Journal of Physiology (1991), **433**, *pp.* 259–281
With 11 *figures*
Printed in Great Britain

EVIDENCE FOR MULTIPLE TYPES OF Ca^{2+} CHANNELS IN ACUTELY ISOLATED HIPPOCAMPAL CA3 NEURONES OF THE GUINEA-PIG

BY DAVID J. MOGUL AND AARON P. FOX

From the Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, IL 60637, USA

(*Received 8 May 1990*)

SUMMARY

1. Current through Ca^{2+} channels was studied in acutely isolated guinea-pig pyramidal neurones from the CA3 region of the hippocampus. Both the whole-cell and single-channel patch-clamp configuration were used.

2. Both whole-cell and single-channel currents displayed holding potential sensitivity indicative of two high-threshold currents similar to L- and N-type Ca^{2+} currents.

3. A low-threshold whole-cell current, similar to T-type current seen in dorsal root ganglion (DRG) neurones, activated at -60 to -50 mV and was blocked by nickel (100 μM) and amiloride (500 μM). Exposure to 50 μM-cadmium left a fraction of the T-type current intact but blocked N- and L-type current. This T-like component needed extremely negative holding potentials to be completely reprimed.

4. Whole-cell N-type Ca^{2+} channel current was blocked by ω-conotoxin (1 μM). From a holding potential of -90 mV, ω-conotoxin decreased the peak whole-cell current by 33%.

5. A slowly inactivating high-threshold Ca^{2+} current (L-type) that was present at depolarized holding potentials, displayed dihydropyridine sensitivity. From a holding potential of -50 mV, addition of the dihydropyridine Ca^{2+} channel antagonist nimodipine (2 μM) to the bath decreased whole-cell peak current by 45%. Interestingly, at negative holding potentials nimodipine worked as an agonist. From a holding potential of -90 mV, nimodipine (2 μM) increased peak current at test potentials from -50 to -20 mV and shifted the peak of the current–voltage relationship in the hyperpolarizing direction similar to the effect of Ca^{2+} channel agonist Bay K 8644. Exposure to Bay K 8644 (2 μM) increased peak current and single channel open probability independent of holding potential while shifting the peak of the whole-cell current–voltage relationship 11 mV in the hyperpolarizing direction. Our experiments suggest that there are approximately the same number of L-type as ω-conotoxin sensitive N-type Ca^{2+} channels in CA3 neurones.

6. A high-voltage-activated whole-cell current was still present in cells exposed to both nimodipine and ω-conotoxin (2 and 1 μM, respectively) suggesting the existence of a fourth type of Ca^{2+} channel in these neurones or that a population of either L-type or N-type Ca^{2+} channels did not respond to dihydropyridine antagonists or ω-conotoxin, respectively. From a holding potential of -50 mV, the combination of

nimodipine and ω-conotoxin substantially blocked the Ca^{2+} channel currents of some cells while others showed little effect. These results indicate that the multiple types of Ca^{2+} channels were differentially distributed rather than incompletely blocked by one of the drugs.

7. Single-channel current in cell-attached patches from CA3 neuronal somas showed three different unitary conductance levels of 7, 12 and 23 pS with 90 mM-Ba^{2+} as the external charge carrier. Ensemble average currents resembled currents recorded in the whole-cell configuration. No evidence for a fourth channel type was seen at the unitary level, suggesting the fourth class of Ca^{2+} channels, if it exists, must have a unitary conductance similar to L- or N-type Ca^{2+} channels or that these channels are located primarily outside the soma.

8. Thus, hippocampal pyramidal neurones from the CA3 region possess at least three, and possibly four different types of Ca^{2+} channels, with properties similar to T-, N-, and L-type Ca^{2+} channels.

INTRODUCTION

Electrophysiological studies of CNS neurones in slices using the voltage-clamp technique have traditionally been difficult to perform because of limited capacity to properly space clamp cells possessing significant processes. Synaptic currents from neighbouring cells also complicate the interpretation of recordings obtained from individual cells. However, many of these problems may be avoided or minimized by utilizing an enzyme dissociation technique (Kay & Wong, 1986) that provides viable, acutely isolated cells and that presents an excellent model for studying membrane currents in CNS neurones.

Using this technique, we sought to study current through Ca^{2+} channels in pyramidal neurones acutely isolated from the CA3 region of the guinea-pig hippocampus. Ca^{2+} ions entering cells via voltage-dependent Ca^{2+} channels play a fundamental role in many cellular processes. Multiple types of voltage-sensitive Ca^{2+} channels have been found in a variety of cells (for review, see Llinás, 1988; Tsien, Lipscombe, Madison, Bley & Fox, 1988; Bean, 1989*a*). Understanding what types of Ca^{2+} channels exist in hippocampal neurones is important because voltage-dependent Ca^{2+} channels contribute to electrical activity and may be involved in such processes as pacemaker depolarizations, paroxysmal depolarizing shifts in epileptiform activity and full blown Ca^{2+} spikes (Wong & Price, 1978; Schwartzkroin & Wyler, 1980; Llinás & Yarom, 1981). However, published reports of the types of Ca^{2+} channels found in hippocampal neurones have yielded conflicting results. Studies carried out on cells voltage-clamped with a one-microelectrode switching clamp suggested that there are two components of Ca^{2+} currents, a dihydropyridine sensitive current that activated at high thresholds (similar to the L-type Ca^{2+} channel in DRG neurones) and a current that activated at low thresholds but needed negative potentials to be reprimed (T-like; Brown, Canstanti, Docherty, Galvan, Gahwiler & Halliwell, 1985; Thayer, Murphy & Miller, 1986). Gray & Johnston (1985*a*, *b*) showed that there were probably three different types of Ca^{2+} channels present in hippocampal granule cells. Similarly, a study of the effects of adenosine on whole-cell Ca^{2+} current in CA3 neurones found evidence for three different channels (Madison, Fox & Tsien, 1987). In contrast, others have found no evidence for T-type channels in acutely isolated

CA1 neurones (Kay, 1988; ffrench-Mullen, Slater & Barker, 1989). Recent studies of Ca^{2+} channels in hippocampal cells have found that low-threshold-activated channels did not appear until after the first few days of culturing (Meyers & Barker, 1989; Ozawa, Tsuzuki, Iion, Ogura & Kudo, 1989) or were limited in acutely isolated cells to animals less than two weeks old (Thompson & Wong, 1989). In contrast, others have found T-currents to exist in adult CA1 neurones (Takahashi, Tateishi, Kaneda & Akaike, 1989).

In this study both whole-cell and single-channel patch clamp data from Ca^{2+} channels in acutely isolated hippocampal pyramidal neurones from the CA3 region of young adult guinea-pigs are presented which indicate the presence of at least three, and possibly four, types of Ca^{2+} channels (both low- and high-threshold activation). Single-channel records displayed three different unitary slope conductances. Whole-cell currents showed pharmacological and kinetic properties similar to T-, N-, and L-type currents characterized in other preparations (Nilius, Hess, Lansman & Tsien, 1985; Nowycky, Fox & Tsien, 1985*b*; Fox, Nowycky & Tsien, 1987*a*, *b*; Hirning, Fox, McCleskey, Miller, Olivera, Thayer & Tsien, 1988; Tsien *et al.* 1988; Bean, 1989*b*). Nevertheless, a high-threshold whole-cell Ca^{2+} current component was recorded that was insensitive to blockers of the N- and L-type Ca^{2+} channels suggesting that a fourth type of Ca^{2+} channel may be present. The identification and characterization of the channels underlying Ca^{2+} current is an important step in understanding the physiology of the hippocampus. The possible existence of a fourth type of Ca^{2+} channel suggests that Ca^{2+} channel physiology in this region may be even more complex than previously suspected.

METHODS

Cell preparation

Freshly dissociated hippocampal CA3 pyramidal neurones were prepared from young adult guinea-pigs (200–250 g). The technique was adapted from Kay & Wong (1986). Briefly, guinea-pigs were decapitated and the brains were removed rapidly and placed in a cold (~ 5 °C) oxygenated piperazine-*N*-*N*′-bis(2-ethanesulphonic acid) (PIPES)-buffered saline solution containing (mM): 120, NaCl; 5, KCl; 1, $CaCl_2$; 1, $MgCl_2$; 25, D-glucose; 20, PIPES and pH, 7·0. The hippocampus was rapidly dissected out, followed by the dissection of the CA3 region. Slices (450–500 μm thick) were cut with a microtome. The slices were then placed in a circular chamber, held at 32 °C, and the chamber was filled with 15 ml PIPES solution and 10·5 mg of trypsin (Sigma XI). Oxygen was continuously perfused through the chamber (1·2 ml/s). The slices were stirred slowly for 90 min; the enzyme-containing solution was then replaced by an oxygenated enzyme-free PIPES saline at room temperature in which the slices were stored. When needed, two slices were removed from the chamber and triturated in a 1 ml Dulbecco's modified Eagle's medium (DMEM) solution using four pipettes with progressively smaller bores. The cells were then transferred to an experimental bath for use. Cells were plated onto glass cover-slips fixed to the bottom of the bath. The cover-slips were pretreated with concanavalin-A, which allowed the neurones to anchor firmly within 5 min of plating.

