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SINGLE CALCIUM CHANNELS IN RAT AND GUINEA-PIG HIPPOCAMPAL NEURONS

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SUMMARY

1. Calcium (Ca^{2+}) channels were studied under whole-cell clamp and in cell-attached patch recordings from acutely isolated and tissue-cultured rat hippocampal neurons. Under whole-cell voltage clamp of tissue-cultured neurons the current–voltage plot for Ca^{2+} channel current was biphasic, with a 'hump' on the descending phase of the plot when the cell was held at −80 mV and stepped to various command potentials. When determined from a holding potential of −40 mV the plot was no longer biphasic.

2. In cell-attached patch experiments extracellular isotonic potassium gluconate was used to zero the cell membrane potential. When the patch electrode solution contained monovalent cations (150 mM-Li^+ in most experiments) and no Ca^{2+}, a small channel (~ 13 pA) could be clearly distinguished in tissue-cultured neurons from a large dihydropyridine-sensitive channel (~ 36 pS). With Ba^{2+} as the charge carrier it was more difficult to resolve small channel openings. The small channel was activated during voltage steps from negative holding potentials (−80 to −100 mV) over a range of potentials (−70 mV and less negative). The channels inactivated rapidly (time constants of 20–40 ms) during the voltage step. Steady-state inactivation and activation functions were well fitted by single Boltzmann equations of the form $y = 1/[1+\exp((V-V_{0.5})/k)]$ and $y = 1/[1+\exp((V_{0.5}-V)/k)]$, where $V_{0.5} = -82.9 \pm 2.4$ and -55.2 ± 1.5 mV and $k = 4.5 \pm 0.6$ and 4.9 ± 0.6 mV, respectively. These small channels were not found in acutely isolated adult cells.

3. Ensemble averages of small-channel activity in numerous sweeps were very similar in time course to the T currents recorded in the whole-cell mode. Often small channels occurred in clusters, with many channels in a single patch. In these multichannel patches the voltage dependence of the kinetics of the channel was clearly revealed.

4. Small channels were insensitive to the dihydropyridine nifedipine (20 μM) and to TTX (1–5 μM), but the Li^+ current through this channel was readily blocked by including 2 mM-Ca^{2+} in the recording pipette. Small-channel activity persisted for minutes in off-cell patches, and in cell-attached patches was not affected by phorbol esters.

5. The large channel was studied with electrodes filled with 110 mM-Ca^{2+} or Ba^{2+} (single-channel conductance ~ 24 pS in Ba^{2+}). Activity of this channel was

dramatically increased by the dihydropyridine Bay K 8644 and suppressed by nifedipine. Bay K 8644 was used in most experiments. Prolonged channel openings often lasted beyond the end of the activation voltage step in the presence of Bay K 8644. Averages of many sweeps containing large single Ca^{2+} channels revealed a slowly inactivating inward current. Low concentrations of cadmium caused a flickery block of the channel. Activation of the large channels was strongly voltage dependent.

6. Inactivation of the great majority ($\sim 95\%$) of large channels was only weakly voltage dependent. Channel activity during an activating voltage step was reduced only about 30% when the channels were held at -40 mV as compared to activity elicited from a holding potential of -80 mV. Inactivation of a few large channels was strongly voltage dependent. Activity of these channels decreased by more than 80% when they were held for several seconds at -40 mV; repolarizing the patches to -80 mV quickly removed inactivation of the channels.

7. No unequivocal evidence was found for an intermediate-sized rapidly inactivating 'N-type' channel in either acutely isolated or tissue-cultured neurons. Single channels other than the T and L channels were observed, however. These channels (conductances of ~ 9 and ~ 14 pS, assuming selectivity to Ba^{2+} equal to that of the L channel) had long openings and did not inactivate even when held at -50 mV. They were not studied in detail.

8. In the absence of external Ca^{2+}, monovalent cations carried current through the large Ca^{2+} channels. Sodium (Na^+; 150 mM) current in Ca^{2+} channels was flickery, as though subject to rapid transient blocking events. However, Li^+ (150 mM) yielded well-resolved channel openings in tissue-cultured cells.

9. Li^+ current through Ca^{2+} channels in acutely isolated cells was very noisy, and clean single-channel openings could not be distinguished during single sweeps, although dihydropyridine-sensitive Ca^{2+} channel current was clearly identified in ensemble averages. The difference in Li^+ current through Ca^{2+} channels between tissue-cultured and acutely isolated neurons was attributed to the enzyme used during the acute isolation procedure. No difference in channel properties between tissue-cultured and acutely isolated cells was observed with divalent cation charge carriers.

10. In the majority of cells tested, the phorbol ester phorbol-12,13-dibutyrate (PDBu) caused a dramatic and persistent increase in large Ca^{2+} channel activity. The kinase inhibitor 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7) blocked the effects of PDBu. The inactive phorbol ester 4α-PDBu had no effect. In several cases PDBu depressed channel activity by causing an apparent shift in the hyperpolarizing direction of the voltage dependence of channel inactivation. In the remaining cells PDBu had no effect.

11. We conclude that the small Ca^{2+} channel recorded from tissue-cultured hippocampal neurons is fundamentally the same as the T-, or low-voltage-activated, Ca^{2+} channel recorded in peripheral neurons. Its voltage dependence, kinetics and pharmacological properties indicate that this channel can account for the T-type Ca^{2+} current in these cells. The high-voltage-activated, L-type Ca^{2+} channel in hippocampal neurons has properties very much like those of the L channel in peripheral neurons. This channel appears to be regulated by protein kinase C, but

only some of the effects of protein kinase C activators were consistent with predictions based on earlier whole-cell voltage-clamp experiments.

INTRODUCTION

Multiple components of voltage-dependent Ca^{2+} current have been found in many cell types (Carbone & Lux, 1984; Armstrong & Matteson, 1985; Fox, Nowycky & Tsien, 1987*a*; Kostyuk, Shuba & Savchenko, 1988*a*; Kostyuk, Shuba, Savchenko & Teslenko, 1988*b*; Plummer, Logothetis & Hess, 1989), including central neurons (Yaari, Hamon & Lux, 1987; Akaike, Kostyuk & Osipchuk, 1989; Coulter, Huguenard & Prince, 1989; Crunelli, Lightowler & Pollard, 1989). While it is often assumed that the various Ca^{2+} current components in brain cells are due to the action of distinct Ca^{2+} channels, as is the case in other cells, little direct experimental evidence has been offered to support this assumption, and there are difficulties associated with identifying channel types from whole-cell currents (Thompson & Coombs, 1988). The issue is an important one because of the key roles played by voltage-dependent Ca^{2+} channels in numerous neuronal processes (Miller, 1987) and the likelihood that selective regulation of Ca^{2+} channel classes constitutes a significant cellular control mechanism.

We have begun to investigate the regulation of single Ca^{2+} channels by protein kinase C in hippocampal neurons. We focused on two well-defined channel types. In whole-cell voltage-clamp recordings from tissue-cultured hippocampal neurons a small-amplitude transient current can be elicited by moderately depolarizing voltage steps from strongly hyperpolarized membrane potentials (Yaari *et al.* 1987; Doerner, Pitler & Alger, 1988*a*, Meyers & Barker, 1989). This current, called 'low-voltage-activated' (Carbone & Lux, 1987) or 'T current' (Fox *et al.* 1987*a*), is carried by a distinct class of Ca^{2+} channels in peripheral neurons. Principal defining characteristics of T channels are their voltage sensitivity, their rapid inactivation after opening, relatively low conductance and, usually, their dihydropyridine insensitivity (but see, e.g. Akaike *et al.* 1989). Our first major goal was to determine if there is a T-type Ca^{2+} channel in these cells, if so how its properties compare to those in other cells, and if it is regulated by protein kinase C.

High-voltage-activated current is mediated by one or more channel types. One type is markedly influenced by both agonist and antagonist dihydropyridines and has a comparatively large, single-channel conductance (Fox, Nowycky & Tsien, 1987*b*; Miller, 1987). The channel, called the 'high-voltage-activated' or 'L' channel, is notoriously metabolically sensitive (Armstrong & Eckert, 1987; Yaari *et al.* 1987; Kostyuk, Akaike, Osipchuk, Savchenko & Shuba, 1989) and is subject to regulation of various kinds (Sanguinetti & Kass, 1984; Dolphin, Forda & Scott, 1986; Macdonald, Skerritt & Werz, 1986; Macdonald & Werz, 1986; Dolphin & Scott, 1987; Sperelakis, 1988; Ewald, Pang, Sternweis & Miller, 1989). Voltage-clamp recordings in hippocampal neurons have identified a high-threshold, slowly inactivating Ca^{2+} current (Johnston, Hablitz & Wilson, 1980; Brown & Griffith, 1983; Gray & Johnston, 1987; Kay & Wong, 1987; Yaari *et al.* 1987) that is sensitive to dihydropyridines (Docherty & Brown, 1986; Doerner *et al.* 1988*a*). Protein kinase C is a regulator of Ca^{2+} channels, depressing whole-cell Ca^{2+} current in chick sensory

neurons (Rane & Dunlap, 1986) and enhancing it in *Aplysia* (DeRiemer, Strong, Albert, Greengard & Kaczmarek, 1985) by facilitating the appearance of a novel Ca^{2+} channel (Strong, Fox, Tsien & Kaczmarek, 1987). Protein kinase C is present in large quantities in mammalian hippocampus (Nagle & Blumberg, 1983; Baraban, Snyder & Alger, 1985; Nishizuka, 1988), and protein kinase C activation reduces whole-cell Ca^{2+} current there (Doerner *et al.* 1988*a*; Doerner, Abdel-Latif, Rogers & Alger, 1990). At least part of the hippocampal high-voltage-activated current reduced by protein kinase C appears to be mediated by the L channel, although there is no single-channel evidence that the L channel is involved. Another class of Ca^{2+} channels activated by strong depolarizing steps is the N channel (Nowycky, Fox & Tsien, 1985*b*; Fox *et al.* 1987*a*,*b*). This channel is insensitive to dihydropyridines and has a conductance intermediate between the L- and T-channel conductances. Originally described as rapidly inactivating (Fox *et al.* 1987*b*), 'N-type' channels now appear to be classifiable as slowly, as well as rapidly, inactivating (Hirning, Fox, McCleskey, Olivera, Thayer, Miller & Tsien, 1988; Aosaki & Kasai, 1989; Lipscombe, Kongsamut & Tsien, 1989; Plummer *et al.* 1989). Previous whole-cell voltage-clamp experiments suggested that high-voltage-activated, rapidly inactivating N channels may be present in hippocampal neurons and may be regulated by protein kinase C (Doerner *et al.* 1988*a*, 1990). Our second major goal was to address the questions: does the L channel contribute to high-voltage-activated current in hippocampal pyramidal neurons, and is it subject to regulation by protein kinase C, as expected on the basis of previous whole-cell voltage-clamp studies? Answers to these questions will contribute to our understanding of the function of these channels in an important central nervous system structure. Preliminary reports of these findings have appeared as abstracts (O'Dell & Alger, 1989, 1990).

