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Inhibition of slow Ca^{2+}-activated K^+ current by 4-aminopyridine in rat hippocampal CA1 pyramidal neurones

*,[1]Mogens Andreasen

[1]Department of Physiology, University of Aarhus, DK-8000 Århus C, Denmark

1 The effect of 4-aminopyridine (4-AP) on the slow afterhyperpolarization (sAHP) seen after high frequency dendritic or somatic firing was investigated in rat hippocampal CA1 pyramidal neurones (PC). Intracellular recordings were obtained from the distal apical dendrites and somata and suprathreshold depolarizing current pulses were used to evoke a sAHP. The sAHP was blocked by low concentrations of carbacholine (Cch) but insensitive to high concentrations of apamin.

2 In the presence of extracellular 4-AP, the first dendritic sAHP evoked was reduced compared to a maximal sAHP evoked in the absence of 4-AP. The reduction was evident at submillimolar concentration and increased to about 80% with 4 mM 4-AP.

3 The stability of the 4-AP-induced block was affected by the type of anion used in the electrode solution. With K^+ acetate (KAc) or K^+ methylsulphate ($KMeSO_4$) containing electrodes, the block was progressively removed during the initial 300–400 s of recordings. With KCl containing electrodes, the block remained stable and was 10% larger than that obtained with acetate. Detailed investigations showed that intracellular acetate promotes the removal of the 4-AP-induced block in an activity-dependent manner.

4 Intracellularly applied 4-AP also induced an acetate-sensitive block of the dendritic sAHP.

5 4-AP also blocked the somatic sAHP and the stability of the block showed the same sensitivity towards anions as the dendritic sAHP.

6 Thus 4-AP appears to block the slow Ca^{2+}-activated K^+ current underlying the sAHP in a complex manner which is sensitive to certain types of anions.
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Keywords: 4-aminopyridine; Ca^{2+}-activated K^+ current; sAHP; acetate; $MeSO_4$; chloride; dendrites; pyramidal neurones; CA1; hippocampus

Abbreviations: AP5, DL-2-amino-5-phosphonovaleric acid; 4-AP, 4-aminopyridine; BIC, bicuculline; Cch, carbacholine; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DAP, depolarizing afterpotential; GABA, γ-aminobutyric acid; HEPES, N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]; KAc, K^+ acetate; $KMeSO_4$, K^+ methylsulphate; NMDA, N-methyl-D-aspartate; PC, pyramidal neurone; R_{in}, membrane input resistance; RMP, resting membrane potential; sAHP, slow afterhyperpolarization; V_m, membrane potential.

Introduction

Most types of neurones contain a large number of different types of K^+ currents which are involved in the regulation of many aspects of neuronal behaviour like membrane excitability, repolarization of action potentials, firing frequency, transmitter release and transmitter action (Halliwell, 1990; Storm, 1990). Furthermore, the particular composition of different types of K^+ currents is often one of the key factors in determining the characteristics of a given type of neurone and therefore ultimately of the different functional areas in the central nervous system. It is therefore important to study the individual types of K^+ currents to gain more knowledge about their kinetics, pharmacology and molecular structure. An important tool in the investigation of ionic currents is the use of chemical compounds to selectively activate or block individual currents. However, one of the problems with many of the compounds used to block K^+ currents are their general lack of specificity and variable potency in different types of preparations (Cook & Quast, 1990), which makes interpretation of results obtained with these drugs at best difficult. One group of compounds, which are used extensively as K^+ current blockers are the aminopyridines and in particular the compound 4-AP, which together with tetraethylammonium is one of the most commonly used K^+ channel blocker. The selectivity and potency of 4-AP depends greatly on the type of neurone and K^+ current investigated (Cook & Quast, 1990). In mammalian neurones, several types of voltage-dependent K^+ currents are blocked by 4-AP, but with a marked difference in sensitivity. At submillimolar concentration, 4-AP primarily blocks a fast activating-noninactivating K^+ current, termed I_D. In millimolar concentration 4-AP also blocks a transient K^+ current, termed I_A, and at concentrations above 5 mM the delayed rectifying K^+ current is affected (Storm, 1990). As regards to the different types of Ca^{2+}-activated K^+ currents in neurones, aminopyridines are generally reported to be either without effect or to enhance this type of K^+ current (Segal & Barker, 1986; Cook & Quast, 1990; Storm, 1990; Osmanovic & Shefner, 1993). The enhancing effect is most likely indirect and caused by an increase in Ca^{2+} influx during firing as a result of either

*Author for correspondence; E-mail: MA@FI.AU.DK

blocking the aforementioned voltage-dependent K^+ currents or a direct effect on high threshold voltage-dependent Ca^{2+} currents (Segal & Barker, 1986). There is, however, a few examples of Ca^{2+}-activated K^+ currents which are blocked by 4-AP. In rat magnocellular neurosecretory cells a transient Ca^{2+}-activated K^+ current is blocked by 4-AP with an IC_{50} of about 1 mM (Bourque, 1988). Also in human pregnant nonlabour myometrical cells a large conductance Ca^{2+}-activated K^+ current has been described which is blocked by 4-AP (0.1–1 mM) (Khan *et al*., 1997).

As in many types of neurones, the hippocampal CA1 PCs are in the possession of a slow Ca^{2+}-activated K^+ current that is activated by the Ca^{2+} influx during somatic firing. This current is responsible for the spike frequency adaptation observed during high frequency firing and also for the following sAHP (Lancaster & Adams, 1986; Storm, 1990). A similar type of current is also observed in intracellular recordings from the apical dendrites of CA1 PC (Andreasen & Lambert, 1995b). The present study is based on the preliminary observation that whenever 4-AP was present the amplitude of the sAHP mediated by the dendritic Ca^{2+}-activated K^+ current was smaller than that of sAHP evoked in the absence of 4-AP. This was unexpected considering the pronounced increase in Ca^{2+} influx seen during perfusion with 4-AP (Andreasen & Lambert, 1995a). These observations suggested that 4-AP may have a so far unknown effect on slow Ca^{2+}-activated K^+ currents. Because of the importance of 4-AP as a tool in the investigation of K^+ currents the present study was undertaken with the purpose of investigating this new action of 4-AP in details. Evidence is presented which shows that 4-AP does indeed block both the dendritic and somatic slow Ca^{2+}-activated K^+ current in a concentration-dependent manner. Furthermore, the study revealed that the stability of the 4-AP-induced block was sensitive to the intracellular presence of the anions acetate or $MeSO_4$.

Methods

Slice preparation

Experiments were performed on hippocampal slices prepared from 75 male Wistar rats (250–300 g). After anaesthetizing with chloroform, the rat was decapitated and the brain removed quickly and placed in a HEPES (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]) buffered Ringer solution (see below) at 4°C. The hippocampus was dissected free and slices (400 μm thick) were cut on a McIlwain tissue chopper. One slice was immediately transferred to the recording chamber, and placed on a nylon-mesh grid at the interface between warm (31–33°C) standard Ringer solution (see below) and warm humidified carbogen (95% O_2 and 5% CO_2). Perfusion flow rate was 1 ml min^{-1}. The slice was allowed to rest for at least 1 h before recordings were started. The remaining slices were placed in HEPES buffered Ringer solution, bubbled with carbogen, and stored at room temperature.

Electrophysiological measurements and data analysis

Intracellular recordings from CA1 PCs were made using borosilicate glass microelectrodes (1.2 mm o.d., Clark Electromedical) filled with either $KMeSO_4$ (2 M), KAc (2–4 M), KCl (2–3 M), or different combinations of KAc and KCl. In some experiments 4-AP (50–250 mM) were applied intracellularly dissolved in 4 M KAc. In all instances the tip resistance was 60–90 MΩ. Penetrations of the distal apical dendrites were made at least 250 μm from the superficial border of stratum pyramidalis and recordings were identified as dendritic on the basis of their similarity to those reported previously from histochemically verified dendritic recordings (Andreasen & Lambert, 1995a). Penetrations were accepted for analysis if the resting membrane potential (RMP) was stable and more negative than −50 mV and the membrane input resistance (R_{in}) was $\geqslant$10 MΩ.

