Rapid Report

Direct demonstration of persistent Na^+ channel activity in dendritic processes of mammalian cortical neurones

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1. Single Na^+ channel activity was recorded in patch-clamp, cell-attached experiments performed on dendritic processes of acutely isolated principal neurones from rat entorhinal-cortex layer II. The distances of the recording sites from the soma ranged from ~20 to ~100 μm.

2. Step depolarisations from holding potentials of −120 to −100 mV to test potentials of −60 to +10 mV elicited Na^+ channel openings in all of the recorded patches ($n = 16$).

3. In 10 patches, besides transient Na^+ channel openings clustered within the first few milliseconds of the depolarising pulses, prolonged and/or late Na^+ channel openings were also regularly observed. This 'persistent' Na^+ channel activity produced net inward, persistent currents in ensemble-average traces, and remained stable over the entire duration of the experiments (~9 to 30 min).

4. Two of these patches contained $\leqslant 3$ channels. In these cases, persistent Na^+ channel openings could be attributed to the activity of one single channel.

5. The voltage dependence of persistent-current amplitude in ensemble-average traces closely resembled that of whole-cell, persistent Na^+ current expressed by the same neurones, and displayed the same characteristic low threshold of activation.

6. Dendritic, persistent Na^+ channel openings had relatively high single-channel conductance (~20 pS), similar to what is observed for somatic, persistent Na^+ channels.

7. We conclude that a stable, persistent Na^+ channel activity is expressed by proximal dendrites of entorhinal-cortex layer II principal neurones, and can contribute a significant low-threshold, persistent Na^+ current to the dendritic processing of excitatory synaptic inputs.

Dendritic integration by central neurones results from the complex interaction of their passive and active properties. It is well known that the transmission of synaptic potentials generated in the dendritic tree to the soma, where the final decision on output encoding modality is made, undergoes passive attenuation due to the cable properties of dendrites (see Rall, 1977). Especially in the cases of highly developed dendritic arbors and of electrotonically remote synapses, the impact of elementary synaptic inputs onto the somatic excitation–inhibition balance would be predicted to be very small on the sole basis of cable theory, unless major temporal and/or spatial summation occurs. On the other hand, it has become increasingly clear that dendrites are also sites of active processing mechanisms (Wong *et al.* 1979; Llinás & Sugimori, 1980; for review, see Johnston *et al.* 1996), including voltage-dependent amplification of excitatory synaptic potentials. The latter function implies the intervention of voltage-dependent conductances active at low- or near-threshold voltage ranges. In different neuronal populations of mammalian central nervous system, a role in boosting excitatory synaptic potentials has been variously attributed to low-threshold Ca^{2+} currents (Sutor & Zieglgänsberger, 1987; Magee & Johnston, 1995*a*; Gillessen & Alzheimer, 1997; Seamans *et al.* 1997; Schiller *et al.* 1997)