METHODS

Hippocampal neurons were studied in two *in vitro* preparations. Animals were anaesthetized with either CO_2 (cell culture) or ether (acute dissociation) prior to surgery. For most of the experiments fetal rat neurons (18–19 days gestation), grown in dissociated tissue culture according to conventional methods (Segal, 1983), were used. Cells were plated on polylysine-coated cover-slips (usually two to three cover-slips per dish), and prior to an experiment a cover-slip was placed in a second culture dish containing extracellular saline. Cells were studied between 3 days and 5 weeks after plating. In some experiments acutely isolated pyramidal neurons were prepared from guinea-pig hippocampus according to the methods of Kay & Wong (1986). Following dissection of one hippocampus from the brain, tissue chunks (1 mm^3) were prepared and placed into a PIPES-buffered saline solution containing (in mM): 125 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 PIPES, and 1·8 mg/ml diphenyl carbamyl chloride (DPCC)-trypsin (pH 7·4) where they were gently agitated for 2–3 h. The enzyme solution was then changed for a HEPES-buffered saline (same composition as above but with 10 mM-HEPES instead of PIPES) without enzyme. One or two chunks were removed, washed in the experimental saline and triturated through fire-polished pipettes into a polylysine-coated tissue-culture dish. Cells were studied from 10 to 90 min after dissociation. All experiments were done at room temperature (20–22 °C).

The whole-cell voltage-clamp and cell-attached patch configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) were obtained using an Axopatch 1C (Axon Instruments). Data were filtered at 1 kHz and sampled at 5 kHz (8-pole Bessel filter, 3 dB down). For whole-cell voltage-clamp studies the standard extracellular solution contained (in mM): 124 NaCl, 5 CsCl, 10 $CaCl_2$, 2 $MgCl_2$, 10 TEA, 10 glucose, 10 HEPES (pH 7·4) and 0·0025 TTX. Low-resistance patch electrodes (2–5 MΩ) were filled with (in mM): 145 $CsCH_3SO_3$, 10 HEPES, 5 MgATP, 11 BAPTA (bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid), 1 $CaCl_2$, 10 TEA, and 0·1 leupeptin (pH 7·4). Both series resistance (approximately 80%) and capacitance

compensation were used. Voltage steps were applied at 0·33 or 0·25 Hz. Whole-cell voltage-clamped currents were not corrected for leak currents; these experiments were performed on thirty cells 3–10 days old.

For single-channel experiments the cells were bathed in an isotonic potassium salt saline containing (in mM): 140 potassium gluconate, 15 NaCl, 3 $MgCl_2$, 10 glucose, 10 EGTA, and 10 HEPES (pH 7·4) to zero the cell membrane potential. Records obtained from cell-attached patches were corrected for leak and capacity transients by subtracting a record generated by averaging records where voltage steps elicited no channel openings. In patches where channel activity was particularly intense we were unable to obtain a sufficient number of 'null' sweeps to generate an average record suitable for subtraction. In these cases hyperpolarizing voltage steps, equal in magnitude to the depolarizing steps used to elicit channel openings, were applied and averaged. This average was then added to the records containing channel openings. All values are given as means ± S.E.M. Cell-attached patch recordings were made from over 360 cells.

To record the small, T-type channels we used patch electrodes filled with a solution containing (in mM): 150 LiCl, 10 EGTA, 10 TEA, 0·0025 TTX and 10 HEPES (pH 7·4), unless noted otherwise. T-channel activity in cell-attached patches was quantified by measuring the peak current in ensemble records generated by averaging numerous records from the voltage steps used to activate the channels.

To study the large channel we used the same methods except that 110 mM-Ba^{2+} (and no EGTA) was usually present in the patch pipette, although we have also recorded this channel with 110 mM-Ca^{2+}. Channel activity was quantified by calculating 'NP_o' values, where N is the number of channels in the patch and P_o is the percentage of time the channels spent in the open state. In this analysis, idealized current records were generated from leak- and capacity-corrected traces using a criterion of a 50% excursion between fully open and fully closed states to determine the occurrence of an opening or closing event. The amount of time the idealized record spent at levels corresponding to amplitudes of one to four channels was summed, and this sum was then divided by the total time of the voltage step to yield NP_o. We did not attempt to determine the number of channels in each patch, although there seemed to be only one to three in most cases. Channel open times were determined using pClamp 5·5 software (Axon Instruments) to analyse and plot the data. Boltzmann parameters were derived from computer-generated fits of the Boltzmann equation to the data. A least-squares minimization procedure was employed, and there were no constrained parameters.

Drugs were added directly to the static recording chamber in known concentration and volume. Unless noted otherwise, all drugs were obtained from Sigma Chemical Corp. (St Louis, MO, USA). Leupeptin was obtained from Boehringer–Mannheim (Indianapolis, IN, USA). Phorbol-12,13-dibutyrate (PDBu, L.C. Services, Woburn, MA, USA, and Sigma Chemical Corp.), Bay K 8644 and nimodipine (gifts from Dr A. Scriabine, Miles Laboratories), and nifedipine and veratrine were dissolved in dimethyl sulphoxide (DMSO), divided into aliquots and stored frozen until use. DMSO in the concentrations used here (< 0·1%) had no effect on cell properties.

RESULTS

As has been shown previously (Yaari *et al.* 1987; Doerner *et al.* 1988*a*; Meyers & Barker, 1989), Ca^{2+} currents recorded in voltage-clamped cultured hippocampal neurons have two distinct components. This is illustrated in Fig. 1*A*, which depicts Ca^{2+} current elicited in a tissue-cultured hippocampal neuron by a 170 ms voltage step from −80 to −30 mV. The current inactivated rapidly. When the cell was held at −40 mV and then stepped to −30 mV there was no evidence of inward current, although steps from −40 to +20 mV elicited an inward current that did not inactivate (Fig. 1*B*). Young (3–5 days) cells were chosen for this experiment to maximize space-clamp control; the currents are often small in young cells. Complete I–V plots from holding potentials (V_H) of −80 mV (○) and from −40 mV (●) are shown for a different cell in Fig. 1*C*. Notice the biphasic nature of the plot when voltage steps were given from −80 mV. With a holding potential of −40 mV the I–V relationship was simpler and there was no sign of a 'hump' on the descending phase

of the plot. These and other characteristics suggest the contribution of multiple Ca^{2+} channel types to the *I–V* plot constructed from −80 mV. In particular, the appearance of a distinct component of current elicited from −80 mV but not from −40 mV implies the presence of the T-type Ca^{2+} channel (Carbone & Lux, 1987; Yaari *et al.* 1987).

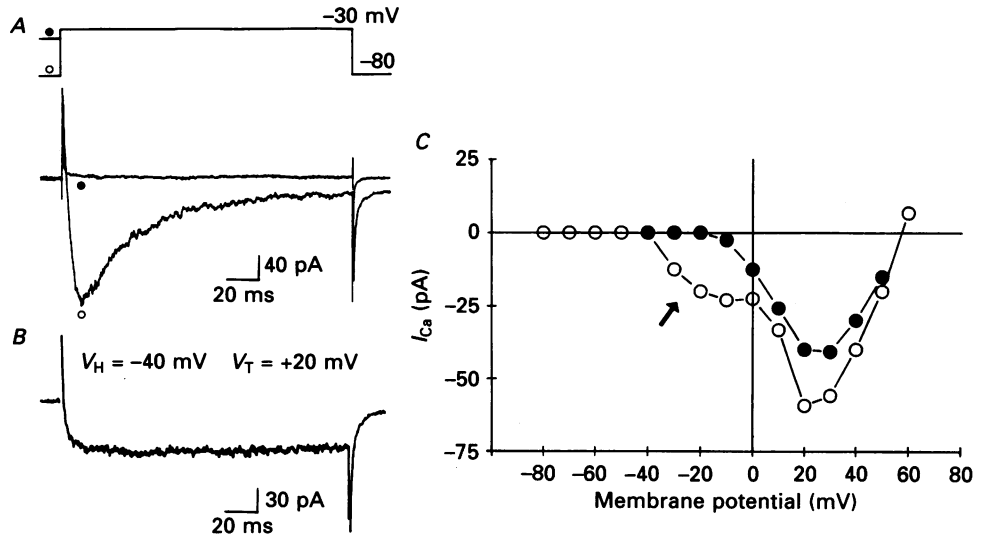

Fig. 1. Whole-cell voltage-clamp recordings of inward Ca^{2+} current in a tissue-cultured hippocampal neuron. *A*, transient, low-voltage-activated current was elicited by stepping the membrane potential to −30 mV from a holding potential of −80 mV (○). This current was totally inactivated when the holding potential was shifted to −40 mV (●). *B*, larger depolarizing voltage steps from V_H of −40 mV elicited a sustained, high-threshold current. V_T, test potential. *C*, *I–V* curve for the peak currents was elicited from V_H of −80 mV (○) or from −40 mV (●). Different cell than in *A* and *B*. Notice the hump on the descending phase of the *I–V* plot obtained with steps from −80 mV (arrow), compared to the smooth curve obtained with steps from −40 mV. Records were obtained with 10 mM-$CaCl_2$ in the bathing solution.

To test the hypothesis that T-type channels are present on these cells we examined Ca^{2+} channel activity in cell-attached patch recordings. With Ba^{2+} (110 mM) as the charge carrier we observed rapid, small-channel openings that tended to cluster near the beginning of the voltage step and larger openings that occurred throughout the voltage step. The large channels had a slope conductance of ~ 24 pS and were sensitive to dihydropyridines (see below), identifying them as L-type Ca^{2+} channels. The smaller-amplitude events (~ 8 pS) were difficult to resolve clearly. While the conductance and transient nature of the small channel are reminiscent of T-type Ca^{2+} channels in peripheral neurons (Carbone & Lux, 1987; Fox *et al.* 1987*a*; Kostyuk *et al.* 1988*a*), we were not able to obtain sufficient unambiguous data for more detailed analysis with Ca^{2+} or Ba^{2+} as the charge carrier.

Monovalent cation current through the small channel

In the absence of divalent cations the conductance of Ca^{2+} channels to monovalent cations can be substantial (Hess, Prod'hom & Pietrobon, 1989; Lux, Carbone &

Zucker, 1989). Hence, we removed all divalent cations from the bathing and pipette-filling solutions, added EGTA and substituted 150 mM-Na^+ or Li^+ for Ba^{2+}. With either Na^+ or Li^+ in the pipette, channel activity was very pronounced. However, with Na^+ the activity was exceedingly 'noisy' and single-channel openings were not

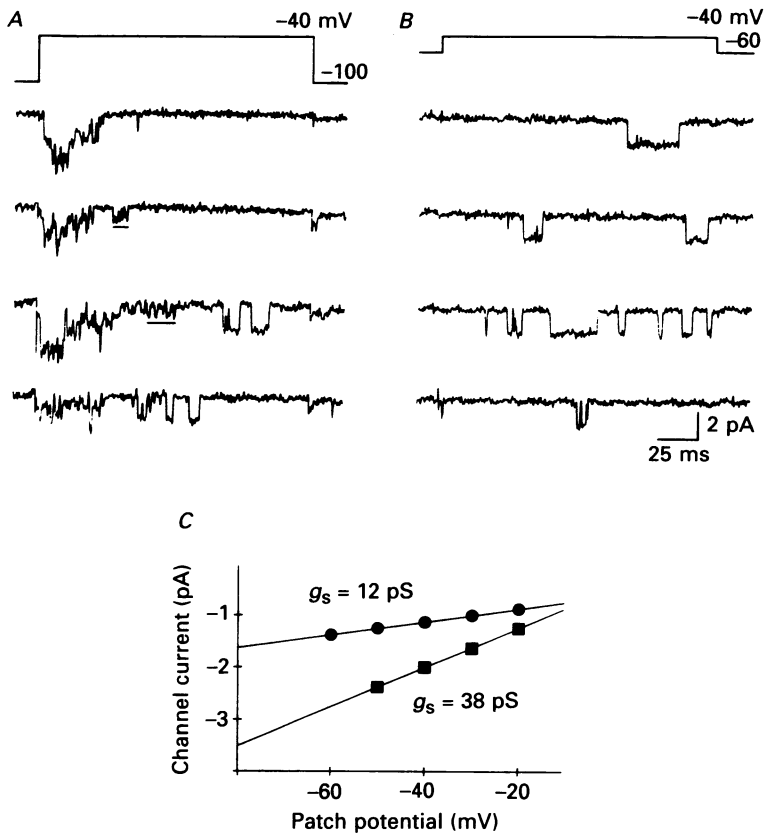

Fig. 2. Consecutive sweeps showing channel activity encountered with 150 mM-Li^+ and 10 mM-EGTA in the recording pipette. The voltage protocol is shown above the current traces in this and succeeding figures. *A*, two types of channel activity were elicited from V_H of −100 mV. *B*, large-channel activity was elicited when V_H was −60 mV; same patch as in *A*. The small, transient channel activity seen when V_H was −100 mV was absent when V_H was changed to −60 mV, while the larger channel continued to gate at this holding potential. Bay K 8644 (5 μM) was present throughout. *C*, single-channel *I–V* plots for this patch indicate two single-channel conductances (g_s) of 12 and 38 pS. The small-channel current amplitudes were measured from single events occurring after most of the transient activity had subsided and single-channel events were observable (e.g. at bars in *A*).

easily resolvable. The noisy aspect of the traces obtained with Na^+ was probably related to the rapid proton block of Ca^{2+} channels that occurs at pH $\leqslant$ 7·4 (Prod'hom, Pietrobon & Hess, 1987). With Li^+ as the charge carrier, however, clear single-channel activity was present at pH 7·4, and hence the remaining experiments on the small channel were performed with 150 mM-Li^+ in the recording pipette.