Electrophysiological studies

Single-channel data was recorded using cell-attached patches. The standard patch-pipette solution contained (in mM): $BaCl_2$, 90; TEA-Cl (tetraethylammonium chloride), 15; CsCl, 15; HEPES, 10 and pH, 7·4. Cs and TEA were included for blocking current through potassium channels in the patch. Unlike other cell types, potassium channels in hippocampal pyramidal neurones were not completely suppressed by 90 mM-Ba^{2+}. The bath solution contained (in mM): potassium aspartate, 140; HEPES, 10; EGTA, 10; $MgCl_2$, 1; pH, 7·4. The cell resting potential was

assumed to be zeroed by bathing in isotonic potassium aspartate (Hess, Lansman & Tsien, 1984; Fox *et al.* 1987*b*).

For whole-cell experiments, the solutions contained (in mM), external: $BaCl_2$, 5; TEA-Cl, 130; HEPES, 10; TTX (tetrodotoxin), 0·35 μM, pH, 7·3, and internal: experiments in which cells were exposed to either Cd^{2+} or Ni^{2+} (Fig. 2) contained CsCl, 100; Na_2-ATP, 2; EGTA 10; HEPES, 40; $MgCl_2$, 5; Na-GTP, 0·35; pH, 7·3. Later experiments were performed with the following (Tris) internal solution which improved the stability of the seal as suggested by Kay & Wong (1987), in mM: Trizma Base, 28; Trizma-PO_4, 70; EGTA, 11; TEA-Cl, 40; Na_2-ATP, 2; Na-GTP, 0·3; $MgCl_2$, 2; pH, 7·3. The only change in current characteristics with the Tris solution was a shift in the peak of the whole-cell current–voltage relationship in the hyperpolarizing direction by 7 mV. For whole-cell experiments, solutions were perfused through the bath at a rate of 1–4 ml/min. All experiments were done at room temperature, 22 °C. Drugs were applied by either changing the perfusion reservoir to one containing the test compound, or the flow was halted and the drugs were applied directly into the bath, e.g. with ω-conotoxin (Peninsula Laboratories). In single-channel experiments, all solutions were applied directly to the bath. Nimodipine and Bay K 8644 were kind gifts of Dr Alexander Scriabine, Miles Pharmaceutical, Inc. Stock solutions of either drug were prepared to a concentration of 10^{-2} M in 100% ethanol and were applied in near darkness to avoid possible degradation of the drugs due to photosensitivity of dihydropyridines. The final bath concentration of ethanol of 0·02%.

Isolated cells were typically less than 100 μm long; the soma constituted between 30 and 50% of total cell length. Cells were chosen in which the cell body possessed a triangular shape characteristic of pyramidal cells. Because pyramidal cells constituted approximately 90% of the total cell population, it was relatively easy to identify a desired target cell. Cells with excessively large projections were rejected in order to maintain space clamp control. Electrodes were fabricated from borosilicate glass using a Narishige two-stage vertical puller (Model PP-83). Electrodes were coated with Sylgard to within 100 μm from the tip and were heat-polished using a Narishige Microforge (model MF-83) to a resistance of 1·7–2·5 MΩ as measured using standard internal and external solutions. Approximately 80% of series resistance (R_s) could be compensated before ringing occurred. Settling time for depolarizations near 0 mV was always less than 1 ms. Set at 1 kHz, our 8-pole Bessel filter settled from a voltage step in ~ 800 μs. The clamp was always significantly faster. Seal resistances for whole-cell experiments were between 1 and 10 GΩ.

For whole-cell recordings, leak sweeps were obtained by averaging sixteen hyperpolarizing test pulses. Leak current was subtracted from the data sweeps by scaling the leak sweep to the data. Frequently, to further reduce the noise introduced by leak subtraction, several leak sweeps were summed and averaged before subtracting from the data sweep. For single-channel data, typically ten leak sweeps were averaged before subtracting from the data sweep. Leak sweeps consisted of data sweeps in which no channel openings were present or depolarizing pulses to potentials at which channels did not open. A moving average was calculated so that only the ten leak sweeps that were the most chronologically proximate to the data sweep were used.

Computer analysis and equipment

Experiments were controlled by an online IBM PC/AT clone computer. The signal from an Axopatch-1C patch-clamp amplifier was filtered by an 8-pole Bessel filter with a corner frequency (f_c) 1 kHz $\leqslant f_c \leqslant$ 2·5 kHz. Data acquisition and analysis was controlled using the AXOBASIC system (AXON Instruments, Foster City, CA, USA). Typically data were sampled at 150 μs intervals.

RESULTS

Voltage dependence of whole-cell Ca^{2+} currents

Isolated cells were voltage clamped in the whole-cell patch-clamp configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Ba^{2+} (5 mM) was used as the external charge carrier. TEA was included in both the internal and external solutions to block K^+ channels and to act as a non-permeant Na^+ substitute. To suppress Na^+ current, TTX (350 nM) was applied externally (see Methods). Under these conditions Ca^{2+} currents were observed in isolation. Figure 1 shows the voltage dependence of

Ca^{2+} current at two different holding potentials. Figure 1*A* shows leak-subtracted currents from a holding potential (HP) of −90 mV (marked by asterisks) or −50 mV to a variety of test potentials (TP), as indicated in the figure. The current–voltage relationship (*I–V*), Fig. 1*B*, plots the peak currents elicited at each

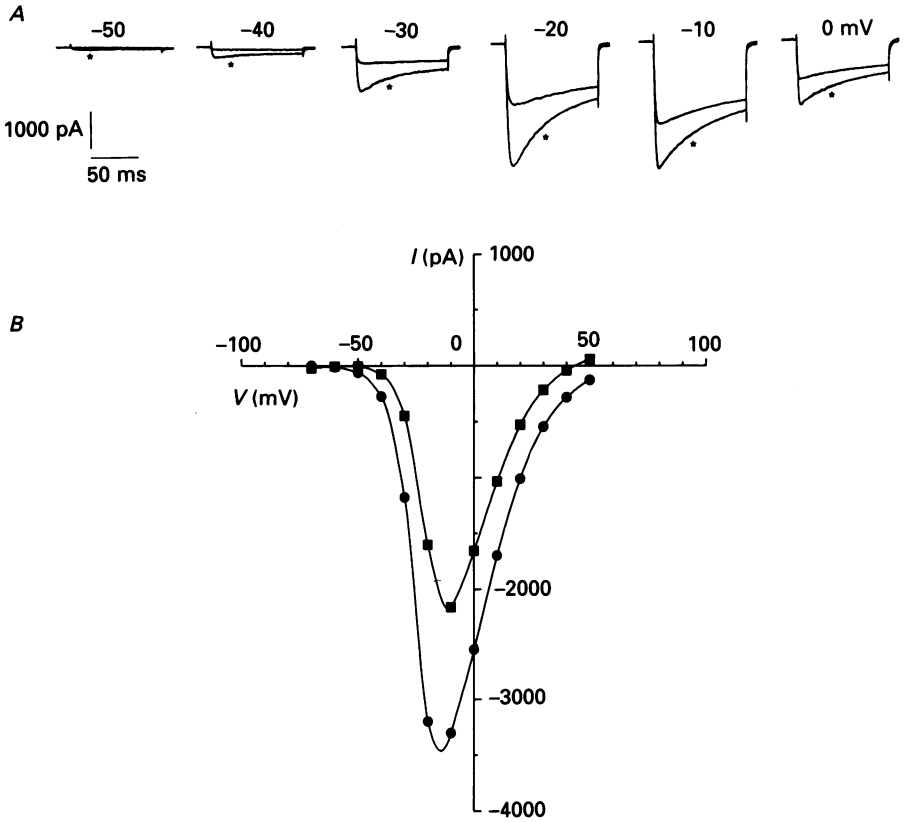

Fig. 1. Voltage dependence of peak whole-cell Ca^{2+} current. *A*, leak-subtracted current recordings at test potentials (TP) from −50 to 0 mV are shown; holding potentials (HP) were −50 or −90 mV (marked with an asterisk). *B*, current–voltage (*I–V*) relationships recorded at HP equal to −90 mV (●) and −50 mV (■). *I–V* data points at each holding potential are connected by a cubic spline. Current was carried by 5 mM-Ba^{2+} (see Methods).