Conventional recording techniques were employed, using a high input impedance amplifier (Axoclamp 2A, Axon Instruments Inc.) with bridge-balance and current injection facilities. Results were digitized on-line using a Labmaster A/D converter and pCLAMP acquisition software (Axon Instruments Inc.) and recorded for off-line analysis using a modified digital audio processor (Sony PCM-701es) and a video tape-recorder. Unless otherwise noted the pulse paradigm used to evoked a sAHP consisted of an initial short hyperpolarizing current pulse (intensity: −0.3 nA, duration: 50 ms) followed by a suprathreshold depolarizing current pulse (duration: 50 or 300 ms). The pulse paradigm was repeated at a standard frequency of 0.1 Hz. The purpose of the initial hyperpolarizing current pulse was to ensure adequate bridge balance and to monitor changes in R_{in} during the experiments. In control conditions the relationship between the amplitude of the sAHP and the current intensity was first determined and from this, an intensity (ranging from 1 to 1.6 nA) was chosen which ensured maximal activation of the sAHP. In the presence of 4-AP, which greatly increases the excitability of the distal apical dendrites (Andreasen & Lambert, 1995a), a fixed intensity of 0.6 nA was used which reliably evoked marked compound spiking in all dendrites tested. Compound spiking is defined as burst-like firing consisting of an initial fast Na^+-dependent spike followed by one or more secondary slow Ca^{2+}-activated spikes of variable amplitude (Andreasen & Lambert, 1995a). An example of compound spiking is shown in Figure 1Ab.

The amplitude of the sAHP was calculated as the difference between the pre-pulse membrane potential (V_m), measured just before the depolarizing current pulse, and the post-pulse V_m measured 150 ms after the current pulse (see Figure 1C). The reason for choosing a post-pulse delay of 150 ms was so as to minimize contamination from the initial fast component of the post-pulse afterhyperpolarization which last for about 50 ms (Andreasen & Lambert, 1995b). All analyses were performed using pCLAMP software. For exponential fitting of the data, a monoexponential function with the following expression was used:

$$y = y_o + a(1 - \exp^{-t/\tau}) \quad (1)$$

where y_o is the amplitude at t equal zero, y is the amplitude at time t, a is defined as $y_{max} - y_o$ (y_{max} is the maximal amplitude) and τ is the time constant. The exponential fitting was performed using Sigmaplot (SPSS Inc.) Values are given as mean ± s.e.m. unless otherwise noted. Data were statistically evaluated using the paired Student's t-test and considered to be significant at $P \leqslant 0.05$.

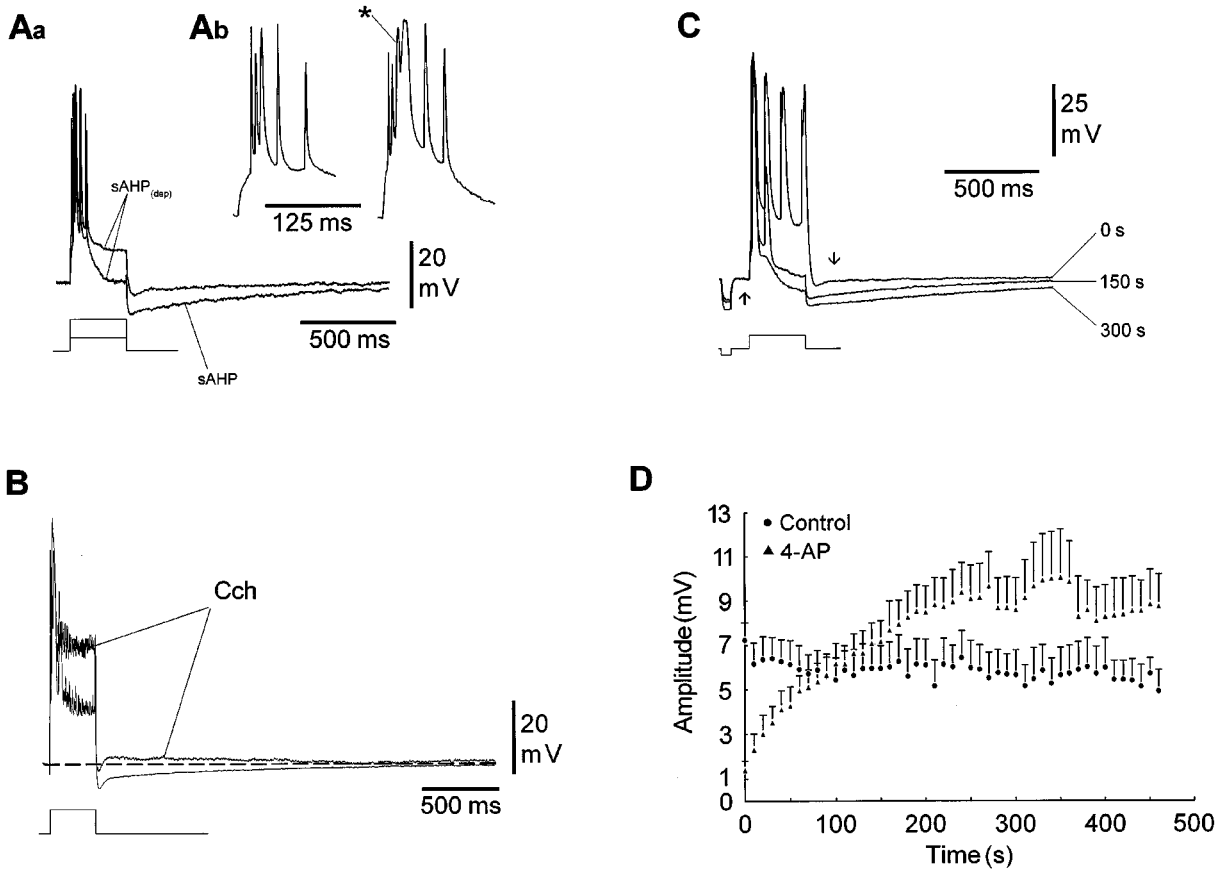

Figure 1 The effect of extracellularly applied 4-AP on the dendritic sAHP. (Aa) Dendritic responses to two depolarizing current pulses (intensity: 0.6 and 1.4 nA; 300 ms). (Ab) The initial cluster of spikes in (Aa) shown on an expanded time scale. Note the presence of compound spiking (marked by *) at high stimulation intensity. (B) Dendritic response to a depolarizing current pulse (1.2 nA) before and after 8 min perfusion with carbacholine (Cch, 1 μM). Note that both the $sAHP_{(dep)}$ and the sAHP are blocked by Cch whereas the initial fast component of the post-pulse afterhyperpolarization is unaffected. (C) In the presence of 4-AP (4 mM), a depolarizing current pulse (intensity: 0.6 nA; 300 ms), initially evoked repetitive compound firing followed by a small sAHP. Repeated stimulation resulted in a progressive increase in both the $sAHP_{(dep)}$ and the sAHP. The depolarizing current pulse is preceded by a small hyperpolarizing current pulse (intensity: -0.3 nA, 50 ms). The numbers after each trace in this and the following Figures indicate the time at which the response was evoked. (D) The averaged change in the amplitude of sAHPs evoked in the absence or presence of 4-AP (4 mM). Each point represents the mean $\pm$ s.d. (*n* equals 10 and 6 in the absence and presence of 4-AP, respectively). RMP: (A) -73 mV; (B) -65 mV; (C) -68 mV.

Drugs and solutions

The composition of the HEPES buffered Ringer solution (in mM): NaCl 120, KCl 2.00, KH_2PO_4 1.25, $NaHCO_3$ 20, HEPES acid 6.6, HEPES salt 2.6, $CaCl_2$ 2, $MgSO_4$ 2, D-glucose 10; bubbled with Carbogen (pH 7.4). The composition of the standard Ringer solution was (in mM): NaCl 124, KCl 3.25, NaH_2PO_4 1.25, $NaHCO_3$ 20, $CaCl_2$ 2, $MgSO_4$ 2, D-glucose 10, bubbled with Carbogen (pH 7.3). All experiments were performed in the presence of DL-2-amino-5-phosphonovaleric acid (AP5, 50 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM), bicuculline methobromide (BIC, 10 μM) and CGP 55845A (2 μM) in order to block N-methyl-D-aspartate (NMDA), non-NMDA, γ-aminobutyric acid $(GABA)_A$ and $GABA_B$ receptors, respectively. Unless otherwise noted 4-AP was perfused for at least 30 min before recordings were started to allow the effects of 4-AP to stabilize.

All pharmacological compounds were made up in aqueous stock solutions of 100–1000 times the required final concentration and diluted in the standard Ringer solution as appropriate. 4-AP, BIC and Cch were purchased from Sigma, AP5 and CNQX from Tocris, sodium acetate were purchased from Merck, apamin was purchased from Alomone Labs and CGP 55845A was kindly provided by Novartis.