Two distinct channel types were also evident in patches where Li^+ was used as the

charge carrier. The traces in Fig. 2 were recorded in the presence of the dihydropyridine Ca^{2+} channel agonist Bay K 8644. When the membrane patch was stepped from −100 to −40 mV there was usually an initial flurry of small-channel activity that ceased before the end of the voltage pulse. In some cases (e.g. traces 3 and 4) a second channel with a larger conductance was also visible. After changing the holding potential of this patch to −60 mV, steps to −40 mV resulted in openings of the large channel only; the initial transient activity was no longer present. The absence of the small, transient openings and continued activation of the large openings from depolarized holding potentials would be expected if the small channels were T channels and the large ones the L channels. Plots of single-channel current *versus* several levels of membrane potential indicated two slope conductances of $13{\cdot}0 \pm 0{\cdot}77$ ($n = 6$) and $35{\cdot}8 \pm 2{\cdot}73$ pS ($n = 5$; e.g. Fig. 2*C*). Both of these conductance values are larger than predicted for the T and L channels with divalent cations as charge carriers, but are comparable to the values expected with monovalent charge carriers in the absence of Ca^{2+} (Lux *et al.* 1989). We therefore postulated that the transient, low-voltage-activated channel was the T channel.

Voltage dependence and kinetics of the small channel

The voltage- and time-dependent inactivation of the T-type Ca^{2+} channels was examined in more detail by eliciting channel activity at various voltages from different holding potentials. An example of this is shown in Fig. 3*A–C*, where channel activity elicited by voltage steps to −40 mV was examined as a function of holding potential. Robust channel activity could be elicited from a holding potential of −100 mV, while no openings were observed when the holding potential was −70 mV. Complete inactivation and activation curves are shown for the same patch in Fig. 3*D*. The plots of peak amplitudes of average current records generated by averaging many sweeps were fitted with Boltzmann equations of the form: $y = 1/[1+\exp((V-V_{0{\cdot}5})/k)]$ and $y = 1/[1+\exp((V_{0{\cdot}5}-V)/k)]$, for inactivation and activation data, respectively, where $V_{0{\cdot}5}$ is the voltage at which half of the maximum current is evoked and k is the slope factor describing the steepness of the relationship. The values were: inactivation, $V_{0{\cdot}5} = -82{\cdot}9 \pm 2{\cdot}4$ mV, $k = 4{\cdot}5 \pm 0{\cdot}6$ mV ($n = 4$); activation, $V_{0{\cdot}5} = -55{\cdot}2 \pm 1{\cdot}5$ mV, $k = 4{\cdot}9 \pm 0{\cdot}6$ mV ($n = 6$).

Channel activity also rapidly inactivated during the 170 ms voltage step. As shown in Fig. 3*E*, the inactivating phase of the ensemble currents could be well fitted by a single-exponential curve with, in this case, a time constant of 24 ms. The time course of inactivation was voltage dependent with time constants of $51{\cdot}4 \pm 6{\cdot}62$ ms ($n = 5$) at −60 mV, $33{\cdot}2 \pm 2{\cdot}51$ ms ($n = 8$) at −50 mV, $26{\cdot}4 \pm 1{\cdot}60$ ms ($n = 11$) at −40 mV and $19{\cdot}8 \pm 1{\cdot}07$ ms ($n = 5$) at −30 mV. The time constants for the inactivation of the channel activity in cell-attached patches were similar to those for the inactivation of macroscopic currents recorded using similar voltage protocols in whole-cell voltage-clamp recordings ($43{\cdot}2 \pm 5{\cdot}14$ ms ($n = 6$) at −60 mV, $23{\cdot}8 \pm 2{\cdot}28$ ms ($n = 5$) at −50 mV, $23{\cdot}4 \pm 1{\cdot}39$ ms ($n = 6$) at −40 mV and $22{\cdot}3 \pm 2{\cdot}16$ ms ($n = 6$) at −30 mV).

Frequently there were so many small channels in a single patch that significant 'ensemble' currents could be obtained with only a few test pulses. With these multichannel patches we were able to examine the voltage dependence of the kinetics of the channel by stepping to a number of test potentials. Examples of such data are

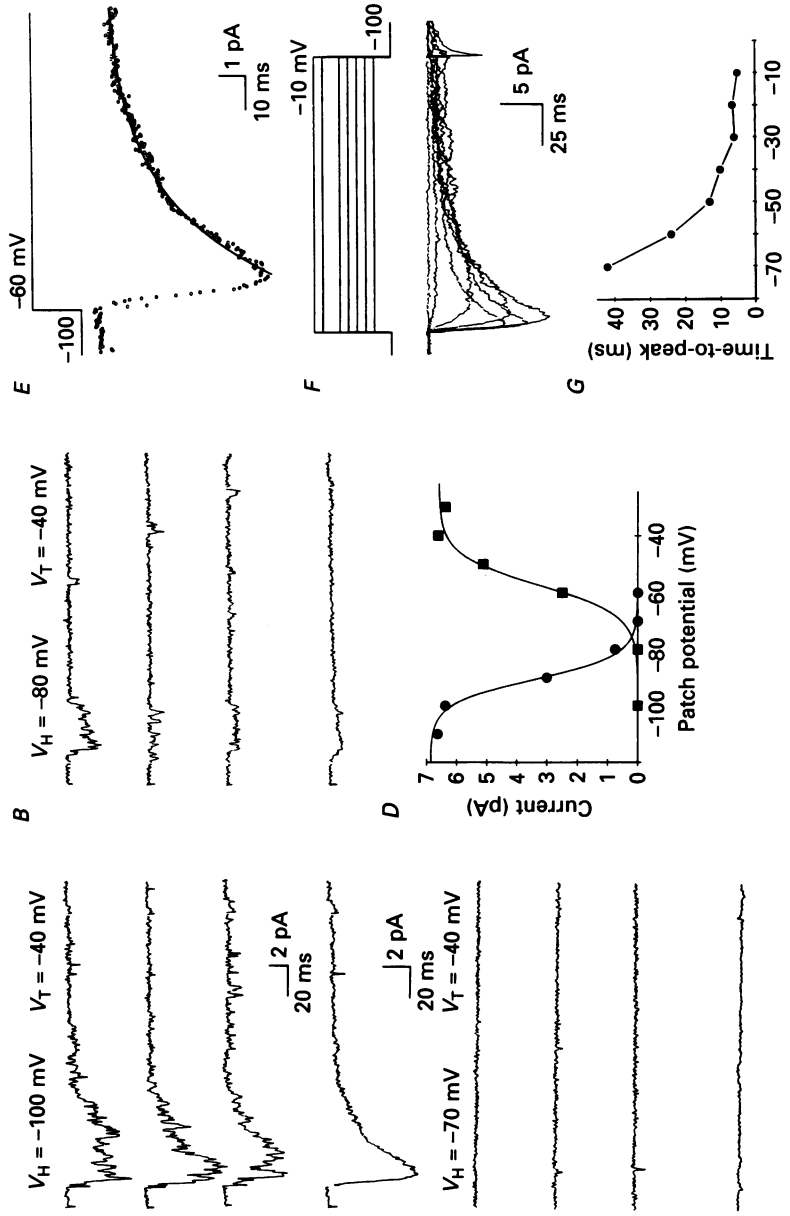

Fig. 3. Voltage dependence and kinetics of the small, transient channel. The first three traces in panels *A*, *B* and *C* are consecutive sweeps showing channel activity elicited by voltage steps to −40 mV from V_H of −100 mV (*A*), −80 mV (*B*) and −70 mV (*C*). The bottom trace in each column is an ensemble current generated by averaging ⩾ twenty sweeps. *D*, activation and inactivation curves from the patch in *A–C*. Steady-state inactivation curves were generated by holding the patch potential at different voltages and stepping to −40 mV. Steady-state activation curves were generated by holding the patch potential at −100 mV and stepping to various voltages. The peak current of ensemble traces generated by averaging at least twenty sweeps was normalized to the maximal current in each case. The continuous lines are single Boltzmann curves fitted to the data using a non-linear least-squares fit. The parameters of the fits were $V_{0.5}$ = −90·1 mV, k = 4·3 mV (inactivation) and $V_{0.5}$ = −57·2 mV, k = 5·3 mV (activation). *E*, inactivation of the low-voltage-activated current is a single-exponential process. The continuous line through the ensemble current is a single exponential with a time constant of 24 ms fitted to the data. *F*, voltage dependence of low-voltage-activated current in a multichannel patch. The patch was held at −100 mV and stepped to potentials from −80 to −10 mV. *G*, time-to-peak for each trace in *F* (above) plotted *versus* the activation step potential.

shown in Fig. 3*F* and *G*. Time-to-peak of the channel was clearly voltage dependent, decreasing with increasing activation steps from −80 to −10 mV.