test potential from a holding potential of −90 (●) or −50 mV (■). The maximum peak current occurred at −10 mV in both cases. The small shift in reversal potential observed in the *I–V* when the holding potential was changed from −90 to −50 mV indicates a small contaminating outward current was present for large depolarizations. Similar experiments performed using a Cs^+-containing internal solution produced similar *I–V* relationships although the maximum peak inward current occurred 7 mV (±1·8 mV; $n = 19$) more positive than in cells containing Tris internal solution, possibly due to surface charge effects at the inner surface of the membrane. It is highly unlikely that the negative shift in the peak of the *I–V* with Tris was due

to series resistance problems for three reasons. First, these experiments yielded consistent and reproducible results as to the potential at which the *I–V* peaked using either internal solution. Second, the maximum currents were recorded at the same potentials regardless of whether the currents were large or small. Third, as stated in

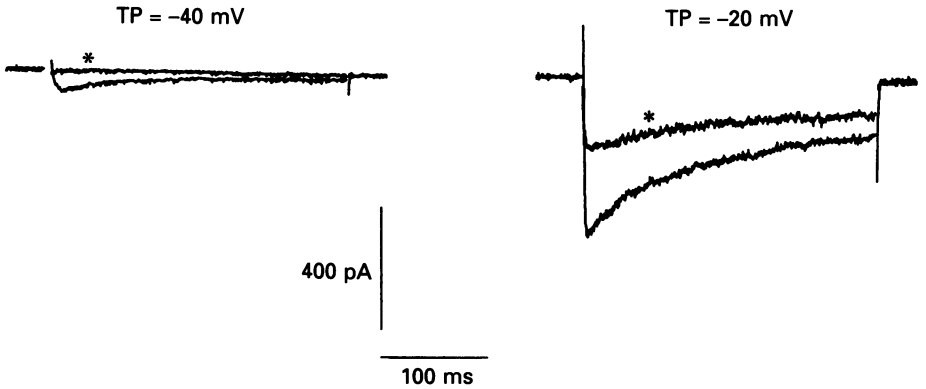

Fig. 2. Effect of Ni^{2+} (100 μM) on low-threshold T-type whole-cell Ca^{2+} current. Currents recorded at HP = −110 mV with test potentials to −40 and −20 mV as indicated. Currents marked by asterisks were recorded with Ni^{2+}.

the methods, the cells possessed limited processes. Because stray capacitance was limited by using Sylgard and low solution levels in the bath, and because series resistance was limited by using large-bore pipettes and R_s compensation, currents were resolved without the characteristic signs of poor clamp control such as precipitous potential-dependence of activation or slow decay of capacitance currents.

Effects of Ni^{2+} and Cd^{2+} on whole cell Ca^{2+} current

Figure 2 shows currents elicited from a holding potential of −110 mV by applying test depolarizations to −40 and −20 mV before and during exposure to 100 μM-Ni^{2+}. At TP = −40 mV, a potential where T-type Ca^{2+} currents were usually observed in relative isolation, Ni^{2+} abolished all the current. At TP = −20 mV, a fraction of the current remained after exposure to Ni^{2+} suggesting the presence of multiple types of Ca^{2+} channels at these potentials. The effect of Ni^{2+} on Ca^{2+} current was most likely due to a differential block by Ni^{2+} on T-type channels versus N- and L-type channels rather than a voltage-dependent blocking effect because complete block occurred at all potentials where only T-channels were active but the block was incomplete at potentials where L- and N-type Ca^{2+} channels were activated. T-type currents have been shown to be preferentially inhibited by Ni^{2+} in rabbit cardiac cells (Hagiwara, Irisawa & Kameyama, 1986), chick DRG (Fox *et al.* 1987*a*), and neuroblastoma cells (Narahashi, Tsunoo & Yoshii, 1987). In contrast, T-Type Ca^{2+} currents found in hypothalamus were not exceptionally sensitive to block by Ni^{2+} (Akaike, Kostyuk & Osipchuk, 1989). The voltage-dependent Ni^{2+} block of the whole-cell current in hippocampal CA3 neurones strongly suggests the presence of T-type channels in CA3 pyramidal neurones.

The effects of 50 μM-Cd^{2+} on the whole-cell current–voltage relationships were

examined. From either HP = −50 or −90 mV, Cd^{2+} blocked nearly all the current at all test potentials from −70 to +50 mV (> 95% block). However, from HP = −90 mV, a fraction of the rapidly inactivating, low-threshold (T-type) current was still present. This effect is similar to that observed in DRG neurones (Fox *et al.*

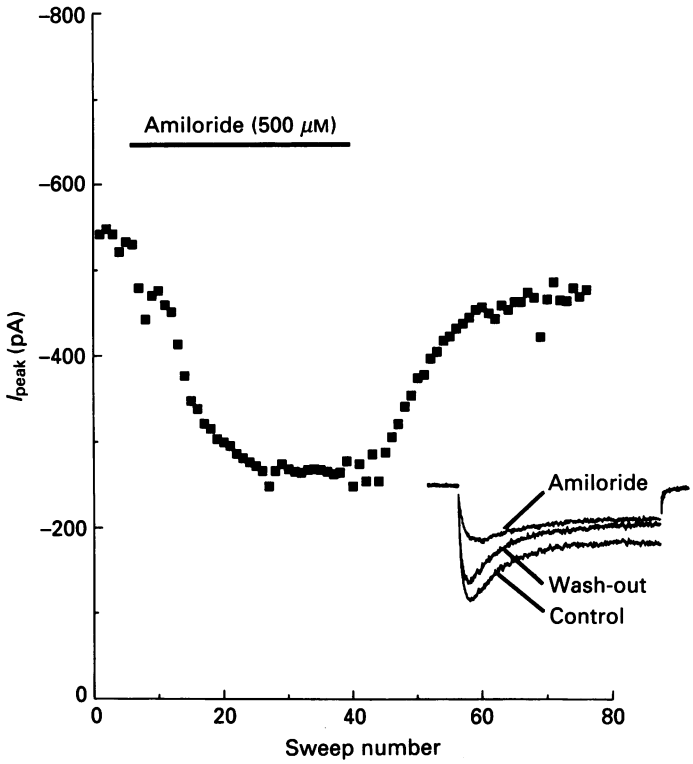

Fig. 3. Amiloride (500 μM) blocked a low-threshold T-type current. Time course of peak current elicited from a HP = −100 mV and TP = −40 mV before, during, and after exposure to amiloride. Peak current after wash-out returned to approximately 86% of original control level. Inset: leak-subtracted currents show the wash-out primarily restored the rapidly inactivating component.

1987*a*) and cultured rat hippocampal neurones (Ozawa *et al.* 1989) where an early, rapidly-inactivating current component (characterized as the T-type Ca^{2+} current) was the component most resistant to block by Cd^{2+}.

Amiloride blocked a low-voltage threshold current

Amiloride has previously been shown to block a low-threshold T-type Ca^{2+} channel in mouse neuroblastoma and chick DRG neurones (Tang, Presser & Morad, 1988). Figure 3 shows the time course of the effect of amiloride (500 μM) on the peak of the whole-cell current elicited from a HP of −100 mV to a TP of −40 mV. Amiloride reversibly blocked an early, rapidly inactivating current component. The inset shows

leak-subtracted currents recorded before, during, and after exposure to the drug. The block of the T-type channel by amiloride was clearly not complete as shown by the drug effect on the currents shown in the inset. Most of the transient current (calculated as peak current minus steady-state current), which was decreased by

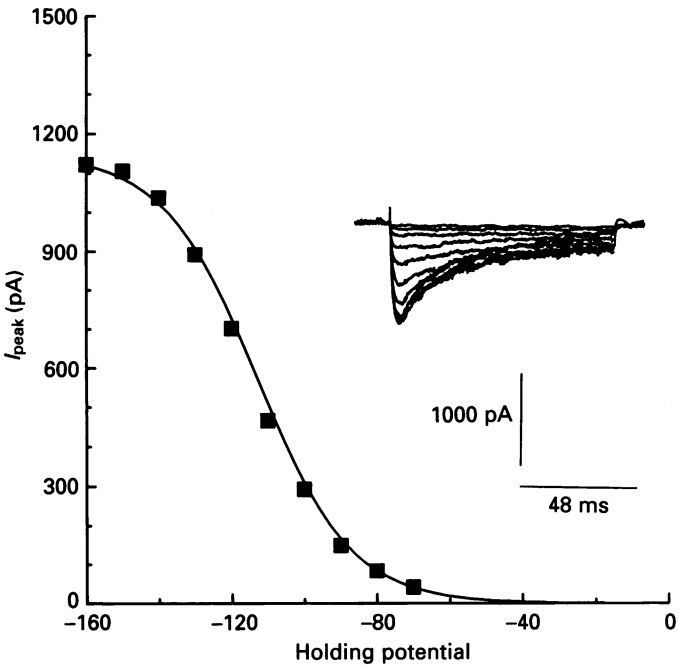

Fig. 4. Steady-state inactivation plot (h_∞) for T-type Ca^{2+} channel current. Eliciting depolarizations to TP = −60 mV, the HP was varied between −70 and −160 mV. Cells were held at each HP for 20 s before a test depolarization was applied. Fitting of the Boltzman equation ($I/I_{max} = [1+\exp((V-V_{\frac{1}{2}})/k)]^{-1}$) to the data, assuming I_{max} = 1150 pA, gave values for $V_{\frac{1}{2}}$ and k of −113 and 13 mV, respectively. Inset: leak-subtracted currents recorded when HP was varied from −70 to −160 mV. The tail current at HP = −150 mV was blanked due to a brief, momentary change in the seal resistance.

