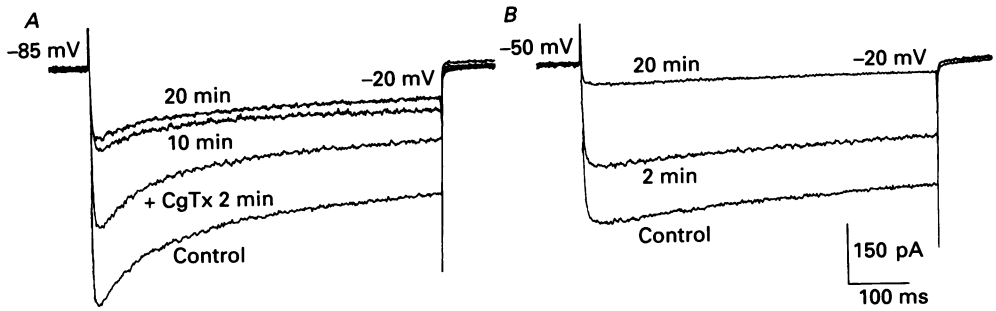

Fig. 10. Effects of CgTx on Ca^{2+} currents in an adult CA1 neuron. High-threshold inactivating and persistent Ca^{2+} currents were elicited with depolarizations to −20 mV after a hyperpolarizing pre-pulse to −85 mV for 3 s (*A*), and high threshold persistent Ca^{2+} currents were elicited with depolarization to −20 mV from the holding potential (*B*). Focal application of CgTx (10 μM) rapidly reduced currents in both cases. This effect could not be attributed to run-down, because the currents were stable after they had been blocked by CgTx (cf. 10 and 20 min in *A*).

bath applied to immature hippocampal pyramidal cells, amiloride was found to markedly reduce the low-threshold component of Ca^{2+} current activated from hyperpolarizing pre-pulses (Fig. 8*A*, *C*). The effect of amiloride was dose-dependent (Fig. 8*D*). Amiloride, however, had no effect on either the high-threshold Ca^{2+} currents in immature cells (Fig. 8*B*, *C*, *D*), or adult Ca^{2+} currents (not shown), at these concentrations.

Ethosuximide and methsuximide, two compounds of interest in the treatment of petit mal epilepsy, have recently been shown to be effective antagonists of the low-threshold Ca^{2+} current in acutely isolated guinea-pig thalamic neurons (Coulter, Huguenard & Prince, 1989*a*). When ethosuximide (350 μM, $n = 9$) or methsuximide (300 μM, $n = 3$) (Fig. 9) were applied to immature hippocampal pyramidal cells, however, they had no significant effect on the low-threshold Ca^{2+} current.

The toxin isolated from the marine snail *Conus geographus* (ω-conotoxin GVIA, CgTx) has been shown to block irreversibly several components of Ca^{2+} current in a

variety of cell types (Fox *et al.* 1987*a*; McCleskey, Fox, Feldman, Cruz, Olivera, Tsien & Yoshikami, 1987; Plummer *et al.* 1989). When applied to adult hippocampal cells by brief pressure ejection from a second patch pipette placed close to the recorded cell, CgTx (10 μM, $n = 5$) was found to rapidly reduce persistent Ca^{2+} currents elicited from the holding potential of -50 mV as well as currents elicited from hyperpolarizing pre-pulses (Fig. 10). These data suggest that CgTx blocks both inactivating and persistent components of the high-threshold Ca^{2+} current. CgTx was not tested on immature neurons.

DISCUSSION

The data presented above suggest that a specific subtype of Ca^{2+} current is expressed in immature hippocampal pyramidal cells which is not present in adult pyramidal cells. We have found that this current is activated close to the resting membrane potential of -60 mV and inactivates rapidly. The threshold for this current was about -57 mV and it inactivated with a time constant of about 25 ms at -20 mV. Half-maximal inactivation was obtained at -76 mV. These kinetic properties thus closely resemble those described for the low-voltage-activated, or T-type, Ca^{2+} current in sensory neurons (Carbone & Lux, 1984, 1987*a*, *b*; Fox *et al.* 1987*a*), chick motoneurons (McCobb *et al.* 1989), mammalian cardiac (Nilius, Hess, Lansman & Tsien, 1985; Droogmans & Nilius, 1989) and skeletal muscle (Gonoi & Hasegawa, 1988), and mammalian thalamic neurons (Coulter *et al.* 1989*b*). In these other cell types T-type Ca^{2+} current is reported to activate between -70 and -50 mV, and inactivate with a time constant of 20–30 ms at a membrane potential of -20 mV. Half-maximal inactivation is reported at voltages between -60 and -80 mV in these cells, comparable to our measured value of -76 mV. We have observed that recovery from inactivation proceeds with a time constant of 820 ms, comparable to previously reported values between 250 ms for thalamic neurons (Coulter *et al.* 1989*b*) and 1·5 s for chick dorsal root ganglion cells (Carbone & Lux, 1987*a*).