Regulation of small-channel activity

T-type Ca^{2+} currents in whole-cell voltage-clamp experiments on cultured hippocampal neurons are insensitive to dihydropyridine Ca^{2+} channel agonists and

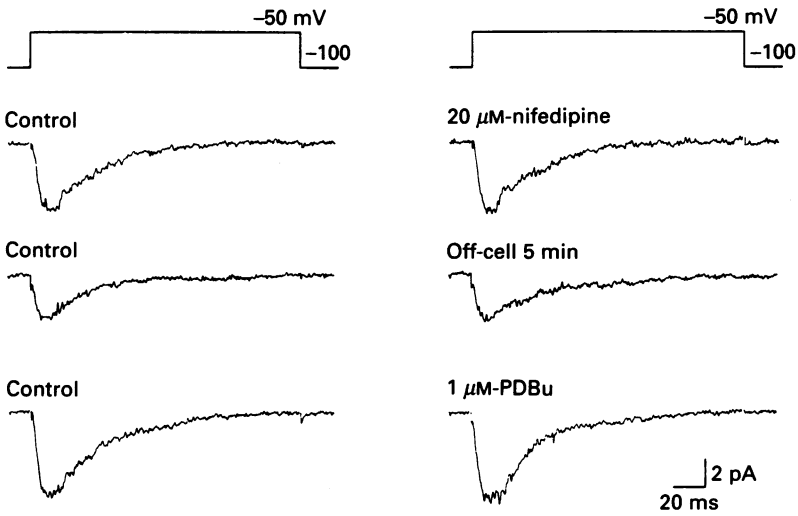

Fig. 4. The low-voltage-activated channel is insensitive to dihydropyridines, is metabolically stable and is unaffected by a phorbol ester. Ensemble averages were generated by averaging ⩾ twenty sweeps before (control) and 5 min after adding 20 μM-nifedipine (top), after removing the patch from the cell in the inside-out configuration (middle) or after adding 1 μM-PDBu to the bath. Experiments were done on different patches.

antagonists and are not sensitive to modulation by phorbol-ester-activated protein kinase C (Doerner *et al.* 1988*a*). As shown in Fig. 4, neither 20 μM-nifedipine ($n = 4$) nor 1 μM-PDBu ($n = 6$) affected T-type channels recorded in cell-attached patches. T channels are much less metabolically sensitive and less subject to second-messenger regulation than either N or L channels, as illustrated by their resistance to 'run-down' and greater persistence in inside-out patches (e.g. Carbone & Lux, 1987; Kostyuk *et al.* 1988*a*). Consistent with the identification of the small transient channel activity recorded in our patches as T channels, we found that small-channel activity could be elicited for up to 5 min (the longest time tested) after patch excision ($n = 3$, e.g. Fig. 4). This is quite different from the behaviour of high-voltage-activated Ca^{2+} channels, which rapidly (⩽ 1·0 min) cease to gate in excised patches (Armstrong & Eckert, 1987; Yatani *et al.* 1987; T. J. O'Dell & B. E. Alger, unpublished observations). Thus several properties of the small channels observed in these experiments resemble those of the T-type currents recorded under whole-cell voltage clamp in these cells.

Distinctions between the small channel and the Na^+ channel

The behaviour of the presumed T channel recorded using Li^+ in some ways resembles Na^+ channels, and Li^+ is permeant in the Na^+ channel. Nevertheless, several features distinguished the presumed T channel from Na^+ channels: (1) T channels were typically recorded in the presence of 2·5 μM-TTX and were insensitive to TTX up to 5 μM, which was the highest dose used and is more than sufficient to block neuronal Na^+ channels. (2) Monovalent cation current through Ca^{2+} channels is readily blocked by Ca^{2+} (Hess *et al.* 1989), and we found that no channel activity was observed in patches where the patch electrode solution contained Ca^{2+} and TTX. Although the number of small-channel-containing patches varied from culture to culture, in most of our cultures small-channel activity was present in a high percentage of our patches (approximately 90%). We used this to compare the occurrence of channels in patches obtained with electrodes filled with solutions that were either Ca^{2+} free or that contained physiological concentrations of Ca^{2+}. When the patch electrode solution contained 2·0 mM-$CaCl_2$ and 0 mM-EGTA, in addition to LiCl, no channel activity was observed in eighteen patches. In contrast, twenty-two out of twenty-four patches from the same cultures contained small channels when the pipette solution was Ca^{2+} free (10 mM-EGTA and no added divalent cations). (3) Finally, in the absence of TTX and presence of 2 mM-Ca^{2+}, a large, rapidly inactivating current carried by Li^+ could be recorded in cell-attached patches (data not shown). Inactivation of this current was markedly slowed by 0·003% veratrine, a mixture of plant alkaloids containing veratridine, a drug that slows Na^+ channel inactivation (Catterall, 1984). Veratrine did not affect the Li^+ current in TTX and 0 mM-external Ca^{2+}, i.e. T-channel current. Together these experiments indicate that the low-voltage-activated channel activity recorded with Li^+ represents the monovalent cation current through T-type Ca^{2+} channels and not Na^+ channels.

Although the T current is readily detectable in tissue-cultured neurons (Yaari *et al.* 1987; Doerner *et al.* 1988*a*), it has not commonly been found in adult neurons acutely isolated from guinea-pig hippocampus (Kay & Wong, 1987; Doerner *et al.* 1988*a*), but see Takahashi, Tateishi, Kaneda & Akaike (1989). We studied adult neurons to test the prediction that the T channels would be absent from these cells. In over fifty patches there was no unambiguous evidence of the highly voltage-dependent, rapidly inactivating T channels. As discussed below (Fig. 10), we did observe other single-channel conductances in the adult cells, however.

Properties of the typical large channels

In the absence of the Ca^{2+} channel agonist Bay K 8644 there is often only slight evidence of single high-voltage-activated Ca^{2+} channels in cell-attached patches with activation steps to -10 mV from a holding potential of -40 mV (Fig. 5*A*). However, as shown in Fig. 5*B*, adding Bay K 8644 (5 μM) to the bathing medium reliably induced robust, large, single-channel activity without apparent change in single-channel conductance.

The lowest trace in each column of Fig. 5 is an ensemble average of forty traces. The paucity of activity evident in the individual sweeps in control is reflected in the absence of averaged current. Bay K 8644 application resulted in a large, essentially

non-inactivating ensemble current during the activation pulse and a tail in the current after the membrane was returned to −40 mV. This tail current is explained by summation of single-channel openings that continued after the end of the pulse (e.g. traces 3–5 in Fig. 5*B*). The effects of Bay K 8644 strongly supported the

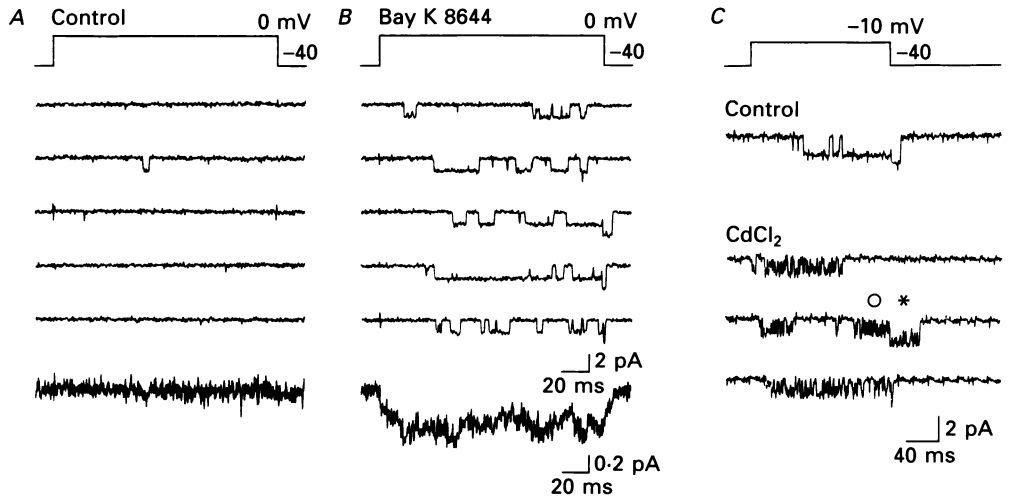

Fig. 5. Properties of a high-voltage-activated Ca^{2+} channel recorded in a cell-attached patch. *A*, five consecutive records collected in the absence of Bay K 8644. The patch potential was held at −40 mV and stepped to 0 mV. The last trace is an average ensemble current from forty records. *B*, five consecutive records collected 2 min after bath application of 5 μM-Bay K 8644. Note the increase in channel activity and the appearance of long-duration openings, some of which outlast the voltage step (traces 3–5). The last trace is an ensemble current from forty records. *C*, typical long openings in another patch. Three selected traces below were from a different patch where the pipette-filling solution contained 20 μM-Cd^{2+} in addition to 110 mM-Ba^{2+}. Note the fast 'flickery' block of the channel openings. Also, the closing flickers during the tail opening in the second trace in Cd^{2+} (*, channel open at −40 mV) were less complete than those interrupting the openings at −10 mV (○). All records are from acutely isolated neurons. Unless stated otherwise, in all subsequent records Bay K 8644 (5 μM) was present in the bathing solution.

identification of the large channel as the L channel (Nowycky, Fox & Tsien, 1985*a*; Miller, 1987). Unless stated otherwise, the remaining experiments on the large channel were done in Bay K 8644. Identical results were obtained from acutely isolated and tissue-cultured neurons, except in the case of monovalent cation current, as discussed below (Fig. 9). With this exception, all remarks pertain to channels from both preparations.

The large channel was blocked by divalent cations that block voltage-dependent Ca^{2+} channels, and, at low concentrations, cadmium ions (Cd^{2+}) produced a characteristic 'flickery block' (Lansman, Hess & Tsien, 1986). The traces in Fig. 5*C* illustrate the rapid opening and closing of the large channel produced when 20 μM-Cd^{2+} was included in the patch pipette.

Trace 2 in Fig. 5*C* also suggests another characteristic of Cd^{2+} block of L channels, namely its voltage dependence. The single-channel closings during activity that continued past the end of the

activation pulse (at −40 mV) were less complete than the closing flickers that interrupted channel openings at −10 mV, as though Cd^{2+} produced a more complete block of the channel at −10 mV (compare ○ and * in trace 2). Similar observations have been previously attributed to a voltage dependence of the Cd^{2+} block (Lansman *et al.* 1986; Swandulla & Armstrong, 1989).