$36 \pm 6{\cdot}7\%$ ($n = 4$) during amiloride application, was recovered upon wash-out indicating that the decrement of this current component was due to inhibition by amiloride. Since a fraction of the steady-state current was also decreased, amiloride may also by affecting another current component, possibly L- or N-type. Alternatively, because amiloride has been shown to block the Na^+–H^+ exchanger, a change in the intracellular pH due to incomplete buffering by the pipette solution may have brought about the decrease in inward current. However, the large quantity of pH buffer available in the patch pipette suggests that the amiloride effect on T-type current was due to a direct action on Ca^{2+} channels. In any case, the specificity of amiloride for the T-type Ca^{2+} channel compared to other types of Ca^{2+} channels is not great and requires a relatively high concentration to block T-type current. Furthermore, in our hands the effects of amiloride were usually not completely reversible. Because the concentrations in these experiments were

somewhat higher than those reported by Tang *et al.* (1988), it may be that hippocampal T-type channels are not blocked as well as those in neuroblastoma or chick DRG cells.

T-channels needed strong hyperpolarizations for repriming

Steady-state inactivation (h_∞) of the low-threshold (T-type) channel was investigated employing a test potential that was fixed at −60 mV while the holding potential was varied between −70 and −160 mV. To assure steady-state measurements, the cell was held at each holding potential for 20 s prior to eliciting a test pulse. The peak current amplitude elicited at each of the holding potentials is plotted in Fig. 4. The fit of the Boltzmann relation to the data yielded a curve with a $V_{\frac{1}{2}}$, the potential at which half the channels were reprimed from inactivation, equal to −113 mV. It is unknown whether these channels *in vivo* possess the same voltage dependence of inactivation as observed in our experiments inasmuch as hyperpolarizing shifts in the activation range following cytosolic access by a patch clamp electrode have been described (Fernandez, Fox & Krasne, 1984). If true, the h_∞ plot suggests that under normal *in vivo* conditions the T-channels would probably never be maximally reprimed from inactivation. A similar relationship for steady-state inactivation was seen in two other experiments. The T-type currents recorded in these cells were similar to that reported in other cells in that it was an inactivating current that required negative holding potentials to be seen, was activated with weak low depolarizations, and was blocked by Ni^{2+} and amiloride. However, some differences were found. Extremely negative holding potentials were required to completely reprime the current, inactivation was slower than that seen elsewhere, and a maintained component of the T-type current was sometimes observed. These differences suggest that different populations of T-type channels may exist.

ω-Conotoxin blocked a high-threshold current

The effect of ω-conotoxin GVIA (ω-CgTx) on components of high-voltage-activated Ca^{2+} current is still somewhat controversial. One report has suggested that both N- and L-type Ca^{2+} channels were inhibited by ω-CgTx in dorsal root ganglion neurones (McCleskey, Fox, Feldman, Cruz, Olivera, Tsien & Yoshikami, 1987), while others have found little or no effect on L-type current in these cells (Kasai, Aosaki & Fukuda, 1987). ω-CgTx has no effect on L-type channels in smooth, cardiac and skeletal muscle (McCleskey *et al.* 1987) or in sympathetic neurones (Plummer, Logothetis & Hess, 1989). In hippocampal CA3 pyramidal neurones, from HP = −90 mV and using a TP to −10 mV, application of ω-CgTx (1 μM) decreased the peak inward current as shown in Fig. 5. Figure 5*A* plots the time course of peak current before and during exposure to ω-CgTx. The current–voltage relationship from HP = −90 mV before and during exposure to ω-CgTx is shown in Fig. 5*B*. No significant reduction of the peak current was observed until the test depolarizations were to −20 mV or greater. Hence, we conclude that low threshold currents were not affected by exposure to ω-CgTx. The mean reduction of peak current with this voltage protocol was 32·6 ± 2·2 % ($n = 7$). Increasing the ω-CgTx to 3 μM did not increase the potency of the block. This data stands in contrast with autoradiographic

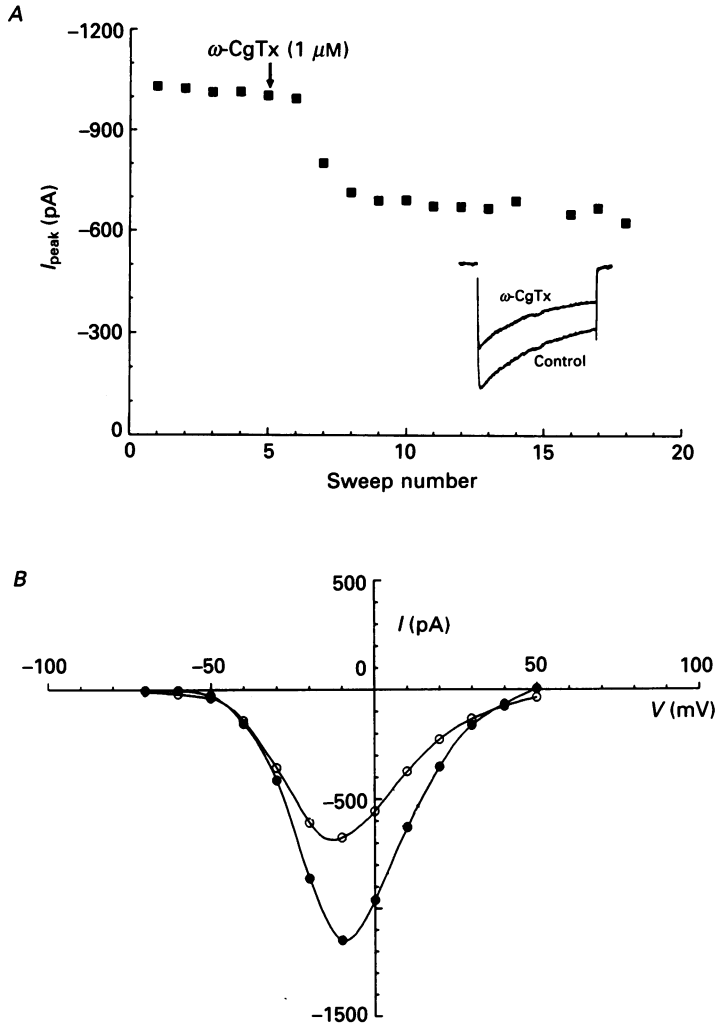

Fig. 5. Effect of ω-CgTx (1 μM) on hippocampal neurone Ca^{2+} currents. *A*, the effect on peak current of ω-CgTx from HP = −90 mV and TP = −10 mV before and during exposure to the toxin. Interpulse interval = 25 s. *B*, the *I–V* relationship from HP = −90 mV, measured before (●) and during (○) exposure to ω-CgTx. ω-CgTx reduced the current with TP = −30 mV to +30 mV. From HP = −90 mV to a TP = −10 mV, ω-CgTx reduced the mean peak inward current by 32·6 ± 2·2 % (mean ± S.E.M., $n = 7$).

analysis of ω-CgTx receptors in the hippocampus that found no ω-CgTx binding sites in the pyramidal cell layer (Takemura, Kiyama, Fukui, Tohyama & Wada, 1988; Jones, Kunze & Angelides, 1989).