The low-threshold Ca^{2+} current in immature isolated hippocampal neurons was reduced by amiloride, as is the T-type Ca^{2+} current of chick sensory neurons (Tang *et al.* 1988) and motoneurons (McCobb *et al.* 1989), further suggesting that the current in hippocampal cell is analogous to other T-type Ca^{2+} currents. We were unable to detect any effect of ethosuximide on the low-threshold current in immature cells although it has been shown to potently block T-type Ca^{2+} current in thalamic neurons (Coulter *et al.* 1989*a*).

The failure to detect low-threshold Ca^{2+} current in acutely isolated adult pyramidal cells in this (Fig. 1) and other studies (Kay & Wong, 1987; Doerner *et al.* 1988) could be due to vulnerability of the channels to enzymatic treatment during the cell dissociation procedure. This seems unlikely, however, because low-threshold Ca^{2+} current can be readily recorded in both immature pyramidal cells (Fig. 2) and adult thalamic neurons dissociated with the same technique (Coulter *et al.* 1989*b*). Rather our data suggest that there is a specific developmental programme that regulates the phenotype of Ca^{2+} currents in hippocampal pyramidal neurons, such that T-type Ca^{2+} channels are no longer expressed after 2–4 postnatal weeks. A

similar developmental loss of low-threshold Ca^{2+} current has recently been observed in mouse skeletal muscle (Gonoi & Hasegawa, 1988) and chick motoneurons (McCobb *et al.* 1989). Interestingly, placing embryonic skeletal myoblasts in culture seems to arrest the further development of the mature phenotype, i.e. T-type Ca^{2+} current is maintained (Gonoi & Hasegawa, 1988). In contrast, dorsal root ganglion neurons apparently increase the density of T-type Ca^{2+} channels during development (Gottmann, Dietzel, Lux, Huck & Rohrer, 1988). Alternatively, we cannot exclude the possibility that T-type Ca^{2+} channels become progressively more restricted to more distal dendritic processes during development, and that these distal dendrites are then amputated during the cell isolation procedure.

T-type Ca^{2+} current has been demonstrated in cultured and acutely isolated embryonic mammalian hippocampal neurons (Bley *et al.* 1987; Yaari *et al.* 1987; Doerner *et al.* 1988; Meyers & Barker, 1989), however, its existence has not been conclusively shown in mature hippocampal neurons. In comparable studies on acutely isolated adult hippocampal CA1 pyramidal cells, only high-threshold Ca^{2+} currents were observed (Kay & Wong, 1987; Doerner *et al.* 1987), as we have confirmed in the present study and extend to CA3 pyramidal cells. Takahashi, Akaike and colleagues (Takahashi, Tateishi, Kaneda & Akaike, 1989*a*; Takahashi, Wakamori & Akaike, 1989*b*) have recently claimed that T-type Ca^{2+} current does exist in acutely isolated adult hippocampal neurons, however, the use of fluoride as the intracellular anion in those experiments complicates interpretation of the results because fluoride drastically alters the kinetics of Ca^{2+} currents (Kay, Miles & Wong, 1986; Carbone & Lux, 1987). Indeed, Takahashi *et al.* (1989*b*) found that dihydropyridines could block this current, unlike T-type Ca^{2+} current in other cells (e.g. Nilius *et al.* 1985; Fox *et al.* 1987*a*).

Single-electrode voltage-clamp studies (Halliwell, 1983; Docherty & Brown, 1986; Gähwiler & Brown, 1987) have also suggested the presence of hippocampal Ca^{2+} currents with more negative activation thresholds, although uncertainty about the adequacy of the voltage-clamp control of remote dendrites makes these results inconclusive, as well. It should be noted that a small, transient overshoot of the membrane potential in poorly clamped portions of a cell with depolarizing step commands from negative holding potentials would be expected to activate inward currents that 'turn off' rapidly and could therefore easily be confused with T-current.