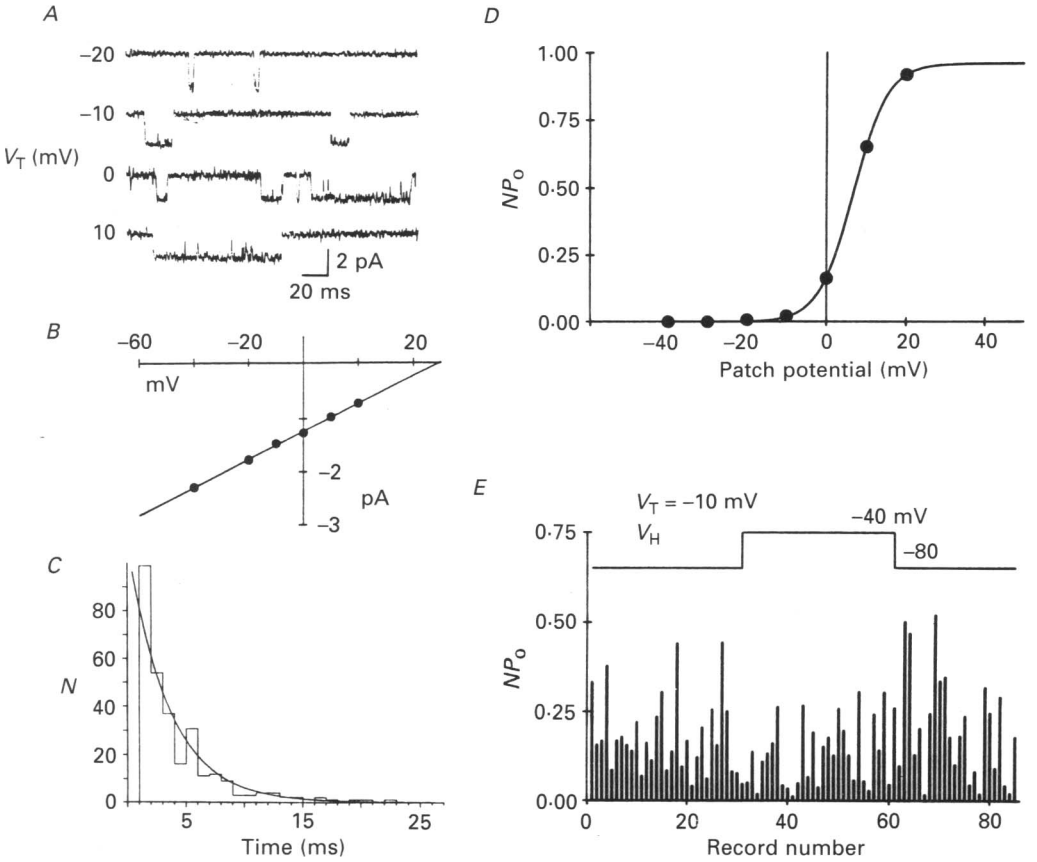

Fig. 6. Characteristics of the large Ca^{2+} channel. *A*, sample traces at different activation potentials; V_H was −40 mV throughout. *B*, single-channel current–voltage plot from a different patch than in *A*. *C*, open-time histogram for a large single channel; test potential was 0 mV. Total *N* was 379 openings. Continuous curve is a single exponential with a time constant of 3·5 ms. *D*, plot of large-channel activity in a single multichannel patch, calculated as mean NP_o from ~ forty individual sweeps at various activation potentials. The data were fitted by a Boltzmann equation (continuous line) by allowing both the slope and maximum of the function to be determined as free parameters (NP_o was not constrained to 1·0, for example); $V_{0.5} = +6{\cdot}8$ mV, $k = 4{\cdot}3$ mV. *E*, dependence of large-channel activity on holding potential. Channels were activated by voltage steps from −80 or −40 mV to −10 mV (V_T). The holding potential was changed to −40 mV for thirty pulses and then returned to −80 mV. The average NP_o values were 0·19 when V_H was −80 mV and 0·13 when V_H was −40 mV. Records were taken from different tissue-cultured neurons.

The slope conductance of the large channel was measured by stepping the patch membrane to a variety of potentials and plotting single-channel current *versus* step potential. Figure 6*A* shows sample traces and Fig. 6*B* a current–voltage plot from

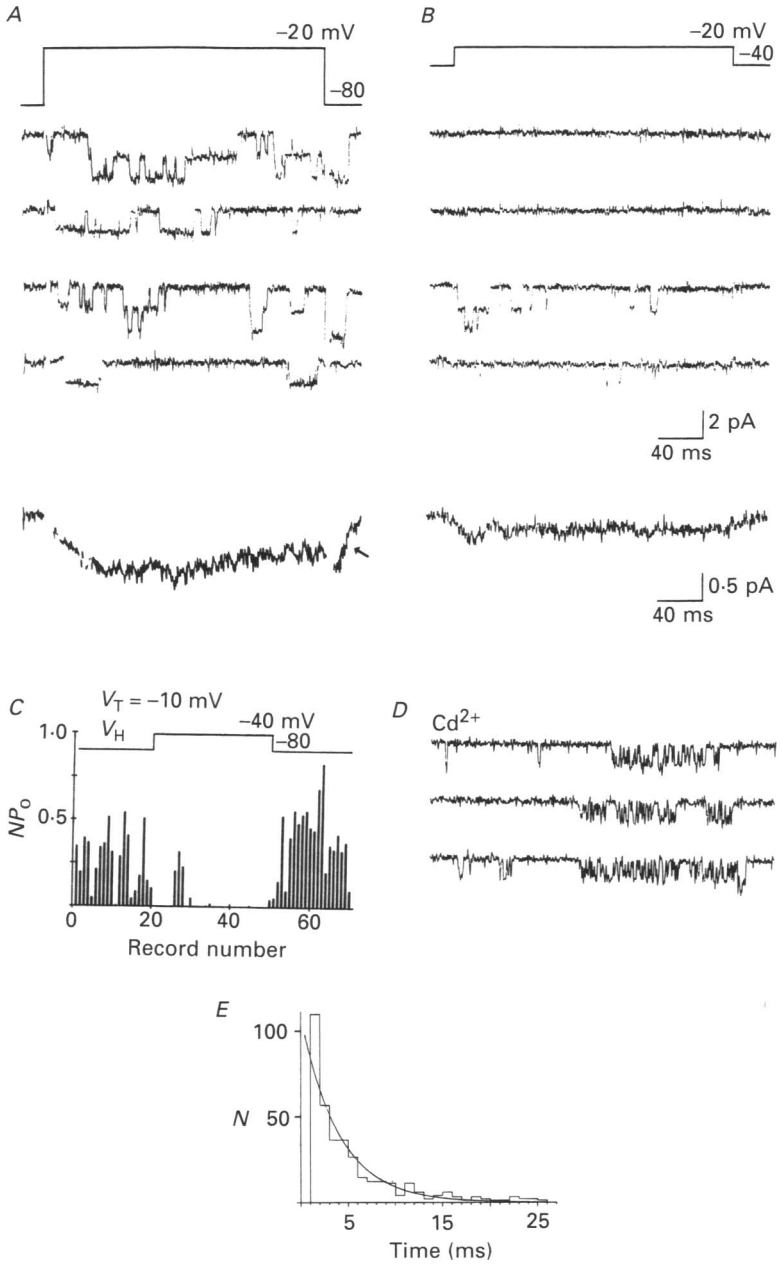

Fig. 7. Characteristics of a large Ca^{2+} channel with strong voltage dependence of inactivation. *A*, four consecutive traces of activity elicited by stepping from V_H of −80 to −20 mV. The lowest trace is the average of thirty traces. *B*, four consecutive traces of activity elicited from the same patch as in *A* from V_H of −40 mV. *C*, NP_o plot of channel activity from a different patch than shown in *A*. Note the decrease in channel activity when the patch potential was changed to −40 mV. The average NP_o values were 0·33 when the holding potential was −80 mV and 0·03 when the holding potential was −40 mV; $NP_{o,-40}/NP_{o,-80} = 0{\cdot}09$. *D*, effect of Cd^{2+} ions on another channel that showed a

a different patch. The single-channel conductance in this case was 25·3 pS, and the mean from twenty-two patches was 24·2 ± 2·9 pS (± S.E.M.). The mean open time of the channel was assessed by plotting the histograms of open times. The distributions were well described by a single exponential with a time constant of about 4 ms (e.g. Fig. 6*C*). When plots of NP_0 (see Methods) *versus* command step potential were made, the voltage dependence of L-channel activation became evident (Fig. 6*D*). To compare hippocampal L channels with others, we fitted L-channel activation data from several patches with Boltzmann equations. Because we could not determine the exact number of channels in a patch, we did not constrain the computer-generated fits; both the slope and maximum of the function were derived by the program. Nevertheless, the data were consistent and in good agreement with those of others. An example of this analysis is shown in Fig. 6*D* (continuous line). The Boltzmann parameters for a group of patches were: $V_{0.5} = 9{\cdot}8 \pm 1{\cdot}7$ mV and $k = 7{\cdot}6 \pm 1{\cdot}1$ mV ($n = 4$).

Inactivation of the large channel was not very voltage dependent in most cases. In these cells it mattered little whether the membrane was held at −80 or −40 mV for many seconds before being briefly stepped to −10 mV to activate the channel. Figure 6*E* illustrates this for a typical patch that was alternately held at −80 and −40 mV while being stepped from either potential to −10 mV. It is clear from the NP_0 plot that holding at −40 mV caused only a moderate reduction in activity. The ratio of the average NP_0 at −40 mV to NP_0 at −80 mV was 0·68 in the example shown. For five patches the ratio was 0·72 ± 0·12.

A large, inactivating channel

The voltage dependence of the patch shown in Fig. 6*E* is typical of about 95% of our patches. The remaining few per cent gave a rather different picture. As shown in Fig. 7, large channels were occasionally found that were much more voltage sensitive than the majority. In these cases, holding the patch at −40 mV and stepping to a more depolarized potential for many seconds elicited much less channel activity than when the patch was held at −80 mV. This is also evident in the ensemble traces shown at the bottom of the columns in Fig. 7*A* and *B*. Other characteristics of these channels are shown in Fig. 7*C–E*. As indicated by the NP_0 plot (Fig. 7*C*), this patch was almost completely inactivated after changing the holding potential (V_H) from −80 to −40 mV while stepping at intervals to −10 mV. Activity was quickly restored upon returning to −80 mV. The unitary conductance was the same whether channel activation was elicited from −80 or from −40 mV. The ratio of NP_0 at −40 mV to NP_0 at −80 mV was 0·09 in the example and 0·17 ± 0·05 for the group ($n = 6$). This channel had a conductance of 22 pS and was blocked in a flickery way by 20 μM-Cd^{2+} (Fig. 7*D*). An example of this type of channel had a mean open time of 4 ms (Fig. 7*E*).

As noted, this channel was rare, and we were not able to test its dihydropyridine sensitivity rigorously; nevertheless we never observed it in the absence of

high sensitivity to holding potential. Compare with Fig. 1*C*. *E*, open-time histogram for a large voltage-dependent channel; test potential was 0 mV. Total N was 429 openings. Continuous curve is a single exponential with a time constant of 4·0 ms. Different cell than that in *A–D*.

Bay K 8644, and the durations of single openings were as prolonged as those of the typical L channel. Prolonged 'tail openings' in Bay K 8644 (e.g. traces 1 and 3 in Fig. 7*A*) explain the tail current evident in the ensemble average.

Monovalent cation current through large channels in tissue-cultured cells

As noted above, in the absence of external divalent cations, Ca^{2+} channels conduct monovalent cations quite well (Hess *et al.* 1989; Lux *et al.* 1989). To investigate this

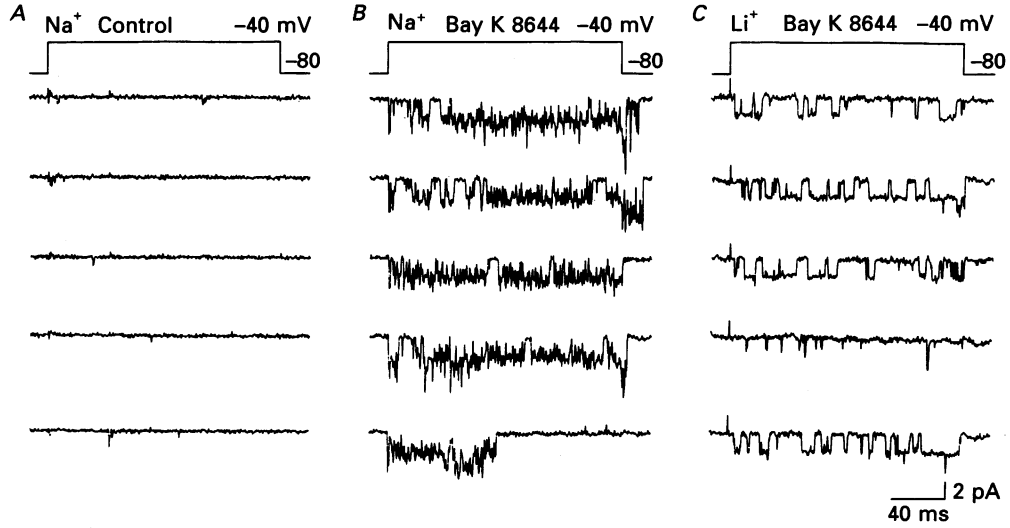

Fig. 8. Monovalent cation current through dihydropyridine-sensitive Ca^{2+} channels in tissue-cultured hippocampal neurons. *A*, control records in the absence of Bay K 8644; 150 mM-NaCl, 2·5 μM-TTX, 10 mM-EGTA and 10 mM-HEPES, pH 7·4, in the recording pipette. *B*, same patch as in *A* after adding 5 μM-Bay K 8644 to the bath. *C*, another patch in the presence of Bay K 8644 recorded with 150 mM-Li^+ in place of Na^+ in the pipette.

property of the large channels in hippocampal neurons we substituted 150 mM-Na^+, or Li^+, and 10 mM-EGTA for divalent cations in the pipette. Under these conditions, channels appeared to gate at the normal holding potential of −50 mV, and it was necessary to hold the patch potential at −80 mV to prevent the activity. The vigorous activation of the channels at −50 mV, where these channels are ordinarily not active, apparently results from a shift in the voltage dependence of activation of the channels. This shift is probably due to the reduction of divalent screening of membrane potential caused by the reduced $[Ca^{2+}]_o$. In the absence of Bay K 8644 few channel openings could be resolved (Fig. 8*A*), but after adding 5 μM-Bay K 8644 to the bathing solution vigorous channel activity was readily apparent (Fig. 8*B*). However, as shown in Fig. 8*B*, with Na^+ as the charge carrier it was often difficult to resolve single-channel openings clearly. Rather the openings appeared extremely noisy, as though subject to rapid blocking events. On the other hand, openings with Li^+ as the charge carrier were discrete and well resolved (Fig. 8*C*). The flickery block of the single-channel current carried by Na^+ may be due to the rapid proton block of the channel that occurs with Na^+ at pH 7·4 (Hess *et al.* 1989).