Effects of dihydropyridines on L-type Ca^{2+} current

The primary site of action of 1,4-dihydropyridines is thought to be L-type Ca^{2+} channels (Nowycky, Fox & Tsien, 1985*a*; McCleskey *et al.* 1987; Yatani, Codina, Imoto, Reeves, Birnbaumer & Brown, 1987; Aosaki & Kasai, 1989; but see Cohen &

McCarthy, 1987; Akaike *et al.* 1989). Bay K 8644, a dihydropyridine agonist, has been shown to promote Ca^{2+} current and transmembrane Ca^{2+} flux (Schramm, Thomas, Towart & Franckowiak, 1983; Brown *et al.* 1985; Hess *et al.* 1984; Kokubun & Reuter, 1984; Nowycky *et al.* 1985*a*) although a weak antagonism of an N-like

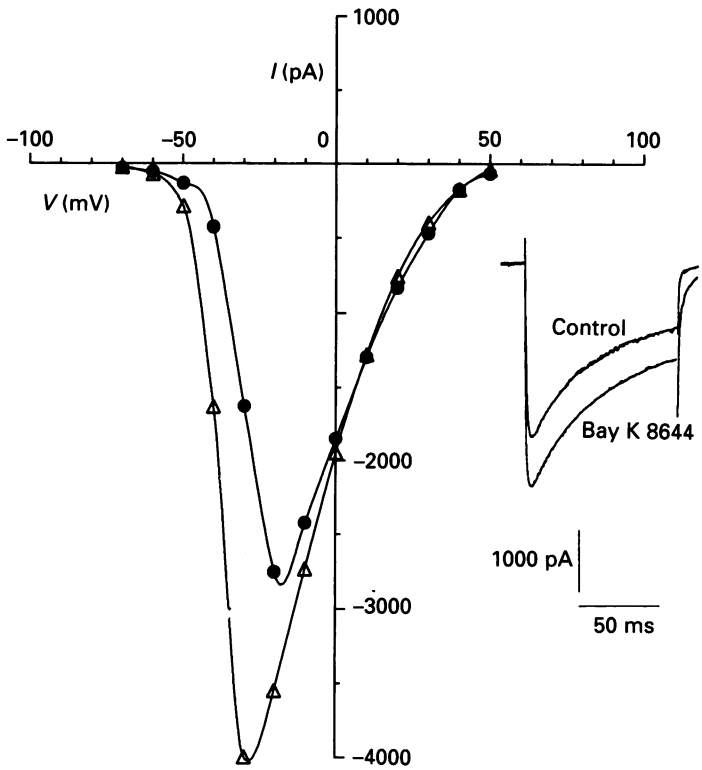

Fig. 6. The Ca^{2+} channel agonist Bay K 8644 (2 μM) potentiated a high-threshold L-type Ca^{2+} current. The *I–V* relationships recorded from HP = −100 mV, before (●) and during (△) exposure to Bay K 8644 is shown. Bay K 8644 increased the peak inward current at test potentials of −50 to 0 mV. In addition, Bay K 8644 shifted the peak of the *I–V* 11 mV in the hyperpolarizing direction. Inset shows leak-subtracted currents at TP = −20 mV before and during exposure to the drug.

current in CA1 neurones has been reported (Docherty & Brown, 1986). The effects of the agonist Bay K 8644 on the current–voltage relationship from HP = −100 mV is shown in Fig. 6. External application of Bay K 8644 (2 μM) resulted in an increase in the peak inward current at test potentials from −60 to 0 mV. The peak of the *I–V* relationship shifted 11 mV in the negative direction consistent with a shift in the voltage dependence of activation seen in heart (Hess *et al.* 1984), rat hippocampal CA1 neurones (Meyers & Barker, 1989), and chick DRG neurones (Fox *et al.* 1987*a*). The inset shows currents obtained in the absence and presence of Bay K 8644, elicited by depolarizations to −20 mV. Tail currents recorded upon repolarizing the cells back to the holding potential were characteristically slowed by Bay K 8644 (Hess *et al.* 1984; Fox *et al.* 1987*a*).

The effect of the DHP antagonist nimodipine on Ca^{2+} current is shown in Fig. 7*A*.

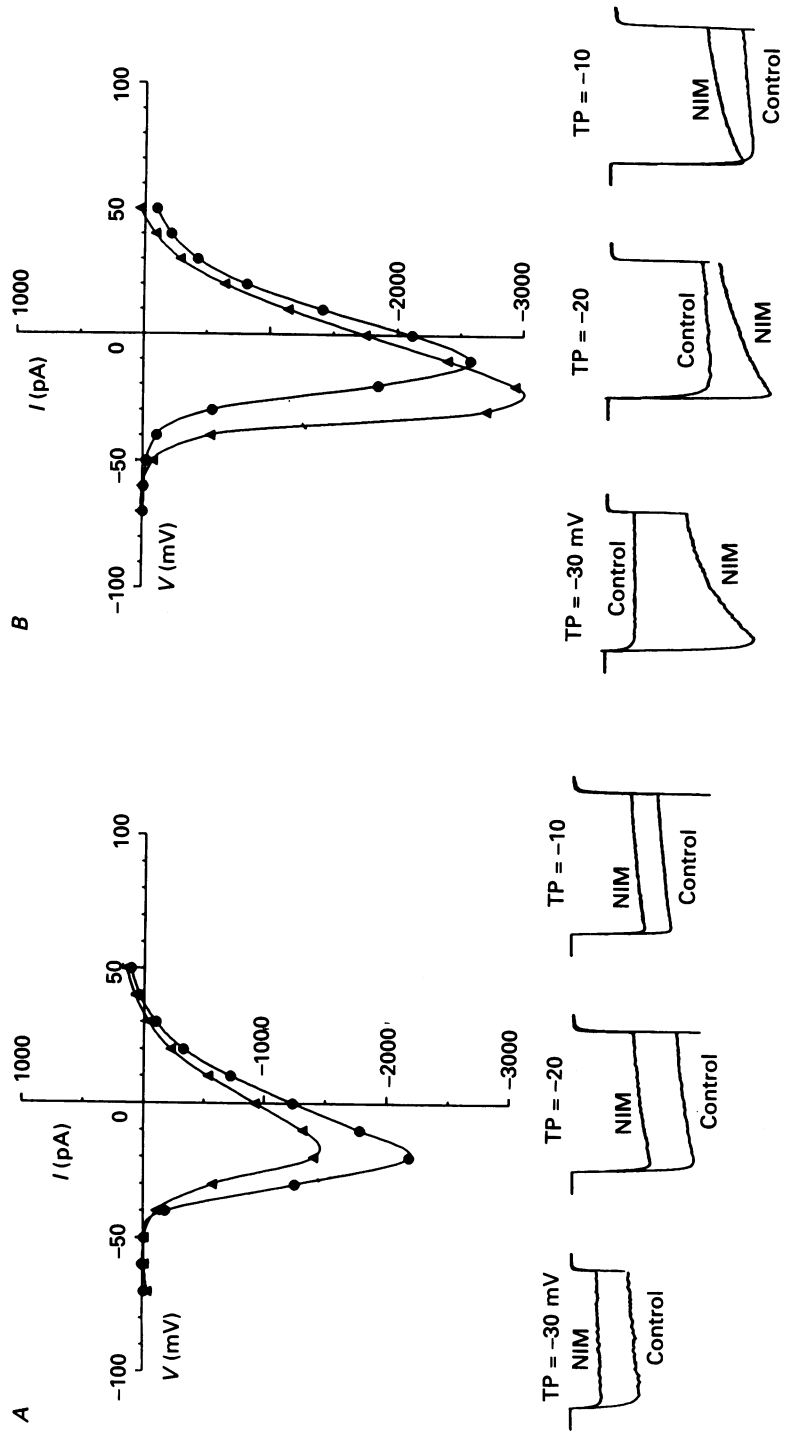

Fig. 7. Holding potential dependence of the dihydropyridine Ca^{2+} channel antagonist nimodipine (NIM; 2 μM). *A*, *I–V* relationship recorded from HP = −50 mV before (●) and during (▲) exposure to NIM. Below the *I–V* are the leak-subtracted currents at HP = −50 mV before and during NIM. At TP = −20 and −10 mV, NIM reduced the mean peak inward current by 42·7 ± 6·2 % and 44·5 ± 7·3 %, respectively (mean ± S.E.M., $n = 6$). *B*, *I–V* relationship recorded from HP = −90 mV before (●) and during (▲) exposure to NIM. NIM augmented the current at test potentials from −50 to −20 mV and caused a shift in the peak of the *I–V* in the hyperpolarizing direction similar to Bay K 8644. At stronger depolarizations (TP = −10 to +50 mV) NIM inhibited the peak inward current. Below the *I–V* are the leak-subtracted currents from HP = −90 mV before and during NIM.

The holding potential was set to −50 mV to facilitate voltage-dependent inhibition (Bean, 1984; Sanguinetti & Kass, 1984). Nimodipine (2 μM) suppressed a slowly inactivating current. This concentration of nimodipine blocked 42·7 ± 6·2 % and 44·5 ± 7·3 % ($n = 6$) of the peak current at test potentials of −20 and −10 mV respectively. While exclusive specificity of dihydropyridines for L-type Ca^{2+} channels has been challenged (e.g. DHP block of T-type Ca^{2+} channels currents (Cohen & McCarthy, 1987; Akaike *et al.* 1989)), these experiments were all performed at HP = −50 mV, a potential where T-currents were completely inactivated, suggesting that L-type Ca^{2+} channels alone were blocked by nimodipine.