Results

The passive membrane properties of CA1 pyramidal neurones

The data presented here are based on 190 intradendritic and 14 intrasomatic recordings from CA1 PCs. Out of the 190 intradendritic recordings 151 were performed with KAc containing electrodes with a mean RMP of -66.5 ± 0.3 mV and a mean R_{in} of 30 ± 0.7 MΩ. Thirty-four recordings were performed with KCl containing electrodes with a mean RMP of -66.8 ± 0.6 mV and a mean R_{in} of 39.8 ± 2.2 MΩ. The remaining five recordings were performed with $KMeSO_4$

containing electrodes with a mean RMP of -67 ± 2 mV and a mean R_{in} of 47.8 ± 6.9 MΩ. There was no significant difference in RMP between the three groups, but R_{in} was significantly higher ($P<0.001$) when KCl and $KMeSO_4$ containing electrodes were used. Nine of the intrasomatic recordings were performed with KAc containing electrodes with a mean RMP of -66.1 ± 0.8 mV and a mean R_{in} of 40.8 ± 2.2 MΩ. The remaining recordings were performed with KCl containing electrodes with a mean RMP of -65.8 ± 1.5 mV and a mean R_{in} of 50.6 ± 5.2 MΩ. There was no significant difference between the two groups.

The effect of extracellular 4-AP on a slow dendritic afterhyperpolarization

In the presence of CNQX (10 μM), AP5 (50 μM), BIC (10 μM) and CGP 55845A (2 μM), which constitute the control medium, a suprathreshold depolarization of the dendritic membrane evoked a complex response consisting of an initial cluster of spikes followed by a slow hyperpolarization ($sAHP_{(dep)}$, Figure 1Aa). After termination of the current pulse the membrane hyperpolarized with respect to RMP for several seconds (Figure 1A). This post-pulse afterhyperpolarization often had an initial fast decaying phase followed by a prolonged slowly decaying phase (sAHP, Figure 1B). The size of the $sAHP_{(dep)}$ and the post-pulse sAHP depends on the number, frequency and type of spikes evoked during the current pulse and is particularly pronounced when Ca^{2+}-spikes are present as in compound spiking (Figure 1A). As exemplified in Figure 1A, there is a close correlation between the size of the $sAHP_{(dep)}$ and the size of the sAHP indicating that the sAHP is due to the continued activation of the same slow Ca^{2+}-activated K^+ current which causes the $sAHP_{(dep)}$ (Andreasen & Lambert, 1995b). Both the $sAHP_{(dep)}$ and the sAHP is blocked by low concentrations of carbacholine (Figure 1B, $n=4$) indicating that the slow dendritic Ca^{2+}-activated K^+ current is similar to that mediating the corresponding somatic sAHP (Lancaster & Nicoll, 1987; Stocker *et al*., 1999).

As reported earlier (Andreasen & Lambert, 1995a) the presence of 4-AP (4 mM) resulted in repetitive firing of compound spikes during the depolarizing current pulse (Figure 1C). In control medium compound spiking was always associated with a very pronounced sAHP (Figure 1A). However, in the presence of 4-AP (4 mM), both the $sAHP_{(dep)}$ and the post-pulse sAHP were initially very small or even absent despite pronounced compound spiking (Figure 1C). In 75% (6/8) of the experiments, performed in the presence of 4-AP, repeated activation at 0.1 Hz resulted in a progressive increase in the size of the $sAHP_{(dep)}$ and the sAHP with a concomitant reduction in firing during the current pulse (Figure 1C). In the remaining 25% of the experiments, the $sAHP_{(dep)}$ and sAHP were blocked during the entire recording period (300–400 s). Whereas V_m remained constant during the recordings, R_{in} often decreased after the first few stimulations as shown in Figure 1C. However, there was no correlation between the change in R_{in} and the progressive increase in the $sAHP_{(dep)}$ and sAHP. On average, the mean amplitude of the first sAHP evoked in the presence of 4 mM 4-AP was 1.4 ± 0.4 mV ($n=6$, Figure 1D) which was significantly ($P<0.001$) smaller than the maximal amplitude of sAHPs evoked in the absence of 4-AP (7.22 ± 0.8 mV, $n=10$). Furthermore, within the initial 450 s of recordings the sAHPs evoked in the control medium showed a slight run-down to an average value of 5.7 ± 0.7 mV ($n=6$) which was, however, not significant. In the presence of 4-AP, however, the sAHP increased progressively to a maximal plateau value of 10.1 ± 2.2 mV after 350 s of recordings followed by a slight run-down (Figure 1D). With respect to the first sAHP evoked in the control medium, the first sAHP evoked in the presence of 4-AP was reduced in a concentration-dependent manner as shown in Figure 2A. Here the averaged amplitude of the first sAHP evoked in the control medium was set to 100%. In the presence of 0.25 and 0.5 mM 4-AP, the amplitude of the initial sAHP was reduced to 82.8 ± 3.7% and 80.7 ± 6.7%, respectively, which was not significant ($P>0.05$). However, with 2, 4 and 8 mM, the amplitude of the first sAHP was significantly ($P<0.001$) reduced to 56 ± 6.7%, 19.4 ± 5.8% and 6.1 ± 6.25%, respectively, of the sAHPs evoked in the control medium. In Figure 2B, the averaged amplitude of sAHPs recorded in the absence or presence of different concentrations of 4-AP is plotted in relation to the recording time. The data collected in the absence of 4-AP were averaged ($n=10$) and normalized with respect to the first sAHP evoked. In order to compensate for the aforementioned run-down the data collected in the presence of 4-AP were averaged (n between 8 to 5) and normalized with respect to sAHPs recorded in the control medium at a corresponding recording time. The time course of the initial increase in the sAHP was adequately fitted by a monoexponential function (see Methods) giving the following time constants: 46.0 s (0.25 mM), 60.2 s (2 mM), 136.9 s (4 mM) and 62.1 s (8 mM). Except for the time constant at 4 mM 4-AP, these results indicate that the time-course of the initial increase is not concentration-dependent. The reason for the larger time constant in the presence of 4 mM 4-AP is not at the moment apparent. In the presence of 4-AP, up to 4 mM, the amplitude of the sAHP reached a similar maximal plateau about 40–50% larger that the amplitude of sAHPs evoked in the absence of 4-AP. In the presence of 8 mM, the plateau was somewhat lower but still higher than that obtained in control medium after 300 s of recording (Figure 2B). The larger amplitude attained in the presence of 4-AP is likely to be due to the increase in compound spiking. These results indicate that 4-AP blocks the current underlying the $sAHP_{(dep)}$ and sAHP in a concentration-dependent manner and that the block is removed during the initial 300–400 s of stimulation.

If the activation of the current underlying the sAHP is important for the removal of the 4-AP-induced block it should be possible to re-establish the block by introducing a pause in the activation of the sAHP. To test this prediction experiments were performed in which stimulation was continued until the sAHP amplitude had reached a plateau at which point the stimulation was stopped for several minutes and then continued. These experiments were done in the presence of either 4 or 8 mM 4-AP. One example of such an experiment is shown in Figure 2C. Here the sAHPs were evoked in the presence of 4 mM 4-AP and the pause lasted for 6 1/2 min. Following the pause a large part of the 4-AP-induced block had re-established and stimulation resulted in renewed removal of the block. An estimate of the amount of block re-