Monovalent cation current through large channels in acutely isolated cells

The traces shown in Fig. 8 were obtained from a tissue-cultured neuron. While the large Ca^{2+} channels appeared virtually identical in tissue-cultured and acutely isolated cells with Ba^{2+} or Ca^{2+} in the pipette, single-channel activity was different

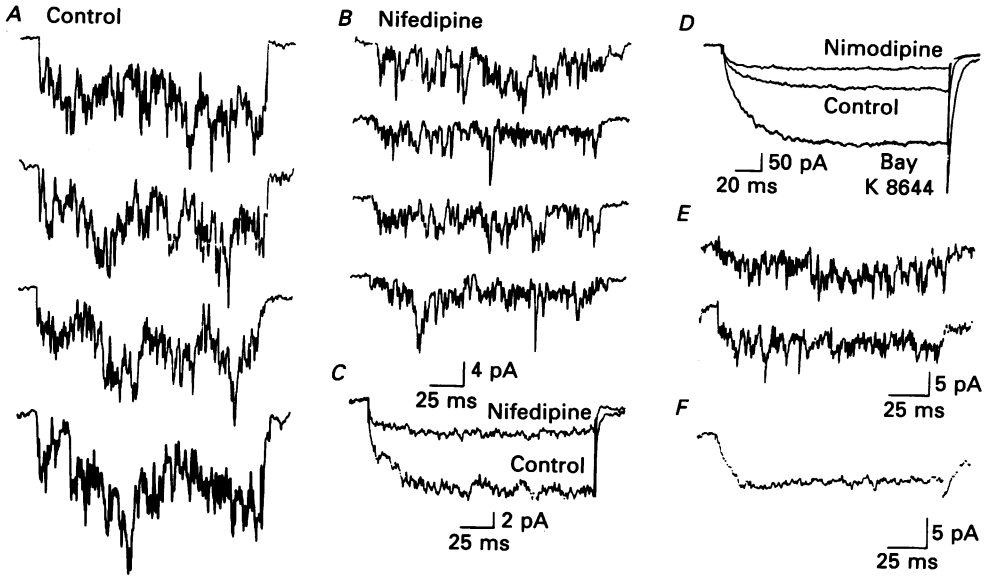

Fig. 9. Li^+ conductance in dihydropyridine-sensitive Ca^{2+} channels in acutely isolated hippocampal neurons. *A*, sample consecutive traces of noisy inward current induced by a step to −40 mV from V_H of −80 mV. No Bay K 8644 was present. *B*, sample consecutive traces from the same patch as in *A* 3 min after adding 10 μM-nifedipine to the bathing solution. *C*, ensemble averages of forty traces from the same patch in control solution and after bath application of 10 μM-nifedipine. *D*, ensemble currents from a different patch, first in control solution, then following application of nimodipine (20 μM) and finally after adding Bay K 8644 (5 μM) to the bath. *E* and *F*, Li^+ current in a tissue-cultured neuron after 45 min of treatment with standard saline containing trypsin (1·6 mg/ml). *E*, two consecutive current sweeps during a voltage step to −40 mV from −80 mV. *F*, ensemble average (forty sweeps) of currents evoked from steps to −40 mV from −80 mV. Same patch as in *E*.

in acutely isolated cells when Li^+ carried the current. Holding a patch at −50 or −60 mV with a Li^+-filled pipette resulted in a pronounced inward current that appeared to be very noisy. Upon holding the patch at a more negative level (−100 to −80 mV) we found that the current trace was quiet until a depolarizing step was made. During the step, large, noisy inward currents were again evoked (Fig. 9*A*). Although initially this might seem to represent some type of 'membrane breakdown' it seems instead to reflect the activity of numerous Ca^{2+} channels. The best evidence for this is that the current is dihydropyridine sensitive and can be reduced by nifedipine (e.g. Fig. 9*B*) or nimodipine and enhanced by Bay K 8644 (Fig. 9*D*). Ensemble averages of thirty sweeps revealed a large, slowly activating and nearly non-inactivating current that was affected by dihydropyridines (Fig. 9*C* and *D*).

Figure 9*D* (from a different cell than in Fig. 9*A–C*) is especially informative since it illustrates that the tail current is markedly prolonged by Bay K 8644 and outlasts the activation pulse, a characteristic of L channels in Bay K 8644.

Although many differences between fetal cultured neurons and adult acutely dissociated neurons might explain the differences noted with Li^+, the enzymatic

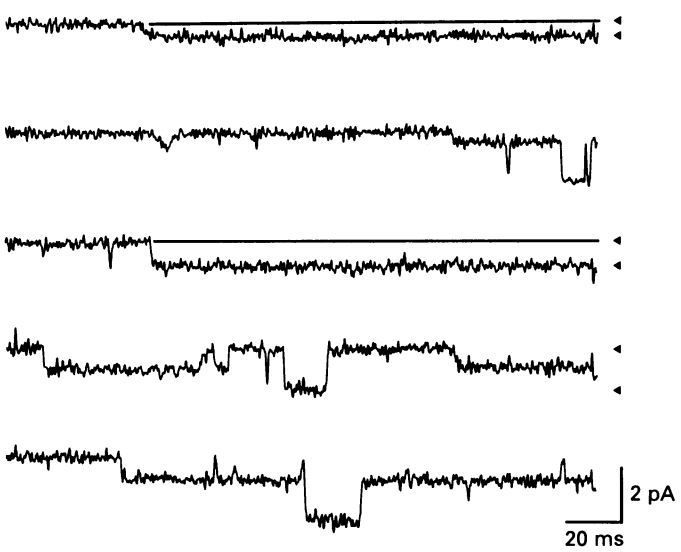

Fig. 10. Other single-channel conductances in hippocampal neurons. Selected traces from a single patch showing three distinct conductance levels. Representative isolated openings of small and intermediate channels marked with bars. Small channel (top two traces; ~ 9 pS; assuming it is Ba^{2+} dependent, as the large channel is), medium channel (~ 14 pS; lower three traces) and large channel (~ 24 pS) were all visible and seemed to occur independently of one another. None of the channels showed obvious time-dependent inactivation. Holding potential in all cases was −50 mV, the step potential was to −20 mV, and Bay K 8644 was present. Arrow-heads point to closed and open states of representative openings. Experiment performed by Dr Diane Doerner.

treatment used during acute isolation was a particularly obvious and readily tested one. We bathed tissue-cultured neurons with trypsin as in the standard dissociation protocol ($n = 6$). This abolished distinct single-channel activity. Current traces appeared noisy (Fig. 9*E*), and yet, in the ensemble averages, a non-inactivating inward current, with a clear tail current in Bay K 8644, appeared (e.g. Fig. 9*F*). In the absence of enzyme, discrete L-channel activity was always observed with Li^+-filled pipettes (e.g. Fig. 8*C*).

In preliminary experiments we have attempted to determine whether trypsin modifies the monovalent cation flux through Ca^{2+} channels by increasing the single-channel conductance. Channel conductance was estimated from increases in the variance of the current noise following voltage steps to activate the channels and calculated from the equation:

$$\gamma = \sigma_i^2 / I\,\Delta V,$$

where γ is the single-channel conductance, σ_i^2 is the difference between the variance of the baseline current noise and the noise during a 512 point interval prior to termination of the voltage step, I

is the mean current through the patch, and ΔV is the driving force. The reversal potential for Li^+ current through Ca^{2+} channels ($\sim +40$ mV) was estimated from *I–V* plots of single-channel current amplitudes from patches on untreated cells where single Li^+ currents through Ca^{2+} channels were observed. In sixteen determinations from four patches on trypsin-treated cells the mean

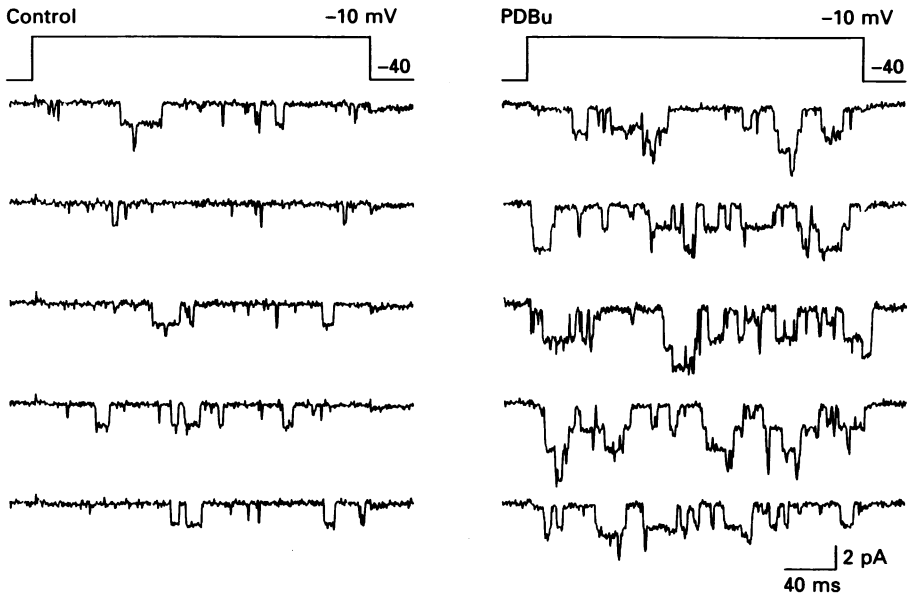

Fig. 11. The protein kinase C activator, PDBu (1 μM), can enhance large Ca^{2+} channel activity. The sequential traces in the left column were obtained in control saline and those in the right column in the same patch on an acutely isolated neuron 3 min after adding 1 μM-PDBu to the bath. Note the increased activity in the presence of PDBu.

single-channel conductance was $28{\cdot}7 \pm 2{\cdot}7$ pS, a value not statistically different from the normal L-channel conductance ($P \leqslant 0{\cdot}05$). Although these results are preliminary, they suggest that it is unlikely that increased L-channel conductance accounts for the large, noisy Li^+ currents seen in patches from trypsin-treated, cultured hippocampal neurons.