In contrast to its blocking effect on whole-cell currents observed from HP = −50 mV, at HP = −90 mV nimodipine exerted an agonist effect during weak depolarizations. The *I–V* shown in Fig. 7*B* demonstrates that 2 μM-nimodipine potentiated the currents dramatically at test potentials between −40 and −20 mV, in a manner analogous to that seen with Bay K 8644. In contrast to the effects of Bay K 8644, however, larger depolarizations to test potentials ⩾ −10 mV always showed antagonist effects. The leak subtracted currents show (Fig. 7*B*) that while nimodipine caused a large increase in the peak current, this augmented current component decayed rapidly (although at TP = −30 mV there was still a significant fraction remaining after 100 ms).

Assuming that no L-type Ca^{2+} channels were reprimed by changing the HP from −50 to −90 mV, we would expect approximately 30 % of the current available at −90 mV (i.e. the magnitude of current blocked by nimodipine when HP = −50 mV) to be L-type, on average ($n = 4$). This number is almost identical to our estimate of N-type Ca^{2+} channels in these cells. This represents a substantial fraction of the whole-cell current and is contrary to other reports suggesting that L-type current represents an insignificant fraction of whole-cell current in neurones (Kasai & Aosaki, 1988; Plummer *et al.* 1989). However, we tested four CA3 pyramidal cells that showed no response to nimodipine, perhaps indicating that even within this cell heterogeneity of channel distribution may be important.

High-threshold current insensitive to ω-CgTx and nimodipine

Figure 8*A* shows that the addition of nimodipine (2 μM) and ω-CgTx (1 μM) decreased peak inward current by approximately 60 % at HP = −50 mV. Doubling the concentration of nimodipine and tripling the concentration of ω-CgTx had no additional effect. This was a consistent finding in all cells utilizing this protocol indicating that the original concentrations of nimodipine and ω-CgTx were sufficient to produce a maximal effect. In contrast, Fig. 8*B* shows data from another cell in which a similar protocol showed only a slight decrease in current upon exposure to both drugs. The data in Fig. 8 shows that sometimes there were large effects of ω-CgTx and nimodipine when applied simultaneously (the maximum block observed was 67 %), while in other cells there was hardly any effect suggesting that one type of high-threshold Ca^{2+} channel found in hippocampal CA3 neurones is insensitive to either ω-CgTx or dihydropyridines.

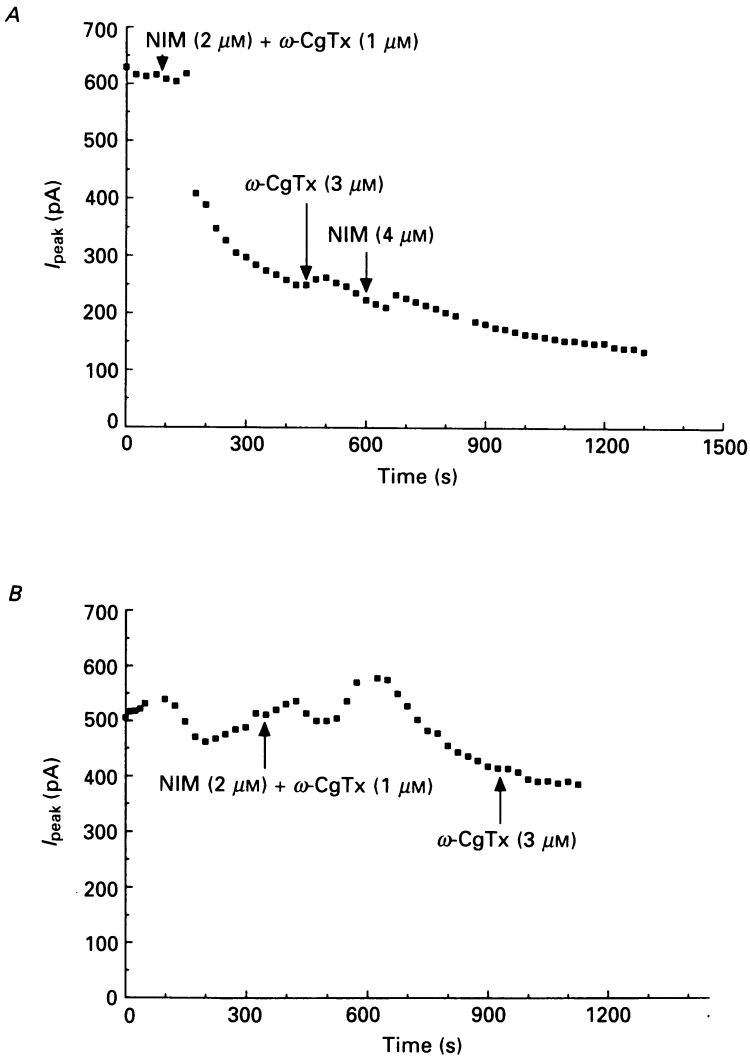

Fig. 8. Hippocampal CA3 pyramidal neurones exhibited a high-threshold Ca^{2+} current insensitive to NIM (2 μM) and ω-CgTx (1 μM). *A*, the time course from cell in which HP = −50 mV and TP = −10 mV. Addition of NIM (2 μM) and ω-CgTx (1 μM) decreased the peak inward current by 60%. Increasing drug levels ([NIM] = 4 μM; [ω-CgTx] = 3 μM) had no additional effect on inward current. *B*, the time course from a second cell at HP = −50 mV and TP = −10 mV. Addition of NIM and ω-CgTx had virtually no effect on the peak inward current.

Single-channel currents showed three different conductance levels

Single-channel currents were recorded in the cell-attached patch configuration with Ba^{2+} (90 mM) as the external charge carrier. Evidence for the presence of at least three different types of Ca^{2+} channels with three different unitary current levels was observed in hippocampal CA3 neurones as shown in Fig. 9*A*. Leak-subtracted

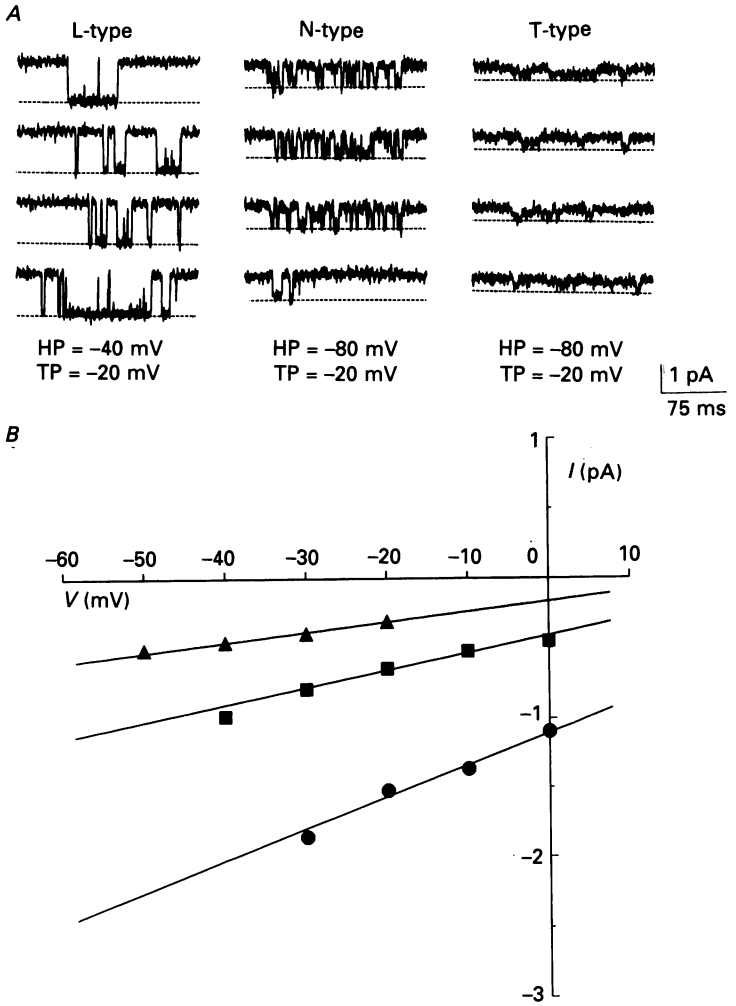

Fig. 9. *A*, single-channel recordings showed three different unitary current levels. Shown are data from three different cell-attached patches. Ba^{2+} (90 mM) in the pipette electrode was the charge carrier in all single-channel experiments. TP = −20 mV for all three patches. HP was either −80 or −40 mV. Dashed lines indicate unitary current level for the channel in each patch equivalent to 1·41 pA, 0·83 pA, and 0·42 pA for the L-, N-, and T-channels, respectively. Bay K 8644 (1 μM) was in the bath for the current elicited from HP = −40 mV (left column). *B*, unitary *I–V* relationships for three different channels. Conductances were measured by least-squares fits to the data and yielded values of 23 pS for the L-channel (eight patches, ●), 12 pS for the N-channel (five patches, ■), and 7 pS for the T-channel (four patches, ▲). Standard errors are within the size of the respective symbols.

current records from three different cell-attached patches are shown. In all three cases the test potential was the same (−20 mV) and the holding potential was either −80 or −40 mV. A dihydropyridine sensitive 23 pS channel was recorded in the presence of Bay K 8644. The single-channel current–voltage relationships for the three different types of channels are shown in Fig. 9*B*. The three different

conductance levels corresponding to the T-, N-, and L-type channels were 7, 12, and 23 pS, respectively (averaged from fifteen patches). Bay K 8644 caused an increase in both the channel open time and the probability of opening of the 23 pS channel.