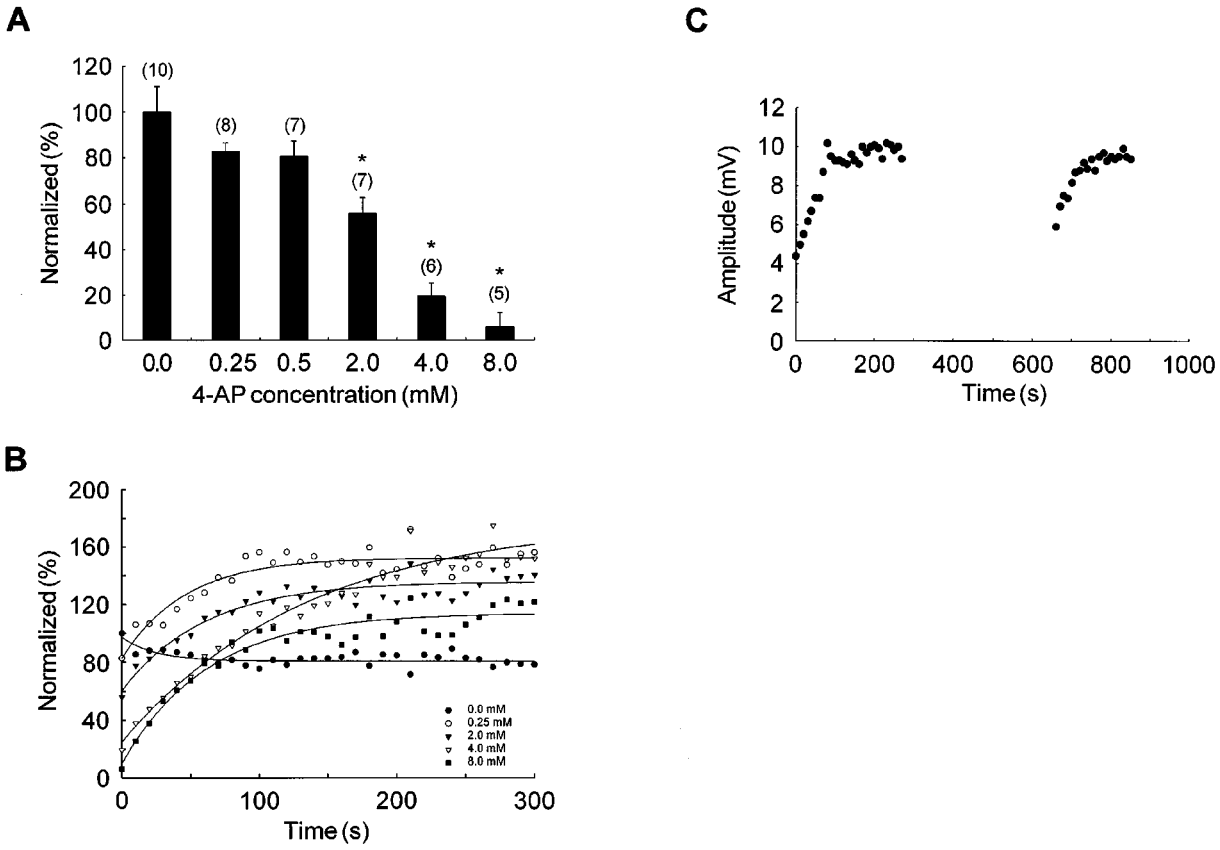

Figure 2 The effect of 4-AP is concentration- and activity-dependent. (A) The 4-AP-induced reduction in the amplitude of the first sAHP evoked. Here all data (mean ± s.e.m., *n* is indicated in parenthesis above each bar) has been normalized with respect to the first sAHP evoked in control medium. (*) indicates statistical significance. (B) Shows the averaged change in the amplitude of sAHPs evoked in the presence of different concentrations of 4-AP. Error bars have been omitted for clarity and the lines represent the best monoexponential fits. (C) This shows the time-dependent change in sAHPs recorded in the presence of 4-AP (4 mM). Following the establishment of a maximal plateau, stimulation was stopped for 6½ mins and then continued. During the pause 70% of the block was re-established. RMP: −65 mV.

established after the pause was calculated using the following equation:

$$(B - C)/(B - A) \quad (2)$$

where *A* and *B* is the amplitude of the first and last sAHP, respectively, evoked before the pause and *C* is the amplitude of the first sAHP evoked after the pause. This method disregards any run-down which may have occurred during the pause which would tend to bias the results towards a slight overestimation of the amount of block re-established. However, as seen in Figure 2C, the amount of run-down during the pause was minimal. In the example in Figure 2C, the pause had resulted in about 70% of the block being re-established. On average a 5 min pause resulted in 39.8 ± 11.1% (mean ± s.d., $n = 5$) of the initial block being re-established. Increasing the pause to between 5 to 10 min increased the amount of block re-established to 61.3 ± 18.5% ($n = 3$) and in the one experiments in which the dendritic recording was kept long enough to introduce a 16 min pause the block was nearly completely (91.7%) re-established. The amount of block obtained during the pause was independent of whether 4 or 8 mM 4-AP was used. Together, these results indicate that the activation of the current underlying the sAHP is necessary for both the induction and maintenance of the unblocking process.

The effect of intracellular 4-AP on the dendritic sAHP

From studies of 4-APs effect on voltage-dependent K^+ currents it appears that 4-AP primarily works from the cytoplasmic site of the membrane (Thompson, 1982; Kirsch & Narahashi, 1983; Kirsch & Drewe, 1993a; Kirsch *et al.*, 1993b; Yao & Tseng, 1994; Russell *et al.*, 1994; Stephens *et al.*, 1994; Yamane *et al.*, 1995; Jerng *et al.*, 1999). It was therefore tested whether intracellularly applied 4-AP had a similar effect as extracellular 4-AP. The following experiments were performed with electrodes containing 4-AP (50–250 mM) and stimulation was started as soon as the penetration had stabilized. Exemplary traces from one such experiment is shown in Figure 3A. Here stimulation was started 80 s after penetration of the distal apical dendrite with an electrode containing 100 mM 4-AP. The changes seen was similar to those seen with extracellular 4-AP, with a progressive increase in the post-pulse sAHP and a concomitant reduction in firing (Figure 3A). Intracellularly applied 4-AP also enhanced the activation of large Ca^{2+}-spikes, however, this effect was somewhat unreliable resulting occasionally in a response with a small or absent Ca^{2+}-component and a correspondingly reduced sAHP. Figure 3B

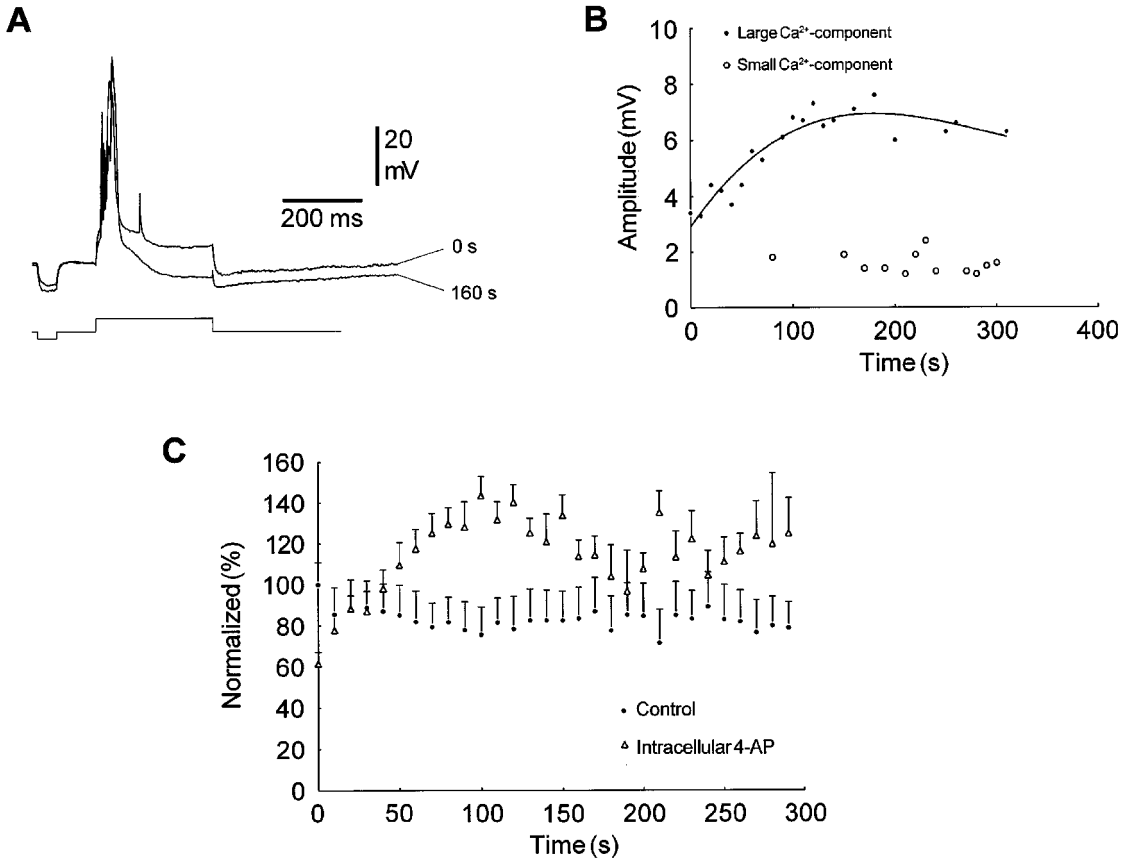

Figure 3 Intracellularly applied 4-AP also blocked the dendritic sAHP. (A) Superimposition of response number 1 and 17 recorded with a 4-AP (100 mM) containing electrode. The first response was evoked 80 s after penetration. RMP: −62 mV. (B) Shows the time-dependent change in the amplitude of the sAHPs shown in (A). The closed and open circles indicate responses in which compound spiking contained a large or small Ca^{2+}-component, respectively. The sAHPs seen after compound spiking with a large Ca^{2+}-component changed in a similar way as sAHPs evoked in the presence of extracellular 4-AP. The line is just a trend-line. (C) Shows the averaged (mean±s.d., $n=7$) time-dependent change in the amplitude of sAHP's recorded with 4-AP (50–250 mM) containing electrodes. The control data are identical to those shown in Figure 2A. The data, measured in the presence of 4-AP were normalized with respect to sAHPs evoked at a similar time but in the absence of 4-AP.