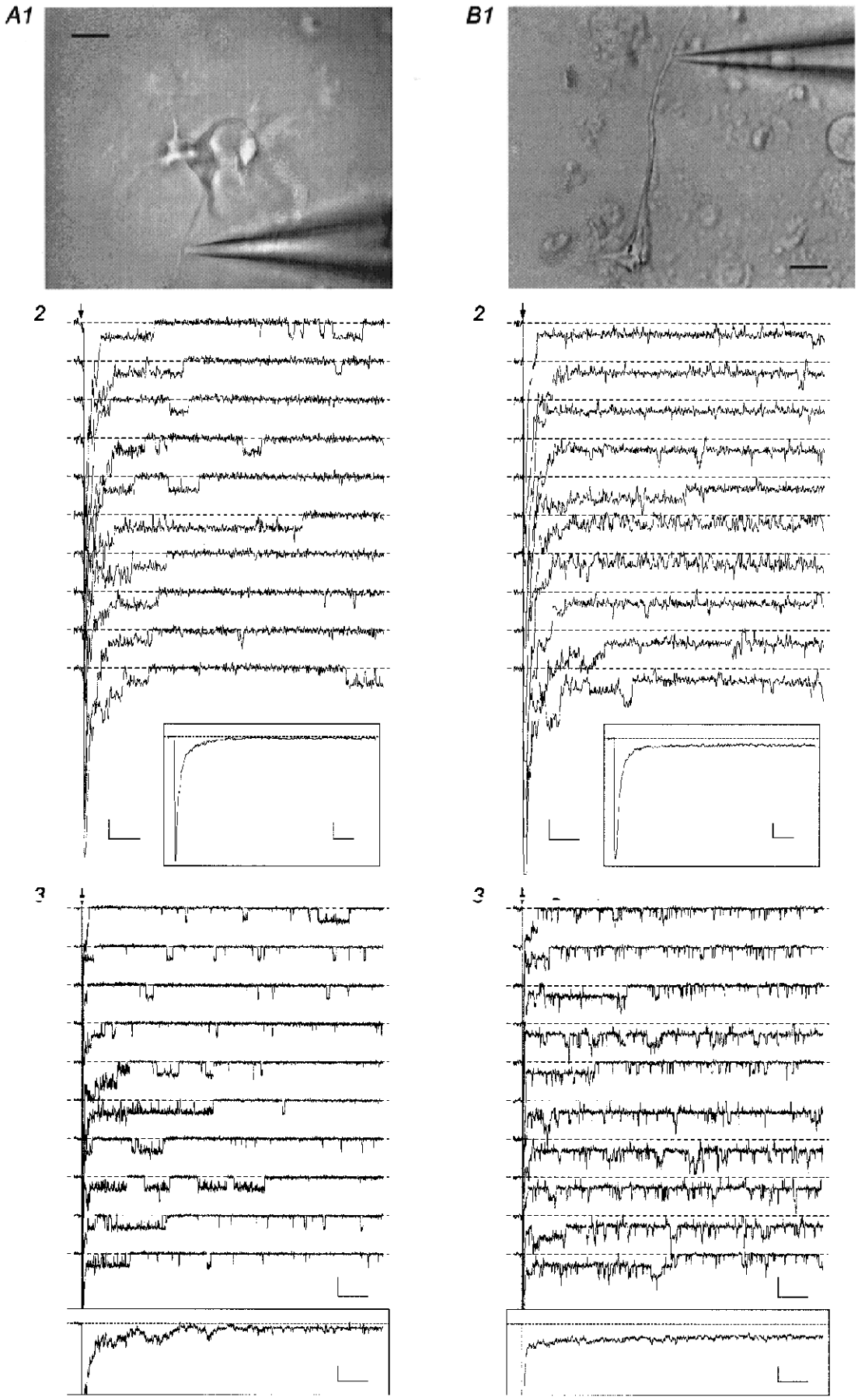

Figure 1. For legend see facing page.

or Na^+ currents. In particular, low-threshold, persistent Na^+ currents (I_{NaP}s: for review, see Taylor, 1993; Crill, 1996) have been implicated in amplification processes of dendritic synaptic inputs in hippocampal (Lipowsky *et al.* 1996) and neocortical (Stafstrom *et al.* 1985; Deisz *et al.* 1991; Stuart & Sakmann, 1995; Schwindt & Crill, 1995) pyramidal neurones. However, whereas some of these reports supported the idea of a dendritic localisation of I_{NaP}, close to the site of generation of synaptic inputs potentially requiring active boosting (Schwindt & Crill, 1995; Lipowsky *et al.* 1996), other data favoured a substantial intervention of axosomatic Na^+ channels in a downstream process of synaptic-potential amplification (Stuart & Sakmann, 1995).

Previous experimental work has demonstrated that the principal neurones of entorhinal-cortex (EC) layer II express a robust I_{NaP} (Magistretti & Alonso, 1999*a*, *b*), which has a key role in sustaining low-threshold, theta-like membrane-voltage oscillations generated at the single-cell level (Alonso & Llinás, 1989; Klink & Alonso, 1993). Single-channel studies revealed an underlying channel activity characterised by a stable, high opening probability during prolonged depolarisations and a single-channel conductance significantly higher than that of classical, transient Na^+ channels (Magistretti *et al.* 1999*b*). Moreover, whole-cell recordings performed in both *in situ* and acutely dissociated EC layer II neurones revealed that, whereas absolute I_{NaP} amplitude is much higher in the former cells than in the latter, I_{NaP} current density is not significantly different in the two conditions (Magistretti & Alonso, 1999*b*). On one hand, these observations indicated that the dissociation procedure employed does not alter the specific amount of persistent Na^+ conductance present in the membranes of the neurones under study; and on the other hand, they strongly suggested that the dendrites, as well as the soma, are sites of significant I_{NaP} expression.