Holding potential dependence of single-channel Ca^{2+} current

The holding potential dependence of the high-voltage-activated N-type Ca^{2+} channel was investigated. Figure 10 shows currents from the 12 pS channel recorded

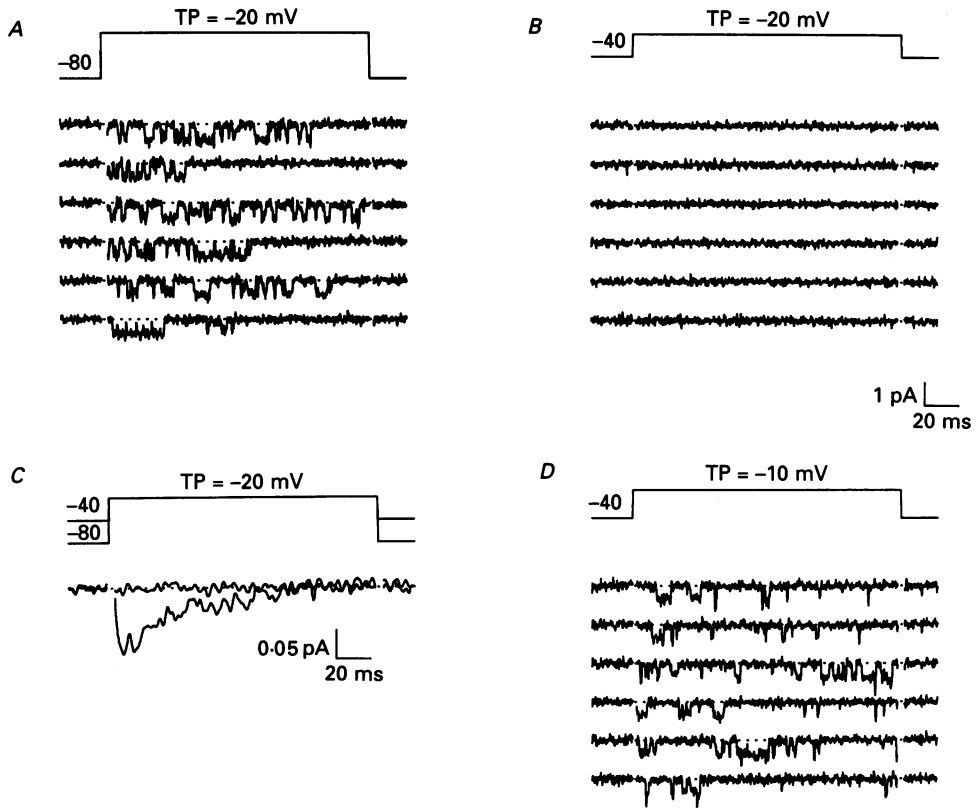

Fig. 10. Holding potential dependence of the N-type Ca^{2+} channel. *A*, from HP = −80 mV, depolarizations to −20 mV produced single-channel openings from the 16 pS channel. *B*, in the same patch, from HP = −40 mV, depolarizations to −20 mV elicited no single-channel openings. *C*, ensemble average currents from the cell-attached patch at the two holding potentials. Ensemble average currents were smoothed using a binomial averaging technique. *D*, in a different patch from above, with a TP = −10 mV from a HP = −40 mV an intermediate conductance channel was still very active, in contrast to the current shown in *B*.

from a cell-attached patch. From a holding potential of −80 mV, depolarizations to −20 mV opened the channel. When the holding potential was changed to −40 mV (Fig. 10*B*), no single channel openings were observed. Figure 10*C* shows ensemble average currents from the same patch in which the test potential was either −80 or −40 mV. From a holding potential of −80 mV, the summed current activated rapidly and decayed to the zero current level by the end of the 175 ms test pulse. The

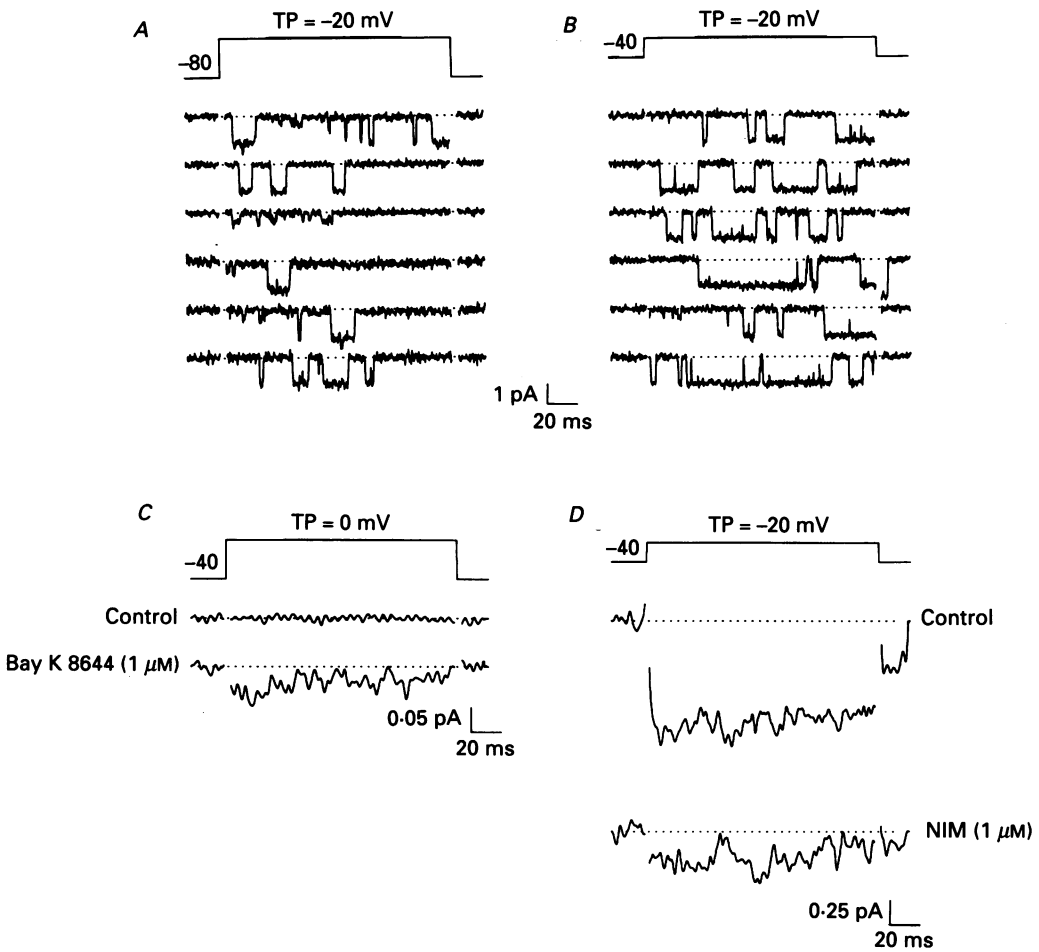

Fig. 11. *A*, holding potential dependence of the T- and L-type single-channel currents. The bath solution contained BayK8644 (1 μM). At HP = −80 mV and TP = −20 mV, openings of both the T- and L-channels (7 and 23 pS, respectively) were seen. *B*, in the same patch as *A*, when HP was changed to −40 mV with the same TP, the T-type channel activity was virtually abolished while the L-type channel remained active. *C*, effects of dihydropyridines on ensemble averages of single-channel current. Ensemble current before (sixty-one sweeps) and during exposure to BayK8644 (1 μM; sixty-two sweeps). HP = −40 mV and TP = 0 mV. BayK8644 increased open time and open probability of the 23 pS channel. Ensemble currents show that the effect of BayK8644 on single channels resembles the potentiation observed in whole-cell currents. *D*, ensemble averages of a different patch from *C* in which HP = −40 mV and TP = −20 mV. This was a multi-channel patch consisting primarily of L-type channels. Addition of nimodipine (1 μM) to the bath decreased the ensemble current. (103 sweeps were averaged for the control current; 113 sweeps were averaged for the nimodipine current.)

ensemble average currents show that, from HP = −80 mV (173 sweeps), the N-type channel mimicked the time-dependent inactivation seen in whole-cell recordings. When the holding potential was changed in −40 mV (105 sweeps) the ensemble current was zero, as expected from the individual current records. In whole-cell

currents significant N-type current was still seen from HP = −40 mV. In a different experiment, however, single-channel recordings at TP = −10 mV from HP = −40 MV (Fig. 10*D*) showed a similar probability of opening when compared to HP = −80 mV. While the sustained openings at depolarized holding potentials were only seen in two patches, these differences may indicate the presence of two different channel types with similar conductances.