shows the time-dependent change in the sAHP shown in Figure 3A. As with extracellular 4-AP, the amplitude of the first sAHP evoked was reduced compared to that of sAHPs evoked in control medium without 4-AP. Furthermore, the sAHP also increased in amplitude until reaching a maximal plateau after about 150–200 s. Note the lack of change in the sAHP following a small Ca^{2+}-component. Out of 15 experiments, three were excluded from further analysis because the compound spiking increased in parallel to the sAHP. Five experiments were excluded because of unstable sAHP and/or fast run-down of the sAHP. In the remaining seven experiments, the sAHP increased in amplitude although the preceding Ca^{2+}-component remained unchanged or even decreased. Figure 3C shows the average change in the sAHP amplitude in these seven experiments. The data obtained in the presence of 4-AP were normalized with respect to sAHPs evoked in control medium at a corresponding recording time (as in Figures 1D and 2B). The amplitude of the first sAHP evoked was significantly ($P<0.05$) reduced to 60.9±5.8% ($n=7$) of sAHP evoked in the absence of 4-AP. On average, the activation of the first sAHP took place 82.9±37.7 s (mean±s.d.) after the penetration. The sAHP amplitude reached a maximal plateau after about 100 s which was almost as large as that obtained with extracellular 4-AP (compare Figures 2B and 3C). Fitting the initial 120 s to a monoexponential function gave a time constant of 81.3 s which was slightly larger than that measured at most concentrations of extracellular 4-AP.

Acetate modulates the 4-AP induced block of the dendritic sAHP

There are several lines of evidence which indicate that anions can interfere with many aspects of neuronal behaviour such as the activation of Ca^{2+}-activated K^+ channels and delayed rectifying K^+-channels (Adams & Oxford, 1983; Cullen & Carlen, 1992; Zhang *et al*., 1994), intracellular release of Ca^{2+} (Morita *et al*., 1980) and activation of G-proteins (Nakajima *et al*., 1992). Furthermore, organic and inorganic anions often seem to have opposite effects (Morita *et al*., 1980; Adams & Oxford, 1983; Zhang *et al*., 1994). The results so far described were all obtained with electrodes containing the organic anion acetate. To investigate the importance of the type of anion used, experiments were repeated with KCl (2–3 M) and $KMeSO_4$ (2 M) containing electrodes. With both types of anion the amplitude of the first sAHP evoked in the

presence of 4-AP (4 mM) was small considering the pronounced compound spiking evoked during the depolarizing current pulse. However, there was a marked difference in the amount of unblocking observed. With $KMeSO_4$ containing electrodes the sAHP increased progressively as seen with KAc containing electrodes from an initial value of 2.3 ± 0.7 mV ($n=5$) to 8.6 ± 0.7 mV after 300–400 s of recording (not shown). With KCl containing electrodes, however, the amplitude of the first sAHP was unchanged even after 300–400 s of recording (Figure 4A). These results therefore indicate that there is some kind of interaction between the anions and the 4-AP-induced block, however, they do not provide any clues as to whether acetate or $MeSO_4$ is promoting the removal of the block or if Cl^- is stabilizing it. To investigate this a comparison between acetate and Cl^- containing electrodes was made. Because loading the cells with Cl^- may have a depressant effect on the sAHP (Zhang *et al*., 1994) the dendritic sAHP was first characterized in the absence of 4-AP. Overall the control sAHPs obtained with Cl^- were similar to those recorded with acetate, except that their averaged size was somewhat smaller (4.75 ± 0.42 mV, $n=6$). This difference was, however, not significant ($0.05 < P < 0.1$). In the presence of 4-AP (4 mM), the compound spiking was similar to that observed with acetate (compare Figure 1C and Figure 4Ab). However, despite the large increase in Ca^{2+} influx, the response was followed by an afterdepolarization (DAP) and not a sAHP (Figure 4Aa). In most cases there was a small sAHP initially which reversed over time to a DAP. Because there was no difference between results obtained with 2 and 3 M KCl all data from these experiments were pooled. In Figure 4B, the average results with ($n=11$) and without ($n=6$) 4-AP are plotted in relation to recording time. The amplitude of the first sAHP evoked in the presence of 4-AP was 0.4 ± 0.6 mV, which was significantly different ($P<0.001$) from that of a maximal sAHP evoked in the absence of 4-AP. The average reduction of the first sAHP evoked was larger with Cl^- than with acetate containing electrodes (91.5% compared to 81.5%) indicating a more effective block with Cl^- as anion. Furthermore, the block remained stable over time and in fact showed a tendency to increase resulting in a convertion of the sAHP into a DAP after about 200 s of stimulation (Figure 4B). To decide between whether it is acetate that is promoting the removal of the block, or Cl^- which is stabilizing it the above experiments were repeated using electrodes containing different combinations of KCl and

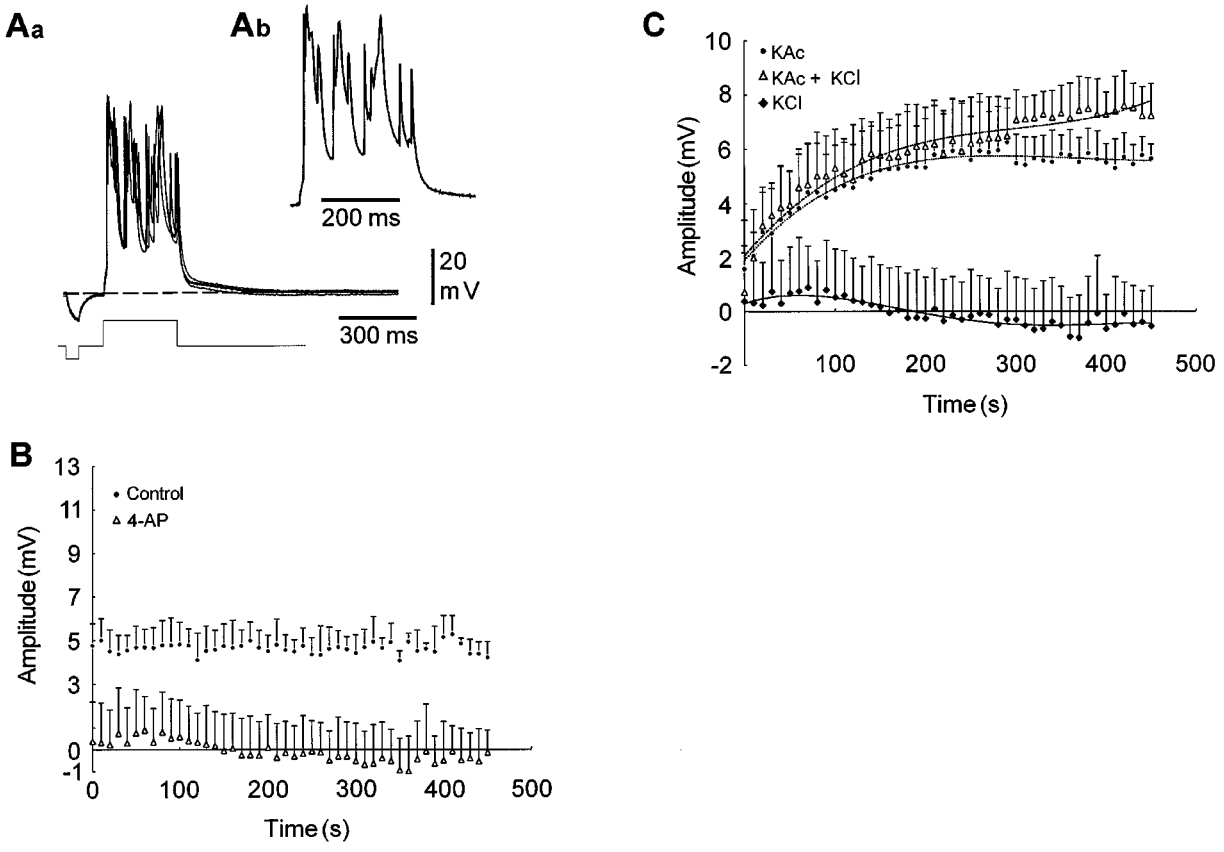

Figure 4 Anions affects the stability of the 4-AP-induced block. (Aa) Superimposition of responses (number: 1, 16, 32 and 48) recorded with a 3 M KCl containing electrode in the presence of 4-AP (4 mM). Note the post-pulse DAP. The stippled line marks the pre-pulse RMP. (Ab) Shows one of the response on an expanded time scale. RMP: −70 mV. (B) Comparison between sAHPs evoked with 2–3 M KCl containing electrodes in the absence or presence of 4-AP (4 mM). In the absence of 4-AP ($n=6$), the sAHP stayed relatively constant with a mean amplitude of about 5 mV. In the presence of 4-AP ($n=8$), the sAHP was nearly blocked and reversed to a DAP after about 200 ms of recording. (C) Shows the time-dependent change in sAHPs evoked in the presence of 4-AP (4 mM) and recorded with electrodes containing either 2 M KAc ($n=5$), 2 M KAc and 2 M KCl ($n=7$) or 2–3 M KCl ($n=11$). A progressive removal of the 4-AP-induced block is only seen when acetate is present in the electrodes. All date are presented as mean ± s.d.