Fisher, Gray & Johnston (1990) have presented evidence of single Ca^{2+} channels in recordings from acutely exposed adult hippocampal pyramidal cells which have a unitary conductance (8 pS) comparable to the channels underlying T-type Ca^{2+} current in other cell types (e.g. Nilius *et al.* 1985; Fox *et al.* 1987*b*). However, they report that this channel is frequently non-inactivating with small depolarizations, unlike T-type Ca^{2+} channels in other preparations (e.g. Carbone & Lux, 1987*b*; Fox *et al.* 1987*a*; Droogmans & Nilius, 1989) and unlike the macroscopic low-threshold current recorded from our immature pyramidal cells (Fig. 4) and in other cells (e.g. Nilius *et al.* 1985; Carbone & Lux, 1987*a*; Fox *et al.* 1987*b*). Additional pharmacological data, and single channel recordings from acutely exposed immature pyramidal cells, would help to confirm that they are in fact T-type Ca^{2+} channels, and to resolve the apparent discrepancy between the presence of such channels and

our inability to detect low-threshold current in whole-cell recordings. It is conceivable that T-type channels may be detected in cell-attached patch recordings, but be of insufficient density to contribute significantly to total cell Ca^{2+} current, particularly given their small unitary conductance. Our data predict that very large numbers of T-type Ca^{2+} channels should be found on immature pyramidal cells.

Hippocampal pyramidal cells at all ages possess high threshold Ca^{2+} current, and it is possible to resolve both inactivating and non-inactivating components of this current (Fig. 4). A similar distinction may be made on high threshold Ca^{2+} currents in cultured hippocampal neurons (Doerner *et al.* 1988; Meyers & Barker, 1989). In the absence of additional single-channel and pharmacological data it is difficult to identify these high-threshold currents as either N- or L-type Ca^{2+} currents. Furthermore, more than one type of Ca^{2+} channel may mediate either macroscopic current. Plummer *et al.* (1989) have recently suggested that only N- and not L-type neuronal Ca^{2+} channels are blocked by ω-conotoxin. In addition, they have suggested that inactivation of N-type channels is often incomplete. Current through N-type channels can thus contribute significantly to non-inactivating whole-cell Ca^{2+} current. We have observed that ω-conotoxin blocks both inactivating and persistent components of pyramidal cell Ca^{2+} current, suggesting the presence of both inactivating and non-inactivating N-type Ca^{2+} channels in our cells. Dihydropyridine-sensitive Ca^{2+} current has also been described in cultured hippocampal pyramidal cells (Gähwiler & Brown, 1987; Doerner *et al.* 1988; Meyers & Barker, 1989) and in acute slices (Docherty & Brown, 1986), indicating that L-type Ca^{2+} channels (Fox *et al.* 1987*b*; Plummer *et al.* 1989) also contribute to total high-threshold Ca^{2+} current. Fisher *et al.* (1990) have recently described two classes of high-threshold Ca^{2+} channels on acutely exposed hippocampal neurons that apparently mediate N- and L-type Ca^{2+} current. The density and distribution of these channels appears to be comparable for CA1 and CA3 pyramidal cells.

Thalamic neurons possess a prominent T-type Ca^{2+} current that is responsible for the characteristic bursting discharge observed in these neurons upon repolarization following hyperpolarizing current injection (Jahnsen & Llinás, 1984; Coulter *et al.* 1989*b*). This type of burst firing is not a common feature of adult hippocampal pyramidal cell discharge, however, consistent with our observation that adult pyramidal cells do not possess a prominent T-type Ca^{2+} current. In contrast to thalamic neurons, hippocampal CA3 pyramidal cells typically fire a burst of Na^+-dependent action potentials, superimposed on a slow depolarizing envelope, in response to depolarizing current injection. This burst envelope is known to be mediated by voltage-dependent Ca^{2+} current(s) since it can be blocked by Ca^{2+} channel antagonists such as Mn^{2+} (Wong & Prince, 1978). Because we are unable to detect low-threshold Ca^{2+} current in mature cells, we conclude that T-type current does not contribute significantly to burst-firing in hippocampal CA3 cells. Indeed, while burst-firing can normally be activated close to the resting membrane potential, much larger depolarizations are needed to activate Ca^{2+}-dependent components of the burst after block of Na^+-dependent action potentials by TTX (Wong & Prince, 1978). This suggests that during normal bursting, Na^+-currents are responsible for depolarizing the cell sufficiently to activate high-threshold Ca^{2+} currents. Burst-firing can also be elicited in acutely isolated adult pyramidal cells (Wong, Traub &

Miles, 1986), which have no low-threshold Ca^{2+} current, further supporting this hypothesis. On the other hand, CA1 pyramidal cells, unlike CA3 cells, do not typically fire bursts of action potentials in response to depolarization. It is therefore somewhat surprising that we could detect no difference in Ca^{2+} currents between CA1 and CA3 pyramidal neurons. Our results thus imply that some other property accounts for the difference in the intrinsic firing properties of the two cell types, such as a difference in the density or location of K^+ channels.

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