The above background prompted us to investigate Na^+ channel activity in the residual dendritic processes that can be found in mildly dissociated EC layer II neurones. We report the finding of a stable, persistent Na^+ channel activity in the majority of the dendritic, cell-attached patches obtained. This persistent Na^+ channel activity shared the basic biophysical properties of the already described persistent Na^+ channel openings observable in the soma of the same neurones (Magistretti *et al.* 1999*b*).

METHODS

Cell preparation

The procedures followed for dissecting rat entorhinal-cortex (EC) layer II, and for acute dissociation of neurones were as previously described (Magistretti *et al.* 1999*a*, *b*). Briefly, male Long-Evans rats (postnatal day (P) 25–35) were decapitated according to a procedure approved by the Animal Care Committee of the Montreal Neurological Institute, and compliant with the Canadian laws on animal research. The brains were quickly removed and submerged into ice-cold dissociation buffer (DB) containing (in mmol l^{-1}): 115 NaCl, 3 KCl, 3 $MgCl_2$, 0·2 $CaCl_2$, 20 Pipes and 25 glucose (pH 7·4 with NaOH, bubbled with O_2). Coronal slices 400 μm thick were then cut with a Vibratome vibroslicer (Lancer, St Louis, MO, USA). Using a fine scalpel, pieces of EC layer II were dissected, transferred to DB plus pronase (protease Type XIV, 1 mg ml^{-1}; Sigma-Aldrich Canada Ltd, Oakville, ON, Canada), and incubated for 15 min at 32 °C with gentle agitation. The tissue fragments were then washed with enzyme-free DB, and stored at room temperature in continuously oxygenated DB. When needed, one tissue fragment at a time was gently triturated with a few passages through Pasteur pipettes with fire-polished tips of progressively smaller inner diameter. Suspensions of dissociated cells were transferred into a recording chamber for electrophysiological experiments. Cells were allowed to settle down for ~10 min before starting the recordings.

Patch-clamp recordings

Na^+ channel activity was recorded using the patch-clamp recording technique (Hamill *et al.* 1981) in the cell-attached configuration. Dissociated cells were initially perfused with a solution containing (in mmol l^{-1}): 140 NaCl, 5 KCl, 10 Hepes, 2 $CaCl_2$, 2 $MgCl_2$ and 25 glucose (pH 7·4 with NaOH, continuously bubbled with O_2). The pipette solution contained (in mmol l^{-1}): 130 NaCl, 35 TEA-Cl, 10 Hepes, 2 $CaCl_2$, 2 $MgCl_2$ and 5 4-AP (pH 7·4 with HCl). Patch pipettes were fabricated from thick-wall borosilicate glass capillaries by means of a Sutter P-97 horizontal puller, and had resistances ranging from 10 to 35 MΩ when filled with the above solution. Every patch pipette was coated with Sylgard (Dow Corning, Midland, MI, USA) from the shoulder to a point as close as possible to the tip, so as to minimise stray pipette capacitance. After performing a mild mechanical dissociation treatment, some isolated neurones retained substantial portions of proximal dendritic processes (see Fig. 1*A*1 and *B*1), which were approached by pipette tips for seal formation. After obtaining the cell-attached configuration, the extracellular perfusion was switched to a high-

Figure 1. Persistent Na^+ channel activity in multichannel patches from stellate- and pyramidal-cell dendrites

Columns *A* and *B* refer to a representative stellate neurone (patch D8716) and a representative pyramidal neurone (patch A8717), respectively. Panels *A*1 and *B*1 are micro-photographs of the two cells in question (scale bars, 15 μm; note the positions of the patch pipettes on the dendritic processes). Panels *A*2 and *B*2 illustrate Na^+ channel currents evoked by 50 ms depolarising pulses at −20 mV (*A*2) and −10 mV (*B*2) starting from the time points marked by the arrows. The current traces shown are consecutive sweeps from families of 20. Scale bars: 2 pA and 5 ms. Insets, ensemble-average currents obtained from the corresponding 20-trace families. Scale bars in insets: 2·5 pA and 5 ms. Panels *A*3 and *B*3 illustrate Na^+ channel currents evoked by 500 ms depolarising pulses at −20 mV (*A*3) and −10 mV (*B*3) starting from the time points marked by the arrows. Scale bars: 2 pA and 50 ms. Insets, ensemble-average currents obtained from the corresponding 20-trace families. Scale bars in insets: 0·5 pA and 50 ms (*A*3), and 1 pA and 50 ms (*B*3).