KAc. First, recordings were done with KAc (2 M) containing electrodes which gave qualitatively similar results as seen with 4 M KAc (Figure 4C). The amplitude of the first sAHP evoked was of similar size (1.6 ± 0.8 mV, $n=5$) as was the time-course of the increase in the sAHP. However, the maximal amplitude obtained after unblocking was somewhat smaller. Similar results were also obtained when KAc (2 M) was combined with KCl (2 M), except that the maximal amplitude now was somewhat larger (Figure 4C). With electrodes containing KAc (1 M) and KCl (3 M) the amplitude of the first sAHP was 2.03 ± 1.3 mV ($n=3$), and in only one case was the sAHP observed to increase over time (not shown). As stated earlier, no increase in the sAHP was observed with electrodes containing only 2 M KCl. Together these experiments therefore indicate that it is the presence of acetate which, in a concentration-dependent way, changes the interaction between 4-AP and the current mediating the sAHP.

Extracellular acetate has been shown to modulate the activation of the sAHP, possibly through an activation of adenosine receptors, an effect which, at least partly, is occluded by loading the cell with acetate (Cullen & Carlen, 1992). However, extracellular perfusion with acetate, at concentrations (0.5–1 mM) which gives maximal effect on the sAHP in granule cells (Cullen & Carlen, 1992), had no effect on the stability of the 4-AP-induced block of the sAHP recorded with KCl containing electrodes ($n=3$, not shown).

From the above experiments it may be predicted that if the PCs are already loaded with the anion when 4-AP is introduced, the sAHP should be blocked when the anion is Cl^- but increase when the anion is acetate. To test this prediction, 4-AP (2–4 mM) was added to the perfusion medium after 10 to 16 control responses had been recorded. With Cl^- containing electrodes, the introduction of 4-AP resulted in a progressive reduction of the sAHP, as predicted, with a maximal effect obtained after about 300 s ($n=5$, Figure 5Aa). The decrease in the sAHP occurred despite a concomitant increase in compound spiking (Figure 5Ab). In the example shown in Figure 5A, the effect of 4-AP was partly reversed, however, this was not a consistent finding. Surprisingly, a similar effect was seen when acetate containing electrodes were used (Figure 5Ba). As expected, the introduction of 4-AP resulted in an increase in both the size of the compound spike and the firing frequency (Figure 5Bb). However, at the same time there was an unexpected reduction in the sAHP until it was blocked in spite of the continued increase in firing ($n=5$, Figure 5B). The block followed a similar time-course as that seen with Cl^- and was also stable for as long as 4-AP was present. No reversal was seen upon returning to control medium.

The effect of apamin on the 4-AP sensitive dendritic sAHP

It has generally been accepted that the slow Ca^{2+}-activated K^+ current mediating the somatic sAHP in CA1 pyramidal neurones is insensitive to the bee-venom toxin apamin (Sah, 1996). However, evidence for the presence of an apamin-sensitive AHP has now emerged (Norris *et al*., 1998; Sah & Clements, 1999; Stocker *et al*., 1999). In the present study, perfusion with high concentration (1 μM) of apamin for more than 25 min had no effect on the dendritic 4-AP-sensitive sAHP ($n=2$, not shown). Futhermore, in five other experiments in which the slice had been incubated with apamin (1 μM) for at least 30 *min*, large sAHPs could still be evoked, which in all respect resembled those evoked in control medium and which were sensitive to 4-AP. Three of these experiments were performed with KAc containing electrodes. These experiments therefore strongly suggest that the 4-AP-sensitive current mediating the dendritic sAHP is insensitive to apamin.

The effect of extracellular 4-AP on the somatic sAHP

The somatic sAHP was similarly affected by 4-AP as the dendritic sAHP. When 4-AP (4 mM) was perfused, the somatic sAHP recorded with either KAc containing ($n=2$) or KCl containing ($n=2$) electrodes was markedly reduced (Figure 6Aa), despite a concomitant increase in firing frequency (Figure 6Ab). As described above for the dendritic sAHP, the stability of the 4-AP-induced block of the somatic sAHP was also sensitive to the type of anion used in the recording electrode. When recordings were started after 4-AP (4 mM) had been present for more than 30 min, the first sAHP recorded with KAc containing electrodes had an averaged amplitude of 4.66 ± 1.4 mV ($n=7$, mean $\pm$ s.d.) and increased progressively during the next 300 s to an average of 8.2 ± 1.9 mV (Figure 6B). With KCl containing electrodes, however, the first sAHP evoked had an averaged amplitude of 1.3 ± 1.3 mV ($n=3$, mean $\pm$ s.d.) and remained stable with an amplitude of 1.7 ± 1.0 mV after 600 s of recordings (Figure 6C).

Discussion

4-aminopyridine blocks a slow Ca^{2+}-activated K^+ current

Two main conclusions can be drawn from the present study: (1) 4-AP blocks the slow Ca^{2+}-activated K^+ currents mediating the somatic and dendritic sAHP in CA1 PC; (2) the anions acetate and $MeSO_4$ interferes in some complex way with the 4-AP-induced block.

The evidence for a 4-AP-induced block of the sAHP are several. Firstly, when the sAHP is evoked in the presence of 4-AP, the amplitude of the first sAHP was reduced compared to that of a maximal sAHP evoked in the control medium. The effect was concentration-dependent with about 44% reduction obtained with 2 mM 4-AP and complete block obtained with 8 mM. Already at submillimolar concentrations, 4-AP reduced the sAHP, although not significantly. However, the experiments performed with KAc containing electrodes indicate that the method used for evaluating the drug action underestimates the effect of 4-AP. The reason for this is as follows, the removal of the 4-AP-induced block reveal a sAHP with a maximal amplitude which is 40 to 50% larger than that of the maximal sAHP evoked in the control medium. The reason for this is the enhancing effect of 4-AP on compound spiking in the apical dendrites (Andreasen & Lambert, 1995a). So when using the sAHP evoked after complete removal of the 4-AP-induced block as reference, the reduction obtained even with submillimolar concentration of 4-AP is considerable.