Other single-channel conductances in hippocampal neurons

Neither of the two large Ca^{2+} channel types discussed above resembled the high-voltage-activated, rapidly inactivating N-type Ca^{2+} channel seen in cultured chick dorsal root ganglion cells (Fox *et al.* 1987*b*). Indeed, in none of the 187 patches tested by stepping from -80 mV to depolarized potentials have we yet observed unambiguous evidence of this channel in either tissue-cultured or acutely isolated CA1 neurons.

Nevertheless we did observe other single-channel conductances under conditions designed to isolate Ca^{2+} channels. In some patches ($\sim 5\%$), in addition to the large openings of L channels, single-channel events with both small and intermediate conductances were also evident, e.g. Fig. 10 (examples of single openings marked with bars). The amplitude of the small openings was $\sim 40\%$, and that of the intermediate openings $\sim 60\%$, of the size of the L channels with, assuming an equal permeability to Ba^{2+}, conductances of ~ 9 and ~ 14 pS, respectively. These channels

usually had long-duration openings and did not obviously inactivate during a voltage pulse or during prolonged periods of holding at −40 to −50 mV. While these other events bore some resemblance to N or T channels, the differences between them and the highly voltage-sensitive N and T channels often reported were sufficiently striking that we chose not to label them and have not studied them further.

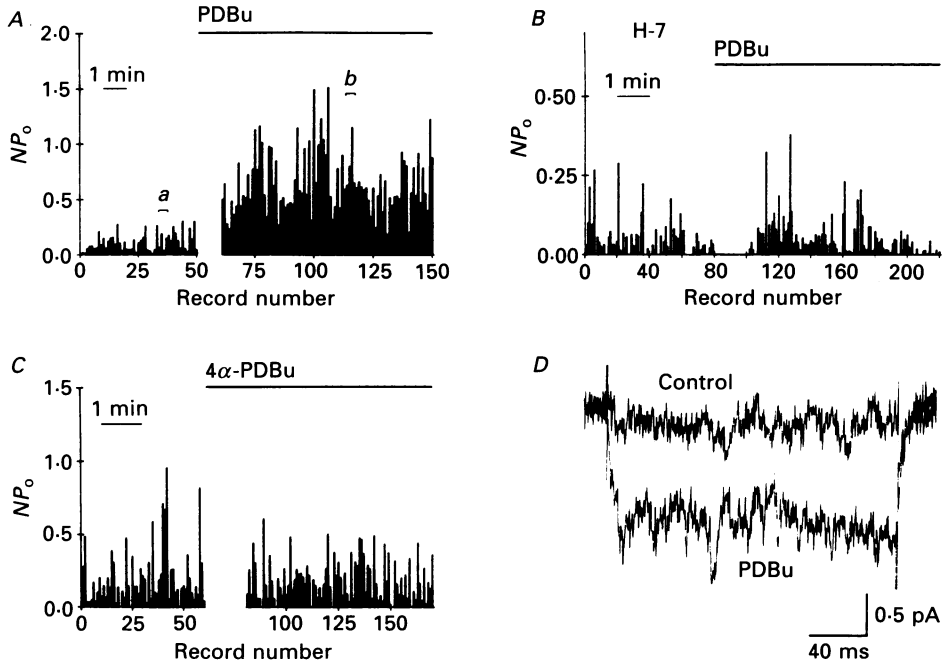

Fig. 12. Evidence that the increase in channel activity caused by PDBu involves protein kinase C activation. *A*, summary of the experiment from which the sample traces in Fig. 11 were taken (control traces in Fig. 11 were from segment marked '*a*', PDBu traces from '*b*', above). The plot indicates NP_o values calculated for each sweep. The gap in the record represents the interval when PDBu was added to the bath. *B*, block of the effect of PDBu by the kinase inhibitor, H-7. H-7 (50 μM) had been present in the bathing medium for 10 min prior to PDBu application. *C*, lack of effect of an inactive phorbol ester, 4α-PDBu (1 μM), on channel activity. Records in *B* and *C* were obtained by voltage steps from −40 to −10 mV. *D*, enhancement of Li^+ current by PDBu (1 μM) in a cell-attached patch in the absence of Bay K 8644. The patch was held at −80 mV and stepped for 170 ms to −40 mV. Note the increase in the magnitude of inward current. Records in *A–D* from different acutely isolated neurons.

Phorbol esters increase large-channel activity

Previous whole-cell recordings had suggested that L-type Ca^{2+} current is reduced by phorbol esters (Doerner *et al.* 1988*a*; 1990). To test the prediction that single-L-channel activity would also be reduced by protein kinase C activation, we applied the protein kinase C activator PDBu to cell-attached patches containing putative L channels. Contrary to expectations, in the majority of cases (thirteen of twenty-one) PDBu (1 μM) caused a clear increase in channel activity. Sample traces from a typical experiment are shown in Fig. 11. The complete experiment from which the traces in

Fig. 11 were drawn is shown in the NP_0 plot of Fig. 12*A*. PDBu increased channel activity with no change in the single-channel conductance. Patches were studied for about 10 min after adding PDBu, and the mean increase in activity (measured at 3–5 min post-application) was 5·6 times control levels ($n = 13$). While we have not

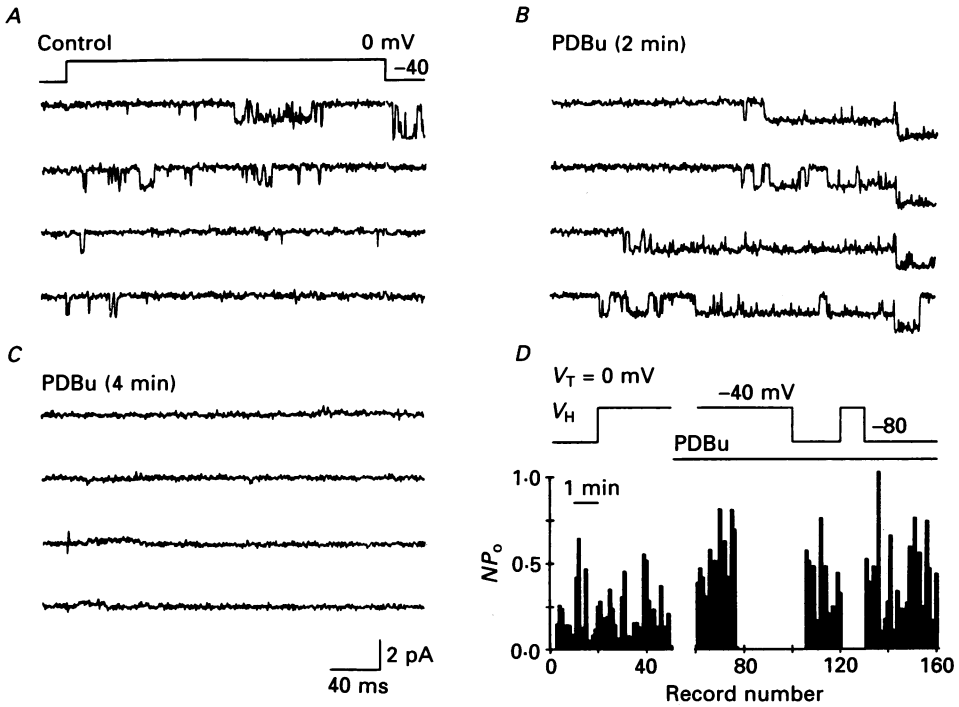

Fig. 13. PDBu can also depress Ca^{2+} channel activity via a hyperpolarizing shift in the voltage dependence of inactivation. *A*, control records from an acutely isolated cell in Bay K 8644 following step to 0 mV from V_H of −40 mV. *B*, same patch 2 min after addition of 1 μM-PDBu to the bath. *C*, 4 min after addition of PDBu to the bath. *D*, summary of the entire experiment from which the sample traces in *A–C* were taken. Note that, prior to PDBu application, the channel was activated equally readily regardless of whether V_H was −80 or −40 mV. After a slight transient increase in activity in PDBu, channel activity could no longer be elicited from V_H of −40 mV, but was always evident when the holding potential was stepped down to −80 mV.

made a detailed study of the mechanism for the increase in channel activity, it appears that a decrease in 'mode 0' behaviour, i.e. a decrease in the number of sweeps with no openings (Nowycky *et al.* 1985*a*), is involved. In the presence of the kinase inhibitor H-7 (50 μM), 1 μM-PDBu had no effect on Ca^{2+} channel activity in three of three patches (e.g. Fig. 8*B*). The inactive phorbol ester, 4α-PDBu, had no effect on single-channel activity in seven of seven patches (e.g. Fig. 12*C*). Figure 12*D* illustrates that the monovalent Ca^{2+} channel current recorded in the absence of Bay K 8644 is also enhanced by PDBu.

In three of twenty-one patches PDBu did cause a decrease in channel activity. To investigate the mechanism by which this occurred we studied one such channel in

more detail. Control records from this channel include sample traces in Fig. 13*A* and the initial fifty records of the NP_0 plot in Fig. 13*D*. The mean NP_0 was 0·12 in control solution. The channel appeared to be a typical L channel and showed little voltage dependence of inactivation; i.e. channel activity was relatively unaffected whether the patch was held at −80 or −40 mV. After PDBu was applied, however, voltage dependence of channel inactivation changed radically. After 2 min in PDBu, channel activity was clearly enhanced; mean NP_0 increased to 0·65, but after 4 min activity could no longer be evoked from −40 mV. Changing the holding potential to −80 mV, however, restored activity. This sequence was repeated many times, and the pattern was clear (Fig. 13*D*). There was no change in the single-channel conductance. Such a change in voltage dependence of inactivation never occurred spontaneously.

In the remainder of the patches (six of twenty-one) PDBu had no apparent effect on single-L-channel activity. There was no obvious difference between the cells in which the activity was affected and those in which it was not.

DISCUSSION

The T channel

The T channel could clearly be distinguished from the dihydropyridine-sensitive, high-voltage-activated, L-type Ca^{2+} channel and from the Na^+ channel. Several distinctive properties of T channels were investigated, including voltage dependence, kinetics, conductance, dihydropyridine insensitivity and phorbol ester insensitivity. The T channel was prevalent in tissue-cultured neurons, but not found in acutely isolated cells.

The properties of ensemble averages of T-channel activity compare favourably with those predicted from studies of T-type currents in a number of cells. T currents activate with a $V_{0.5}$ of between −51 and −63 mV, and a $V_{0.5}$ of inactivation between −95 and −78 mV (Fox *et al.* 1987*a*; Coulter *et al.* 1989; Crunelli *et al.* 1989; Kostyuk *et al.* 1989). These data agree quantitatively with our data. Ensemble averages of T-channel activity indicate that activation and inactivation of this channel are very similar to these properties of the T-type current in these cells. Hence the T channel probably mediates the T current in tissue-cultured hippocampal neurons.

The T channel shares some similarities with the N channel. They are both commonly thought to be dihydropyridine insensitive and are, in many cases, transiently activated (Fox *et al.* 1987*a*), but see Plummer *et al.* (1989), Hirning *et al.* (1988) and Lipscombe, Kongsamut & Tsien (1989). Their conductance is clearly less than that of the L channel. However, a number of differences do exist. The most important are the voltage sensitivity and rate of inactivation of channel activity. The T channel is activated and inactivated at substantially more negative potentials than the N channel, and T currents inactivate much more rapidly than do N currents (e.g. Fox *et al.* 1987*a*). A particularly significant characteristic of N channels is the unusually broad range of potentials over which inactivation occurs, the Boltzmann slope factor being 12 (Fox *et al.* 1987*b*). The small channel we have studied inactivates over a comparatively narrow range of potentials, the slope factor being only 4·4, which is in good agreement with the value found in dorsal root ganglion neurons, 5 (Fox *et al.* 1987*a*), or thalamic neurons, 6·3 (Coulter *et al.* 1989), for example. On the

basis of these features we conclude that the small, rapidly inactivating channel we have recorded is the T rather than the N channel. Although we typically studied the T channel using Li^+, which is permeant in the Na^+ channel, a number of tests showed that the Na^+ channel could be clearly distinguished from the T channel.