potassium solution containing (in mmol l^{-1}): 140 potassium acetate, 5 NaCl, 10 Hepes, 4 $MgCl_2$, 0·2 $CdCl_2$ and 25 glucose (pH 7·4 with KOH, continuously bubbled with O_2), so as to hold the neurone resting membrane potential at near 0 mV. Recordings were performed at room temperature with an Axopatch 200B amplifier (Axon Instruments). Capacitive transients and leak currents were minimised by acting on the respective compensation sections of the amplifier. The holding potential was −100 or −120 mV. Depolarising voltage steps were delivered at 5 s intervals. Current signals were low-pass filtered at 5 or 2 kHz, and digitised at 100 or 10 kHz, when acquiring 50 or 500 ms sweeps, respectively.

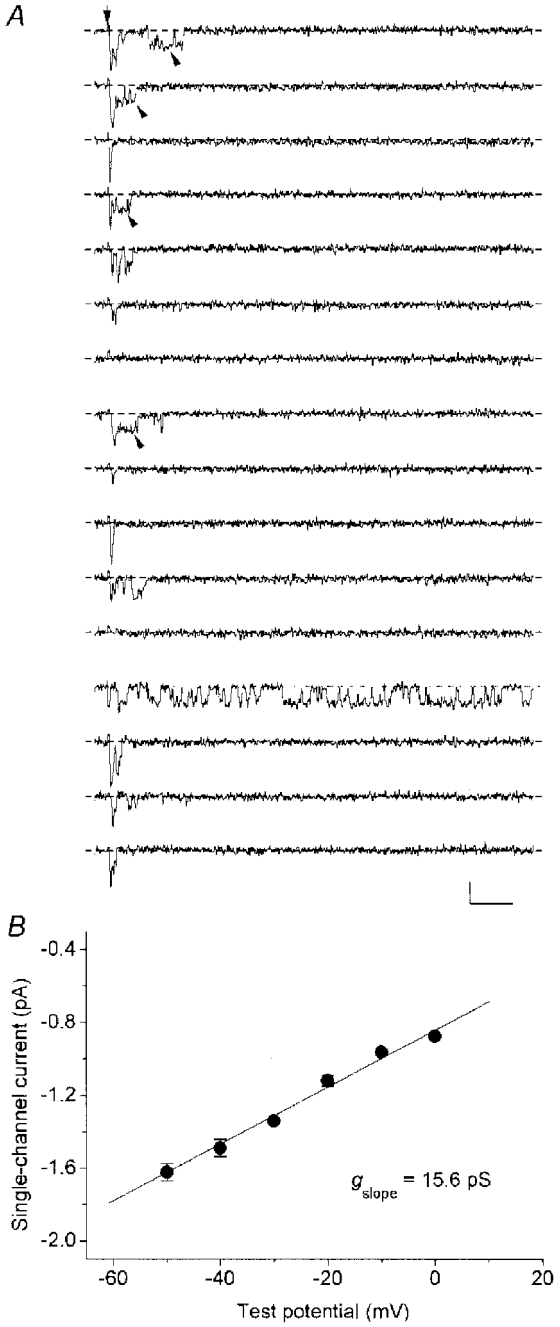

Figure 2. Transient Na^+ channel activity in dendrites
A, Na^+ channel currents evoked by 50 ms depolarising pulses at −30 mV (starting from the time point marked by the arrow) in a patch containing two channels (patch E8710). The current traces shown are consecutive sweeps from a family of 20. Nearly all sweeps exhibited only transient Na^+ channel activity, but occasionally (in 2 out of 110 trials) long-lasting openings were observed (see the fourth trace from bottom). Scale bars: 2 pA and 5 ms. *B*, voltage dependence of the amplitude of early, transient single Na^+ channel currents. Data points are from 4 patches. Where not shown, S.D. values are smaller than the symbol size. The linear best fitting is also shown, along with slope factor (g_{slope}).

Data analysis

Single-channel recordings were analysed using the Clampfit and Fetchan programs of the pCLAMP 6.0.4 software package (Axon Instruments). Residual capacitive transients were nullified by off-line subtracting fits of average blank traces. Residual leakage currents were carefully measured in every single sweep at trace stretches devoid of any channel openings, and digitally subtracted. Average values are expressed as the mean ± S.D.