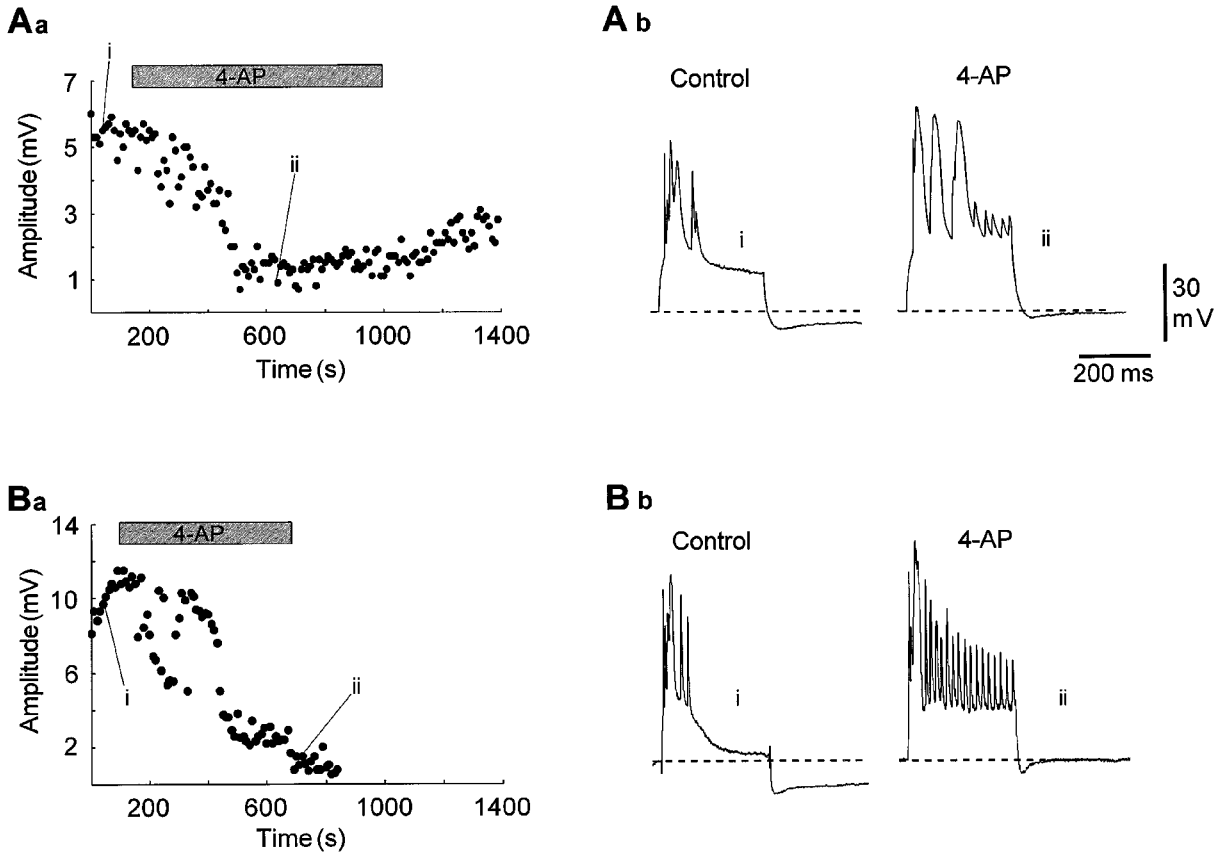

Figure 5 Perfusion of 4-AP induced a stable block independently of the anion used. This shows the effect of introducing 4-AP (4 mM) during recordings with either a KCl (A) or a KAc containing electrode (B). (Aa) Shows the time-dependent change in the sAHP amplitude during perfusion of 4-AP. The time during which 4-AP was perfused is indicated by the hatched bars. (Ab) Shows exemplary responses evoked before and during the perfusion of 4-AP at times indicated by (i) and (ii) in (Aa). (Ba) Shows the time-dependent change in sAHPs recorded with a KAc containing electrode during perfusion of 4-AP. (Bb) Shows exemplary responses recorded at times marked by (i) and (ii) in (Ba). RMP: (A) −70 mV; (B) −68 mV.

Secondly, the perfusion experiments in which 4-AP was introduced during recordings with either acetate or Cl^- containing electrodes clearly showed that 4-AP greatly reduces the sAHP amplitude in spite of a concomitant increase in compound spiking and/or firing frequency (Figures 5 and 6A). Thirdly, when 4-AP was applied intracellularly the first sAHP evoked was also reduced compared to a similar sAHP evoked in the absence of 4-AP. Also here the reduction was very pronounced when compared to the amplitude of the sAHP evoked after the removal of the block (Figure 3). The fast action of intracellularly applied 4-AP is in support of a fast on-rate for the 4-AP binding. The induction of the block does not appear to be use-dependent in that the amount of reduction obtained was very similar irrespective of whether 4-AP had been present from before the experiment started or whether it was applied during the experiment (compare Figure 4B and 5A). So 4-AP appears to block the slow Ca^{2+}-activated K^+ channels mediating the dendritic and somatic sAHP in a concentration-dependent manner with a relatively fast on-rate. The effective concentrations are within the range of concentrations generally used to block different types of voltage-dependent K^+-channels. Furthermore, the lack of use-dependency indicates that 4-AP can bind to the K^+ channels when these are in their closed resting state.

A possible mechanism for the action of 4-aminopyridine

There are several possible mechanisms by which 4-AP could block the slow Ca^{2+}-activated K^+ current. One possibility is that 4-AP reduces the influx of Ca^{2+} during spike firing and thereby the activation of the K^+ current. This does, however, not seem likely considering the marked increase in compound spiking and/or firing frequency in the presence of 4-AP (Figures 4–6). Studies have also shown that 4-AP actually increases the Ca^{2+}-influx through a facilitatory action on high-voltage activated Ca^{2+} channels (Segal & Barker, 1986). A second possibility is that 4-AP changes the Ca^{2+}-binding affinity of the slow Ca^{2+}-activated K^+ channels. Even though the results presented here cannot exclude this possibility, to my knowledge an interaction between 4-AP and calcium-binding proteins has so far not been described. A third, more plausible mechanism is that 4-AP binds directly to the K^+ channel complex to a site that is accessible when the channels are in their closed resting state. From the studies of a large number of different 4-AP-sensitive voltage-gated K^+ currents it appears that 4-AP works primarily from the cytoplasmic side of the membrane in its cationic form also when applied extracellularly (Thompson, 1982; Kirsch & Narahashi, 1983; Kirsch & Drewe, 1993a; Kirsch *et al*., 1993b; Yao & Tseng, 1994; Russell *et al*., 1994; Stephens *et al*., 1994; Yamane *et*

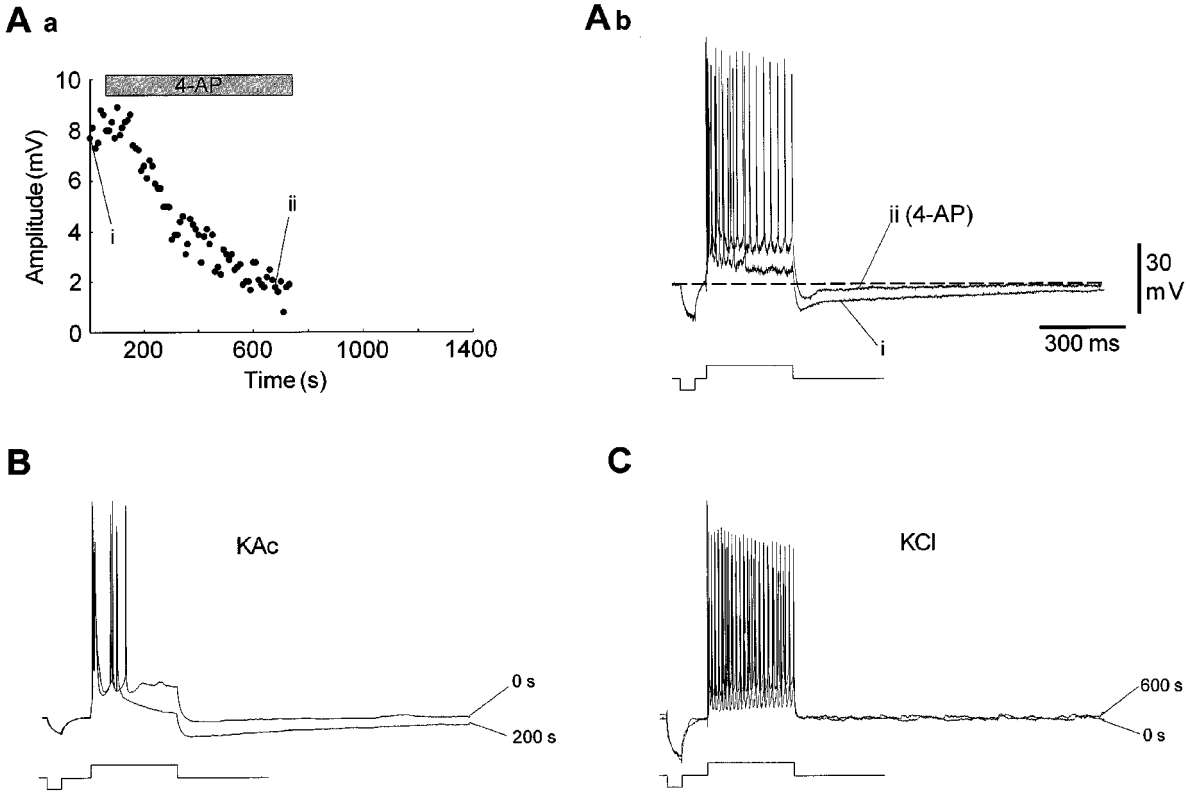

Figure 6 The effect of 4-AP on somatically evoked sAHP. This shows the effect of introducing 4-AP (4 mM) during recordings with a KAc containing electrode. (Aa) Shows the time-dependent change in the somatic sAHP amplitude during perfusion of 4-AP. The time during which 4-AP was perfused is indicated by the hatched bar. (Ab) Superimposition of a responses evoked before and during the perfusion of 4-AP at times indicated by (i) and (ii) in (Aa). (B) Superimposition of the first response and the response evoked after 200 s of recording with a KAc containing electrode in the presence of 4-AP (4 mM). Note the initial small sAHP and the progressive increase in the sAHP amplitude during the following 200 s. (C) Superimposition of the first response and the response evoked after 600 s of recording with a KCl containing electrode in the presence of 4-AP (4 mM). Note that in spite of the high frequency firing the sAHP is blocked for the whole recording period. RMP: (A) −72 mV; (B) −62 mV; (C) −65 mV.

al., 1995). In the present study, there are two observations which indicate that 4-AP also blocks the slow Ca^{2+}-activated K^+ current primarily from the cytoplasmatic site of the membrane. The first one is, that with intracellular application, the reduction of the sAHP was established within 82.9 s after the penetration such fast action is in accordance with a cytoplasmatic binding site because at cytoplasmic pH, most 4-AP molecules will be in the cationic form and only a small fraction would be capable of escaping to the outside. The second one is, that the 4-AP-induced block is affected by acetate only when this is applied intracellularly. It should be pointed out that with the preparation and recording technique used here an additional extracellular binding site cannot be excluded.