Figure 11*A* shows unitary traces from a cell that had 7 and 23 pS (T- and L-type, respectively) Ca^{2+} channels. Bay K 8644 (1 μM) was in the bath. From a holding potential of −80 mV, openings of both channel types were observed. When the holding potential was changed to −40 mV (Fig. 11*B*), the 7 pS channel activity was virtually abolished; only the 23 pS channel remained. Note that the T-type channels clustered at the beginning of the sweep at HP = −80 mV, indicating an inactivating component to the current waveform. A lone sweep in Fig. 11*B*, fifth from the top, still shows T-current openings but this was quite rare (T-channel openings occurred in only four out of sixty-five sweeps at HP = −40 mV). The single-channel data showing a negative holding potential requirement for T-channel repriming is consistent with whole-cell experiments (see Fig. 4).

Dihydropyridines modulate single-channel L-type current

Single-channel recordings provide further evidence for the effect of Bay K 8644 on L-type currents. Figure 11*C* shows ensemble currents before and during exposure to Bay K 8644 (1 μM) in a patch containing only L-type channels. Bay K 8644 increased the ensemble current similar to its effect as seen in whole-cell current recordings. Figure 11*D* shows the effect of nimodipine (1 μM) on the ensemble average current in a different cell from a holding potential of −40 mV to a test potential of 0 mV. The recordings in Fig. 11*D* were made in a multi-channel patch consisted primarily of L-type channels. As the patch had many channels, the control current level seen in *D* was substantially greater than that seen in *C*. With a depolarized holding potential nimodipine decreased the current in a manner similar to that seen in whole-cell experiments.

DISCUSSION

We sought to resolve discrepancies that exist in the literature concerning the presence or absence of various Ca^{2+} channel types in the hippocampus. Our studies focused on the CA3 region exclusively. While various studies of the CA1 region have found a diverse apportionment of Ca^{2+} channels, heterogeneity in channel types between different regions of the hippocampus would not be unexpected as they possess distinct electrical characteristics. For example, when the actions of GABA were blocked by convulsive agents such as bicuculline or picrotoxin, synchronized bursts originated in the CA3 region and propagated to the CA1 region (Schwartzkroin & Prince, 1978; Wong & Traube, 1983; Miles & Wong, 1984). The isolated CA1 region showed a much higher threshold for generation of spontaneous bursting. Similarly, pyramidal cells in the CA1 region were much more susceptible to irreversible damage from ischaemia than were CA3 pyramidal cells (Pulsinelli & Duffy, 1983; Suzuki, Yamaguchi, Choh-Luh & Klatzo, 1983). Our whole-cell and single-channel data confirmed the presence of at least three different types of Ca^{2+} channels in CA3

pyramidal neurones similar to T-, N-, and L-types already described (Nowycky *et al.* 1985*b*; Fox *et al.* 1987*a b*; Hirning *et al.* 1988). Pharmacological evidence suggests the presence of a fourth type of high-threshold Ca^{2+} channel or the existence of an L- or N-type Ca^{2+} channel with unique pharmacological properties.

Physiological implications of T-channels in CA3 neurones

The T-channel has been implicated in bursting and pacemaker activity, characteristics which may have particular relevance to the physiology of neurones in the CA3 region. The low-threshold for activation of these channels would agree with the pacemaker hypothesis, that is, following repolarization, T-channel openings result in membrane depolarization to the threshold level necessary to fire another action potential. However, with extremely negative potentials required for complete repriming, the importance of these channels under physiological conditions is unclear.

To the extent that these channels may contribute to pacemaker potentials (Llinás & Yarom, 1981; Tsien *et al.* 1988), factors which can alter membrane potential may change the electrical excitability of these cells over a fairly large voltage range. In fact, the requirement for such negative repriming potentials may indicate that T-type channels may play no significant role in the electrical properties of these cells or that these channels are regulated in some heretofore unknown way that alters T-channel voltage-dependence.

Effect of dihydropyridines

Nimodipine caused a reduction in almost half of the whole-cell current at a holding potential of -50 mV indicating that there was a significant L-channel population in these neurones. Nimodipine is currently used in stroke management. It has also entered the clinical testing phase as a treatment of Alzheimer's disease. In animal studies, nimodipine injections increased the cognitive abilities of aged rabbits (Deyo, Straube & Disterhoft, 1989). Our experiments on cells from the CA3 region showed that the effect of nimodipine was dependent on holding potential. If neurones were to have their resting potential decrease for whatever reason, L-type Ca^{2+} current would be preferentially blocked by nimodipine. On the other hand, well-polarized cells might have their Ca^{2+} current enhanced. It is difficult to predict exactly what the effects the dihydropyridine antagonists may have on neuronal excitability as there are so many Ca^{2+}-dependent conductances. For instance, if nimodipine were to work as an antagonist, its effect may actually increase excitability by decreasing a Ca^{2+}-dependent K^+ current. Of course, other Ca^{2+}-dependent processes may be inhibited as well. If nimodipine were to work as an agonist, it might increase $[Ca^{2+}]_i$ which may affect Ca^{2+}-dependent mechanisms such as neurotransmitter release and second messenger activity. A parallel between *in vitro* and *in vivo* cellular responses to nimodipine cannot yet be drawn. The large agonist effects described in this paper, to our knowledge, have not been reported previously. This may indicate that hippocampal L-type Ca^{2+} channels are somewhat different than those found elsewhere.

High-threshold current component insensitive to nimodipine and ω-CgTx

A component of the high-threshold current elicited at a test potential of −10 mV was still present after exposure to both nimodipine and ω-CgTx. The identity of this current remains unknown at present. A number of possible explanations exist. First, the remaining current may be a fraction of N- or L-type current that is not completely blocked by the two drugs because of an inadequate blocking dose. However, altering doses of nimodipine by a factor of four (1–4 μM) and ω-CgTx by a factor of three (1–3 μM) did not alter the magnitude of blockade suggesting that a maximal effect on Ca^{2+} currents had been achieved. Because of nimodipine's lipophilic nature with a partition coefficient, $K_p \approx 6000$ (Mason, Rhodes & Herbette, 1990), there is concern that further increases in the concentration of this drug may yield changes in the current independent of its normal action on the channel. A second explanation is that either or both of these drugs are only partial antagonists for their respective channels. If this explanation were valid then one or both of the two channels possess markedly different pharmacological sensitivities than N- and L-channels previously described. In cells in which the L-type channel predominates such as in skeletal or cardiac muscle (Hess *et al.* 1984; Almers & McCleskey, 1984), a similar protocol to the one we utilized employing dihydropyridine antagonists was effective in producing nearly total block. While it has been reported that ω-CgTx fully blocks N-type current in DRG neurones (McCleskey *et al.* 1987), another study found that a significant fraction of the same neurones tested possessed a small percentage of high-threshold current that was insensitive to ω-CgTx at concentrations up to 5 μM (Aosaki & Kasai, 1989). In Purkinje neurones, Ca^{2+} currents were insensitive to either dihydropyridine antagonists or ω-CgTx (Sah, Regan & Bean, 1989). In the CA3 neurones a variable fraction of the total current was blocked by both drugs depending upon the particular cell suggesting that a Ca^{2+} channel insensitive to both nimodipine and ω-CgTx was present in different proportions in these cells rather than incomplete block by these inhibitors. Hence, a reasonable alternative is that the current may represent a fourth type of channel; possibly the P channel described in cerebellar Purkinje cells (Llinás, Sugimori & Cherksey, 1989) or the NEN channel expressed in *Xenopus* oocytes (Lester, Snutch, Leonard, Nargeot, Curtis & Davidson, 1989). Nevertheless, because the channel was not observed as a distinctive unitary conductance in single-channel experiments, this channel if it exists is located away from the soma or it may have a conductance similar to T-, N-, or L-type Ca^{2+} channels. Historically, differential sensitivity to pharmacological agents has been the first evidence of channel or receptor diversity. It seems clear that the complex issue of Ca^{2+} channel diversity in hippocampal neurones has not yet been fully elucidated.

We are grateful to Dr Dorothy Hanck for comments on the manuscript. This work was supported by NIH, Klingenstein Fund, McKnight Foundation, Whitaker Foundation, Sloan Foundation, Miles Inc. and Sprague Foundation awards to A.P.F. and NIH award to D.J.M.

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