For the measurement of the amplitude of single Na^+ channel openings of the transient type, only openings of at least ~1 ms in duration were considered. This precaution introduced a significant safety factor with respect to the effects of low-pass filtering on brief openings, the theoretical time constant of filtered square events being less than 50 μs in our conditions.

Na^+ channel opening probability during long-lasting (500 ms) test voltage pulses was measured by determining channel dwell times via a standard threshold routine of Fetchan. The number of channels present in each patch was estimated on the basis of the maximal number of superimposed channel openings observed. If n channels were estimated to be present in the patch under analysis, the single-channel opening probability (P_o) was calculated, under the assumption that P_o was the same for all of the channels of that patch, as:

$$P_o = 1 - (1 - P_{o\geqslant 1})^{1/n},$$

where $P_{o\geqslant 1}$ is the probability of observing one or more channels open during the entire duration of the pulses. Subsequently, the predicted probability of observing two or more channels open ($P_{o\geqslant 2}$) was derived as:

$$P_{o\geqslant 2} = \sum_{i=2}^{n} nC_i P_o^{\,i} (1 - P_o)^{n-i},$$

where $nC_i = n!/[(n-i)!\,i!]$ are binomial coefficients.

RESULTS

When subjected to mild mechanical dissociation, acutely isolated neurones from rat entorhinal-cortex (EC) layer II frequently maintained well-preserved proximal dendritic processes, which in some cases extended in length for several tens of microns. Figure 1*A*1 and *B*1 shows typical examples of isolated stellate and pyramidal neurones whose dendrites underwent patch-clamp recordings in the cell-attached configuration. Dendritic, cell-attached recordings were obtained from 4 stellate cells and 12 pyramidal cells. The distances of the recording sites from the outer boundary of the soma ranged from 19 to 97 μm, with an average value of 42·5 ± 18·9 μm ($n = 16$).

Depolarising voltage steps 50 or 500 ms long applied from a holding potential of −100/−120 mV to more positive test potentials (−60 to +10 mV) evoked Na^+ channel openings in all of the recorded patches. Half of the patches ($n = 8$; 4 from stellate cells and 4 from pyramidal cells) contained

⩾15 channels, as judged on the basis of the maximal number of superimposed channel openings observed. Figure 1 provides two typical examples of multichannel recordings from stellate-cell (*A*) and pyramidal-cell (*B*) dendrites, respectively. The most prominent channel activity in multichannel patches consisted of early, short-lived channel openings clustered within the first few milliseconds of the depolarising pulses. In addition to these early, transient channel openings, a different Na^+ channel activity consisting of prolonged and/or late openings was also always observed in multichannel patches. This 'persistent' channel activity was remarkably stable. Prolonged and/or late openings of at least 5 ms in duration occurred in nearly 100% of the sweeps at test potentials of −30 to +10 mV, even throughout prolonged periods of recording (~9 to 30 min; ~20 min on average). When many consecutive traces were averaged, the persistent channel activity was able to produce sizeable net inward currents even in 500 ms protocols (Fig. 1*A* and *B*, insets). The ratio between the amplitude of the persistent current component (as measured by averaging the last 400 data points in 500 ms protocols) and that of the peak transient component of average currents was 0·0113 ± 0·0102 at the test potential of −20 mV.

The remaining eight patches contained less than six channels. In six of these 'oligochannel' patches, Na^+ channel activity was almost entirely limited to early, short-lived openings (Fig. 2*A*). Single-channel *I–V* relationships were constructed for these transient Na^+ channel openings (Fig. 2*B* shows the average *I–V* plot). To ensure that conductance measurements were not distorted by brief openings truncated by the low-pass filter, only unequivocal, square single-channel openings (such as those indicated by arrows in Fig. 2*A*) were considered for these measurements. Linear fittings returned an average slope conductance of 14·6 ± 1·93 pS ($n = 4$), a value consistent with that previously reported for dendritic transient Na^+ channels of hippocampal pyramidal neurones (Magee & Johnston, 1995*b*).