Detailed studies of the interaction between 4-AP and different types of voltage-gated K^+ channels have suggested that 4-AP binds to a site on or near the cytoplasmatic half of the S6 segment which forms the cytoplasmatic vestibule of the channels (Kirsch & Drewe, 1993a; Kirsch *et al.*, 1993b; Jerng *et al.*, 1999). So far three genes have been identified, SK1, SK2 and SK3, which codes for small-conductance Ca^{2+}-activated K^+ channels, also termed SK channels (Köhler *et al.*, 1996). The overall structure of these SK channels are very similar to that of voltage-gated K^+ channels with a high degree of homology in the pore region between the S5 and S6 segments (Köhler *et al.*, 1996; Vergara *et al.*, 1998; Doyle *et al.*, 1998). The 4-AP-sensitive Ca^{2+}-activated K^+ channels investigated in the present study seem to be distinct from the SK channels in that they are insensitive to apamin and BIC at concentrations which would block the SK channels (Debarbieux *et al.*, 1998; Khawaled *et al.*, 1999; Shah & Haylett, 2000; Stocker *et al.*, 1999). Even so, single channel studies of the K^+ channels mediating the apamin-insensitive part of the sAHP have indicated that the basic channel structure is similar to that of the SK channels (Sah & Isaacson, 1995; Sah, 1996; Vergara *et al.*, 1998; Hirschberg *et al.*, 1998; Sah & Clements, 1999). It is therefore tempting to suggest that the 4-AP-binding site is to be found within the pore region of the 4-AP-sensitive Ca^{2+}-activated K^+ channels. A similar location has also been suggested for the 4-AP binding to the large conductance Ca^{2+}-activated K^+ channel in human pregnant nonlabor myometrical cells, which also have a pore region that is highly conserved with voltage-dependent K^+ channels (Khan *et al.*, 1997).

Organic anions modify the 4-AP induced block

With acetate or $MeSO_4$ containing electrodes, the 4-AP-induced block of the sAHP was gradually removed during the initial 300–400 s of stimulation (Figures 1C and 6B), which

could indicate some kind of activity-dependent unblocking. This is further supported by the ability to re-establish the block by introducing a pause in the activation of the sAHP. In contrast, when using the inorganic anion Cl^-, the 4-AP-induced block was stable and on average 10% larger than that obtained with KAc containing electrodes. It therefore, seems as if the type of anion used is also of importance for the removal of 4-AP from the slow Ca^{2+}-activated K^+ channels. Using electrodes with different combinations of acetate and Cl^- revealed that removal of the block only occurred when acetate was present and only reliably so with acetate concentrations $\geqslant 2$ M. Furthermore, the stability of the block seen with Cl^- containing electrodes indicates that activation of the Ca^{2+}-activated K^+ channels in it self is not sufficient to promote unblocking. So there are little doubt that loading the cell with acetate or $MeSO_4$ is in some way responsible for the removal of 4-AP from the channel complex. There could be several possible explanations for the action of acetate. One could be through a change in the intracellular pH, which most types of ion channels are very sensitive to (Tombaugh & Somjen, 1996), however, re-establishment of the block should not then be possible in the continued presence of acetate. Another mechanism could be through activation of extracellular adenosine receptors. However, in the present study extracellular acetate had no effect on the 4-AP-induced block, which excludes this possibility and further supports a direct action of intracellular acetate. A third, and more plausible explanation is that acetate directly interacts with the binding of 4-AP to the Ca^{2+}-activated K^+ channels resulting in the removal of 4-AP. If so, how is it then possible to re-establish the block simply by inserting a pause in the activation of the channels? One explanation could be that the binding of acetate rather than the unbinding of 4-AP is activity-dependent. It is possible that binding of Ca^{2+} to the 4-AP-sensitive Ca^{2+}-activated K^+ channel results in a conformational changes, as has been suggested for the SK channels (Keen *et al*., 1999), that exposes a binding site for acetate. Binding of acetate to the channel complex could then, in turn, lead to a change in the affinity for 4-AP. This means that acetate would only be able to bind to the channels after Ca^{2+} has bound. Under the present experimental conditions this would primarily occur during the suprathreshold depolarizing current pulse where intracellular Ca^{2+} would rise above resting level. If stimulation is stopped, the intracellular Ca^{2+} concentration will decrease to resting levels and Ca^{2+} will leave its binding site on the K^+ channels resulting in a reversal of the conformational changes making the acetate binding site inaccessible again. During a pause, acetate would then unbind allowing 4-AP to rebind to the channel despite the presence of acetate. For full recovery of the 4-AP-induced block, the pause needed to be more than 15 min indicating that the unbinding of acetate is a slow process. This hypothesis is not in disagreement with the results obtained with intracellular co-application of 4-AP and acetate. During the delay between penetration and the start of stimulation (83 s), the Ca^{2+}-activated K^+ channels will be in their closed state and 4-AP will have access to its binding site. Once stimulation commence, Ca^{2+} will start to bind to the channels making the binding site for acetate accessible; acetate will then bind and 4-AP will unbind. Anions are known to affect different types of K^+ channels either directly (Adams & Oxford, 1983; Zhang *et al*., 1994) or indirectly *via* second messengers (Morita *et al*., 1980; Nakajima *et al*., 1992). In the case of the delayed rectifying K^+ channels in squid giant axons inorganic anions like fluoride, F^-, and Cl^- reduces this current whereas organic anions do not (Adams & Oxford, 1983). Interestingy, this anion-induced block can be partly antagonized by adding low concentrations of 4-AP or tetraethylammonium ions. So there are precedence for an interaction between anions, 4-AP and K^+ channel proteins.

An interesting observation was that 4-AP, when introduced during recordings with acetate containing electrodes, gave a decrease of the sAHP and, furthermore, the block remained stable for the entire recording period (Figures 5B and 6A). On the other hand, when Cl^- was used, the introduction of 4-AP resulted in a progressive reduction of the sAHP, as expected, to a level similar to that seen when 4-AP was present before the start of the experiment. The fact that, with acetate containing electrodes, the stability of the 4-AP-induced block depended on whether 4-AP was introduced before or after loading with acetate is not easily explained by the above hypothesis. However, one possibility is that the prolonged exposure to 4-AP somehow has primed the slow Ca^{2+}-activated K^+ channels for acetate binding. Recently, 4-AP, in the concentration range used here, was reported to increase the activity of phospholipase C leading to an increase in intracellular inositol triphosphate and diacylglycerol in neurones and astrocytes (Grimaldi *et al*., 2001). This could lead to an increase in the activity of Ca^{2+}-calmoduline kinases and/or protein kinase C which could then, through phosphorylation, prime the slow Ca^{2+}-activated K^+ channels. The reason why unblocking is only seen if 4-AP has been present for some time and not during the initial perfusion of 4-AP could be that it takes some time for the intracellular concentration of 4-AP to become high enough to increase the activity of PLC. One thing which seems to argue against this is the experiments in which 4-AP and acetate was co-injected intracellularly. Here, unblocking was observed even though 4-AP had only been present, on average, for about 90 s. The reason for this could be that because of the higher concentration of 4-AP in the electrode, the intracellular concentration of 4-AP increased much faster than when 4-AP is applied extracellularly.

To summarize, the present study provide evidence for a 4-AP-induced block of the dendritic and somatic slow Ca^{2+}-activated K^+ current in hippocampal CA1 PCs. It is suggested that 4-AP binds directly to the cytoplasmatic sites of this channel and that the binding site is accessible when the channel is in its closed resting state. Even though the 4-AP-sensitive Ca^{2+}-activated K^+ channels seems to be distinct from the SK channels it is important to point out that the present study does not exclude the possibility that 4-AP also affects the SK channels. Intracellular loading of acetate or $MeSO_4$ resulted in a complex activity-dependent removal of the 4-AP-induced block whereas the block was stable when Cl^- was used. The anion-dependent removal was only seen when 4-AP was already present when the recordings were started. When 4-AP was introduced during the recordings a stable block of the sAHP was induced irrespective of the type of anion used in the electrode solution. The present study is not only important with respect to the actions of 4-AP but also emphasize the need to consider the influence of different types of organic and

inorganic anions on the kinetics of ion channels, drug interactions, secondary messenger systems etc. The present study also indicates that the use of different anions in combination with 4-AP could be potentially useful in studying the functional significance and molecular structure of slow Ca^{2+}-activated K^+ currents.

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