No unequivocal evidence for T-type currents has been found in whole-cell voltage-clamp experiments from acutely isolated hippocampal CA1 pyramidal neurons (Kay & Wong, 1987; Doerner *et al.* 1988*a*; Thompson & Wong, 1989), suggesting that T channels either did not exist or were not functional in these cells. Takahashi *et al.* (1989) have identified a 'T-type' current in acutely isolated hippocampal neurons; however, the fluoride they typically use in their patch pipettes is known to affect I_{Ca} kinetics (Kay, Miles & Wong, 1986), so this identification is not yet certain. Our data provide some support for the hypothesis that functional T channels are absent from adult cells, since we were unable to detect them in acutely isolated cells. T channels persist in enzymatically isolated thalamic cells (Kay & Wong, 1987; Coulter *et al.* 1989; Crunelli *et al.* 1989), and Thompson and Wong recently reported (1989) that a T-type current could be recorded from neurons enzymatically isolated from neonatal animals. These observations suggest that the enzyme does not alter T channels, although this cannot absolutely be ruled out. Whether T channels are in fact absent from the adult cells, are present only on the secondary dendrites inevitably removed during the isolation process or have undergone some other alteration has not as yet been determined. Our findings must also be reconciled with observations on hippocampal neurons in the rat slice preparation apparently indicating the presence of T-type current (Docherty & Brown, 1986; Blaxter, Carlen & Niesen, 1989).

The L channel

Identification of the large primary Ca^{2+} channel in hippocampal neurons as the L channel seems quite straightforward as its properties agree closely with those of L channels in many other cell types. Points of similarity include: dihydropyridine sensitivity, flickery block by Cd^{2+}, conductance and voltage dependence. A particularly important characteristic, and one considered diagnostic of L channels (Plummer *et al.* 1989), is that the large-channel openings often outlasted the voltage step in the presence of Bay K 8644. Although the non-inactivating Ca^{2+} current known to exist in hippocampal neurons (Johnston *et al.* 1980; Brown & Griffith, 1983; Segal & Barker, 1986; Gray & Johnston, 1987; Yaari *et al.* 1987) would have been thought to be mediated by L channels, this has not been extensively supported by single-channel studies.

On the other hand, our observation of voltage dependence of steady-state inactivation of an L-like channel appears to be novel. While identification of this channel as an L channel would be bolstered by a direct demonstration of its dihydropyridine sensitivity, the similarity of its conductance, Cd^{2+} block and its open time indicate that this channel more strongly resembles the L channel than it does other Ca^{2+} channel types. Moreover, openings of this channel also frequently outlasted the voltage step in Bay K 8644. Whether it represents a subclass of L channels, or a conventional L channel in an unusual state of behaviour, is not yet clear. The dihydropyridine Bay K 8644 has mixed agonist–antagonist properties, and its antagonist action is voltage dependent, with channel block increasing at less-

negative voltages. We cannot exclude the possibility, therefore, that the properties of the inactivating L channels are due to the Bay K 8644, although we have no explanation for why the drug should affect only a small subset of all the channels studied in its presence.

There were no obvious differences between L channels in acutely isolated cells as compared to those in tissue-cultured cells when Ba^{2+} carried the current. However, in the absence of Ca^{2+}, the Li^{+} current differed strikingly between the two preparations. The difference was ascribed to the enzyme used to isolate adult cells acutely, since trypsin treatment resulted in noisy Li^{+} channel records in tissue-cultured cells. It appears that trypsin produces modifications of the channels that are evident only in specific conditions. The finding that L channels in trypsin-dissociated neurons and untreated cultured neurons behave similarly in the presence of divalent cations suggests that the trypsin-modified sites participate in channel gating only in the absence of divalent cations. Perhaps L-type channels adopt different conformational states depending on whether divalent or monovalent cations permeate the channel, and different domains of the channel protein participate in channel gating in each conformation. Trypsin may preferentially modify portions of the channel protein that are involved in gating in the monovalent-cation-permeable conformation of the channel and thus leave the channel relatively intact in the divalent-cation-permeable conformation. Although this hypothesis is speculative, it has recently been reported that high-voltage-activated Ca^{2+} channels in dorsal root ganglion cells adopt different conformational states depending on whether divalent or monovalent cations permeate the channel and that these different states have different pharmacological sensitivities (Carbone & Lux, 1988). Further work will be necessary to understand the differences in monovalent cation currents between the two preparations.

Our failure to observe intermediate-sized, rapidly inactivating N channels was unexpected. Whole-cell Ca^{2+} current in hippocampal neurons is comprised in part of a high-voltage-activated, rapidly inactivating component that resembles an N-channel-mediated phase (Doerner *et al.* 1988*a*) and a slowly inactivating phase similar to an L current. The N-type component is clearly enhanced by hyperpolarized holding potentials and suppressed by depolarization. Preliminary pharmacological experiments with dihydropyridines appeared to support the segregation of high-voltage-activated current into dihydropyridine-sensitive and -insensitive components, i.e. L and N components (Doerner *et al.* 1988*a*). However, the actions of dihydropyridines are notoriously complex, and the identification of N current was considered tentative. Kay & Wong (1987) argued on the basis of kinetic measurements that the participation of more than one Ca^{2+} channel in the high-voltage-activated Ca^{2+} current was improbable, although they took no position on what type of Ca^{2+} channel was present. There can be many reasons for our not observing inactivating N channels, including: (a) species and/or tissue differences. N channels have thus far been demonstrated to exist mainly in peripheral ganglia of chicks and rats. To date there are very few data on single N channels in central neurons (however, see Gray & Johnston, 1987; and Fisher, Gray & Johnston 1990). (b) Some aspect of our methods suppresses N-channel activity. This factor, if it exists, is unlikely to be the enzyme used in acute dissociation since N channels were

not seen in tissue-cultured cells either. (c) Topographical differences. We have not done a rigorous mapping of these cells to determine channel localization. If the N channels were localized predominantly on dendrites, we might well have missed them. (d) Finally, we note that we have observed channels that do not clearly fit easily into the T, L or N scheme as developed on peripheral cells (e.g. Fig. 10). Because these other channels differed in significant ways from the rapidly inactivating N channels as described in earlier papers, we have not called them N channels, even though the conductance of some of them is approximately correct for N channels. In this context it may be important that the time-to-peak of the ensemble L current that we measured (20–60 ms, cf. Figs 5, 7 and 9) is considerably slower than that of whole-cell Ca^{2+} current ($\leqslant$ 10 ms, cf. Kay & Wong, 1987), a discrepancy not obviously accounted for by Bay K 8644 since this drug does not appear to slow the time-to-peak of whole-cell Ca^{2+} current (Doerner *et al.* 1988*a*). Fisher *et al.* (1990) have recently reported finding T, L and N channels in adult hippocampal neurons from partially dissociated slices. Their nomenclature, based largely on conductance measurements, includes cases in which other channel properties differed from the original definitions as well. Evidence from molecular biological studies suggests that many types of Ca^{2+} channels may exist in brain (e.g. Snutch, Leonard, Gilbert, Lester & Davidson, 1990). It is likely that the categorization of hippocampal Ca^{2+} channels into three groups will not be sufficient to capture the diversity of channels present.

There is a striking similarity between the voltage dependence of the channel shown in Fig. 6 and that of the channel whose voltage dependence was apparently shifted by PDBu (Fig. 13). This suggests that the voltage dependence of steady-state inactivation may be regulated by phosphorylation. Chad & Eckert (1986) have shown that the rate of inactivation during a voltage pulse is regulated by phosphorylation. Our hypothesis would suggest that currents that differ in their voltage dependence of inactivation might be mediated in part by a single channel type in different states of phosphorylation rather than, or in addition to, by different channels.

Effects of protein kinase C activation

A major goal of the present work was to test the protein kinase C sensitivity of high-voltage-activated Ca^{2+} channels. Previous work from our laboratory demonstrated that phorbol esters depress, by about 40%, whole-cell Ca^{2+} channel current via protein kinase C activation (Doerner *et al.* 1988*a*,*b*, 1990). The whole-cell current has both rapidly and slowly inactivating phases and can be influenced by dihydropyridines, suggesting that it is carried at least in part by the L channel. The depressive effect of protein kinase C activation resulted from both a reduction in macroscopic Ca^{2+} conductance and a shift in the hyperpolarizing direction of the voltage dependence of inactivation of the current. Accordingly, we predicted that a single-channel analysis would reveal a down-regulation of L-channel activity by PDBu. Indeed, in 14% of the cases, we found that channel activity was suppressed. Channels like those that evinced an apparent shift in voltage dependence of inactivation (see Fig. 13) are likely candidates to account for the shift in voltage dependence seen at the whole-cell level. Thus, these single-channel data are

compatible with one aspect of the whole-cell findings, although they cannot entirely explain the depression in macroscopic Ca^{2+} conductance.

In any case, it was a surprise to find that L-channel activity was, in the majority of cases, increased, not decreased, by phorbol esters. 'Wash out' of some essential regulatory factor in the whole-cell experiments does not explain the difference between whole-cell and single-channel results since whole-cell Ca^{2+} current is reduced by PDBu when recorded using the nystatin-induced 'perforated patch' method of Horn & Marty (1988) (D. Doerner & B. E. Alger, unpublished observations). The use of Bay K 8644 in single-channel studies cannot explain the difference, since on-cell monovalent patch current was enhanced by PDBu in the absence of Bay K 8644 (Fig. 12*D*).

Lacerda, Rampe & Brown (1988) reported a biphasic effect, increase followed by depression, of the phorbol ester TPA on Ca^{2+} channel activity recorded in on-cell patches from cardiac myocytes. However, these cells clearly differ from hippocampal neurons in several ways: in whole-cell recordings neither phorbol ester nor a diacylglycerol analogue affects cardiac Ca^{2+} current, whereas in hippocampal neurons both do (Doerner *et al.* 1988*a*). In cardiac cells the phorbol-ester-induced depression was thought not to be caused by protein kinase C activation since it was not reproduced by diacylglycerol. In our cells both classes of protein kinase C activators can depress Ca^{2+} currents (Doerner *et al.* 1988*a*; Doerner & Alger, 1990), and effects of both are blocked by the specific peptide protein kinase C inhibitor, protein kinase C pseudosubstrate 19–36 (D. Doerner & B. E. Alger, unpublished observations). We suggest that these differences in protein kinase C effects between recording configurations and between cell types are real and significant. Since protein kinase C appears to be involved in regulation in both the whole-cell and single-channel experiments, selection of different modes of protein kinase C activation (Bazzi & Nelsestuen, 1988; Huang, Yoshida, Cunha-Melo, Beaven & Huang, 1989) and, perhaps, activation of different protein kinase C isozymes (Nishizuka, 1988) under the different conditions will be interesting possibilities for further exploration. An alternative possibility is that different types of Ca^{2+} channel dominate under the different recording conditions. If, for example, the whole-cell Ca^{2+} current in pyramidal cells were not predominantly mediated by dihydropyridine-sensitive L channels, then the single-channel experiments, which clearly involved these channels, would not necessarily be in conflict. More work on the 'other' types of Ca^{2+} channels must be done to test this hypothesis.

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