In two patches of this group, occasional long-lasting openings were also observed. An example of these sporadic events is given in the thirteenth trace of Fig. 2*A*. Prolonged openings occurred in 2 of 110 sweeps in one case, and in 1 of 166 sweeps in the other (test potentials were −40 to +10 mV). A similar, rare occurrence of non-inactivating Na^+ channel openings in patches otherwise exhibiting only the usual, transient Na^+ channel activity has been observed in neocortical pyramidal neurones, and has been attributed to

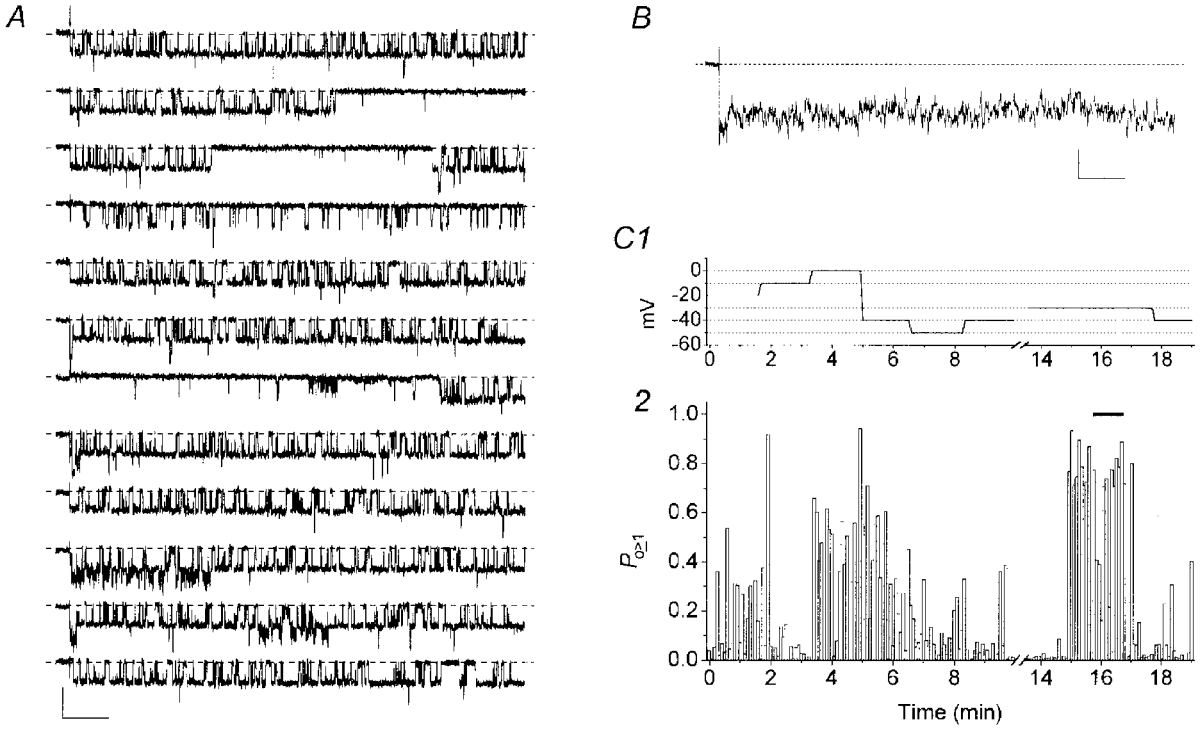

Figure 3. Persistent Na^+ channel activity in a dendritic patch containing three channels (patch A8721)

A, Na^+ channel currents evoked by 500 ms depolarising pulses at −30 mV, starting from the time point marked by the arrow. The current traces shown are consecutive sweeps. Scale bars: 2 pA and 50 ms. *B*, ensemble-average current obtained from 20 consecutive traces, including those shown in *A*. Scale bars: 0·5 pA and 50 ms. *C*, time course of the probability of observing at least one channel open at any time point ($P_{o\geq1}$) in the same experiment as in *A*. Each vertical bar in *C*2 represents the $P_{o\geq1}$ calculated over the whole duration of a single 500 ms depolarising test pulse. The horizontal bar in *C*2 remarks the sweeps shown in *A*. The voltage levels of test pulses are depicted in *C*1. The omitted time interval corresponds to recordings at more negative test potentials (−60 to −80 mV).

short-lived switches of transient Na^+ channels to a non-inactivating gating modality (Alzheimer *et al.* 1993).

The remaining two of the 'oligochannel' patch group showed prominent persistent activity. Figure 3*A* shows typical, consecutive traces from one of these patches, in which no more than three superimposed channel openings were ever observed in a total of 186 sweeps run at test potentials between −50 and 0 mV. In this patch the persistent gating modality was remarkably frequent and intense throughout the experiment. Figure 3*C* shows how the probability of observing at least one channel open ($P_{o\geqslant1}$), measured over the whole duration of 500 ms depolarising pulses, remained consistently high for long periods of recording, with transient episodes of reduced channel activity occasionally interposed. The periods of high $P_{o\geqslant1}$ were most probably due to the activity of one single channel, as indicated by the relatively small probability of observing two or more superimposed channel openings ($P_{o\geqslant2}$). In the case of the patch illustrated in Fig. 3, $P_{o\geqslant1}$ at −30 mV was 0·669 during high-activity phases, whereas $P_{o\geqslant2}$ was 0·021 under the same conditions. Given an estimated number of three channels in the patch, the observed $P_{o\geqslant1}$ value would return a $P_{o\geqslant2}$ of 0·308, assuming the same opening probability for all channels (see Methods), a value higher by more than one order of magnitude than that actually measured. The same was observed in the other oligochannel patch exhibiting prominent persistent activity. These findings strongly suggest that individual Na^+ channels expressed by dendritic membranes of EC layer II principal neurones are able to maintain a persistent gating modality for prolonged periods of time. It thus seems reasonable to assume that the sustained/late Na^+ channel activity observed in multichannel patches must be due to a small number of channels behaving like that of Fig. 3, i.e. as 'persistent' channels.

The voltage dependence of persistent-current amplitude in ensemble-average traces was also analysed. Figure 4*A* shows the *I–V* relationship of the persistent component of average currents obtained from four multichannel patches. A detectable current was already present at −60 mV, whereas the peak value was observed at about −30 mV. The same *I–V* properties have been shown to be typical of the macroscopic persistent Na^+ current (I_{NaP}) recorded in the same cells (see Magistretti *et al.* 1999*b*).

The voltage dependence of the amplitude of 'persistent', single-channel openings was also determined in both multichannel and oligochannel patches. Figure 4*B* shows the average single-channel *I–V* relationship as obtained from six patches. Linear fittings to the data points obtained from individual patches returned an average slope conductance of 19·8 ± 1·75 pS. This value is very similar to that reported for somatic, 'persistent' Na^+ channels (Magistretti *et al.* 1999*b*), and is significantly higher than that found here for transient channel openings ($P < 0·005$).

DISCUSSION

To our knowledge, the present results represent the first direct demonstration of the expression of persistent Na^+ channel activity by dendritic membranes of mammalian neurones. They give strong support to previous studies in which the effect of local application of TTX on the depolarising responses elicited by excitatory-neurotransmitter delivery (Schwindt & Crill, 1995) or input-fibre stimulation (Lipowsky *et al.* 1996) were used to implicate

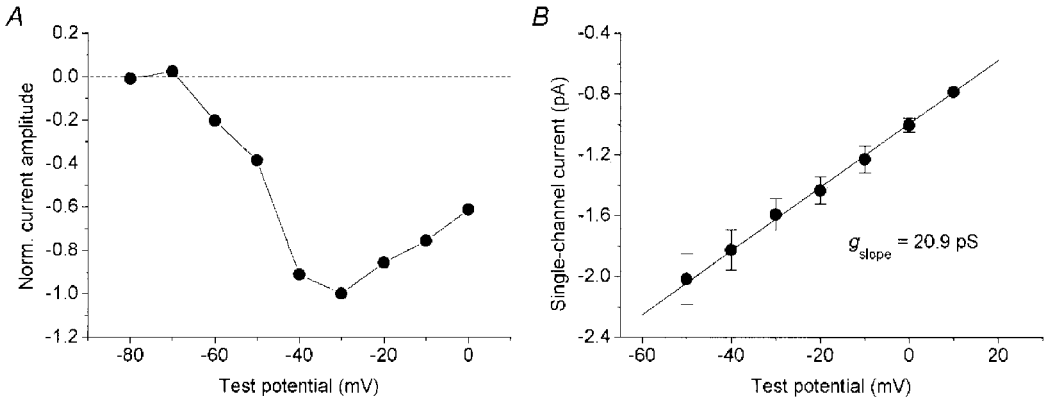

Figure 4. *I–V* relationships of dendritic, Na^+-dependent persistent ensemble-average currents and persistent single-channel currents

A, voltage dependence of the amplitude of the persistent component of ensemble-average currents obtained from both multichannel and oligochannel patches exhibiting persistent Na^+ channel activity. Sixty to seventy-eight 50 ms traces recorded from 4 multichannel patches were averaged per each test potential, and the persistent-current-component amplitude was measured by averaging the data points comprised within the last 25 ms of each ensemble-average trace. The plot was then normalised to the absolute value of its peak amplitude (−0·208 pA). *B*, voltage dependence of the amplitude of late, Na^+-dependent single-channel currents. Only openings occurring after at least 20 ms from the pulse onset were considered. Data points are from 6 patches. The linear best fitting is also shown, along with slope factor (g_{slope}).

the presence of substantial persistent Na^+ conductance in the dendritic arbor of central neurones.

A stable, persistent Na^+ channel activity was observed in the majority of dendritic cell-attached patches, in both stellate and pyramidal neurones from EC layer II. In both multichannel and oligochannel patches, the persistent Na^+ channel activity observed was regular enough to produce sizeable sustained components in ensemble-average traces. Indeed, in multichannel patches the amplitude ratio between the persistent component and the peak, transient component of average currents was very similar to that found in macroscopic, whole-cell Na^+ currents from the same neurones (Magistretti & Alonso, 1999*b*). This suggests that in EC layer II neurones transient and persistent Na^+ channel activity is distributed uniformly across the somatic and proximal dendritic segments. Interestingly, the Na^+ channels expressed by hippocampal CA1 pyramidal cells also appear to be uniformly distributed across the soma-dendritic axis, although a slight decrease in channel density may be present at distances greater than 200 μm from the soma (Magee & Johnston, 1995*b*).

A basic feature that would be expected in a current involved in the processing of subthreshold excitatory synaptic potentials is its low threshold of activation. Importantly, a significant recruitment of persistent Na^+ channel activity at voltage levels as low as −60 mV was actually observed in our study. Indeed, the general features of the *I–V* relationship of persistent currents returned by ensemble averagings, including its low threshold of activation, closely resembled those typical of whole-cell I_{NaP} recorded in the same cells (Magistretti *et al.* 1999*b*; Magistretti & Alonso, 1999*b*).

At least two distinct single-channel behaviours representing potential sources of dendritic persistent Na^+ current were found. First of all, single channels opening with high probability during long-lasting depolarising pulses, and maintaining this 'persistent' gating behaviour throughout prolonged recording periods, were observed. The fact that in some oligochannel patches a stable, persistent Na^+ channel activity could be attributed to one single channel is consistent with the idea that, in mammalian neurones, the task of generating I_{NaP} is mainly entrusted to a distinct subset of Na^+ channels, each of which spends at least a substantial fraction of its time in a persistent gating modality (see Magistretti *et al.* 1999*b*). Indeed, persistent Na^+ channel activity was characterised by a significantly higher single-channel conductance than early, short-lived Na^+ channel openings responsible for transient Na^+ current generation. Basically the same results have also been previously obtained in somatic cell-attached recordings (Magistretti *et al.* 1999*b*). Again, this finding points to the biophysical specificity of Na^+ channels devoted to I_{NaP} generation. Whether this specificity is the consequence of the existence of a molecularly distinct, 'persistent' Na^+ channel species, or of a switch, possibly under modulatory control, to a long-lived, higher-conductance, persistent gating modality of otherwise classical, transient Na^+ channels remains to be established.

A different type of persistent single-channel event was also observed, namely the sporadic occurrence of long-lasting openings in patches otherwise exhibiting only transient Na^+ channel activity. Very similar events have already been reported in a single-channel study carried out in neocortical pyramidal neurones (Alzheimer *et al.* 1993), and have been attributed to occasional, short-lived forays of transient Na^+ channels into a non-inactivating gating modality. Such a behaviour, named 'modal gating', may thus give some additional contribution to I_{NaP} generation in dendritic membranes of entorhinal neurones.

In conclusion, proximal dendrites of EC layer II principal neurones are sites of significant expression of persistent Na^+ channel activity. The single-channel events involved may range from sporadic modal gating of classical, transient Na^+ channels to the activation of a distinct channel pool specifically devoted to stable, persistent gating. Dendritic persistent Na^+ channel activity exhibits the same basic biophysical properties as the persistent activity observed in the somata of the same cells, including low threshold of activation and relatively high single-channel conductance. In the dendrites of EC layer II principal neurones, the low-threshold, sustained Na^+ channel activity we observed may be involved in local amplification of excitatory synaptic inputs, as persistent Na^+ conductances have been shown to be in neocortical and hippocampal neurones, and, perhaps, in more complex dynamic interactions between the soma and the multiple primary dendrites that the stellate cells possess.

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