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Effects on K^+ currents in rat cerebellar granule neurones of a membrane-permeable analogue of the calcium chelator BAPTA

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1 Whole cell recordings of voltage-activated K^+ currents were made with the amphotericin B perforated patch technique from cerebellar granule (CG) neurones of 6–8 days rats that had been in culture for 1 to 16 days. By use of appropriate voltage protocols, the effects of the membrane-permeant form of BAPTA, 1,2-*bis*-(2-amino-phenoxy)ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester (BAPTA-AM), on the transient A current (I_{KA}), the delayed rectifier current (I_{Kv}) and a standing outward current (I_{Kso}) were investigated.

2 Bath application of 25 μM BAPTA-AM inhibited both I_{Kv} and I_{Kso} in cultured neurones, but did not seem to affect I_{KA}. Neither 25 μM BAPTA (free acid) nor 25 μM ethylenediaminetetraacetic acid acetoxymethyl ester (EDTA-AM) had any significant effect on the magnitude of I_{Kso}. Similarly in short-term (1–2 days) cultured CG neurones I_{Kv}, but not I_{KA}, was inhibited by 25 μM BAPTA-AM.

3 BAPTA-AM (2.5 μM) reduced I_{Kv} in short-term culture CG neurones, with further inhibition being seen when the perfusate was changed to one containing 25 μM BAPTA-AM.

4 Tetraethylammonium ions (TEA) (10 mM) reversibly inhibited I_{Kv} in these cells with a similar rate of block of I_{Kv} to that induced by 25 μM BAPTA-AM.

5 The degree of inhibition of I_{Kv} by 25 μM BAPTA-AM was both time- and voltage-dependent, in contrast to the inhibition of this current by TEA.

6 These data indicate that BAPTA-AM reduces K^+ currents in cerebellar granule neurones and that this inhibition cannot be explained in terms of intracellular Ca^{2+} chelation, but is a direct effect on the underlying channels.

Keywords: BAPTA; BAPTA-AM; potassium current; cerebellar granule neurone; TEA

Introduction

Cerebellar granule (CG) neurones grown in explant or primary culture have been shown to have transient and sustained components to their whole cell K^+ current, which correspond to A-type (I_{KA}) and delayed rectifier (I_{Kv}) conductances (e.g. Hockberger *et al.*, 1987; Cull-Candy *et al.*, 1989; Watkins & Mathie, 1994). These components have also been seen when recording from slice preparations (Bardoni & Belluzzi, 1993). CG neurones also possess a K^+ current which is dependent on intracellular Ca^{2+} levels, corresponding to a maxi-K (BK_{Ca}) conductance (Fagni *et al.*, 1991). We have very recently described an additional component of the whole cell K^+ currents of CG neurones which appears with time in culture (Watkins & Mathie, 1996). This current, which we denote as I_{Kso}, does not exhibit appreciable inactivation, being a standing outward current at membrane potentials above −90 mV. The biophysical and pharmacological properties of this current mean that it cannot be considered a 'delayed rectifier' type K^+ conductance.

I_{Kso} is an outwardly rectifying K^+-selective current, with negligible inward current being seen even with strong hyperpolarization to −140 mV; the threshold for I_{Kso} is approximately −90 mV, much more hyperpolarized than that for I_{Kv} in cerebellar granule neurones (Hockberger *et al.*, 1987; Cull-Candy *et al.*, 1989; Bardoni & Belluzzi, 1993; Watkins & Mathie, 1994). The activation and deactivation kinetics of I_{Kso} are very rapid, such that the deactivation of the current when stepping from a holding potential of −30 mV to −80 mV can be fitted with a single exponential function with a time constant of 0.5 ms. Unlike I_{Kv} in these neurones (Hockberger *et al.*, 1987; Cull-Candy *et al.*, 1989; Bardoni & Belluzzi, 1993; Watkins & Mathie, 1994), I_{Kso} is unaffected by 5 mM TEA, but is reversibly and potently inhibited by muscarinic agonists. Muscarinic inhibition of I_{Kso} causes a depolarization of approximately 15 mV, with an associated increase in input resistance; this enhanced excitability is evident under current clamp (see Watkins & Mathie, 1996). Unfortunately, I_{Kso} is very labile such that the current declines rapidly during conventional whole cell recording, and consequently the perforated variant of the patch clamp technique must be used in its study.

The perforated patch variant of whole cell recording was first developed by Horn & Marty (1988), using nystatin to permeabilize the plasma membrane under the pipette tip. More recently, amphotericin B has been used to permeabilize the patch (Rae *et al.*, 1991). This has the advantage of significantly lower access resistances than nystatin, whilst still preventing the dialysis out of intracellular constituents. However, a major drawback to the perforated patch technique is that the manipulation of intracellular constituents cannot be achieved. This is problematic in the study of intracellular signalling pathways, where Ca^{2+} chelation may play a pivotal role. For example, the use of different concentrations of the Ca^{2+} chelator 1,2-*bis*-(2-amino-phenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) in the pipette solution has revealed the importance of the intracellular Ca^{2+} concentration in the modulation of Ca^{2+} and K^+ currents in rat sympathetic neurones (e.g. Bernheim *et al.*, 1991; Beech *et al.*, 1991; 1992; Mathie *et al.*, 1992).

As a consequence of the inability to control the intracellular conditions via dialysis of compounds from the patch pipette when using the perforated patch technique, manipulation of intracellular conditions can be achieved only by use of membrane-permeant chemicals. Such compounds may be inherently active (e.g. okadaic acid, caffeine), or may pass across the membrane in an uncharged form, with the charged compound acting intracellularly (e.g. certain local anaesthetics, see Hille, 1992). Synthetic compounds have been developed such that the membrane-permeant form is inactive, being converted

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into the active form once inside the cell. An important development was that of Tsien (1981) for the loading of Ca^{2+} chelators into cells, namely the addition of an acetoxymethyl (AM) ester onto the parent compound. The AM esterified compound is membrane-permeant, and once inside the cell, the acetoxymethyl ester bond is hydrolysed by cytosolic esterases to reveal the active compound. This technique has been widely used as a method of loading cells with compounds, such as fluorescent dyes for fluorometric and imaging studies (see Grynkiewicz *et al.*, 1985). One widely used acetoxymethyl ester compound is the BAPTA analogue 1,2-*bis*-(2-amino-phenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid acetoxymethyl ester (BAPTA-AM). This compound has been utilized in studies on the effects of chelation of intracellular Ca^{2+} (see e.g. Fredholm & Hu, 1993; Noda *et al.*, 1993; Tymianski *et al.*, 1994).

However, in the course of our studies on the regulation of I_{Kso} in CG neurones, we have found that BAPTA-AM, whilst attenuating the muscarinic modulation of this current (Watkins, Amos & Mathie, unpublished observations), also itself directly affected I_{Kso}. We describe herein some effects of BAPTA-AM on three voltage-activated K^+ currents in CG neurones (I_{KA}, I_{Kv} and I_{Kso}), and discuss why these cannot be explained in terms of chelation of intracellular Ca^{2+} by BAPTA.

Some of the data described here have been presented in preliminary form (Watkins & Mathie, 1995).

Methods

Granule neurones were isolated from the cerebella of decapitated 6–9 day old Sprague-Dawley rats of either sex by previously described methods (Huston *et al.*, 1993). Following dissociation, neurones were plated on poly-ornithine-coated glass coverslips, allowed to adhere and then flooded with a modified essential medium (MEM) with Earles salts, supplemented with 10% foetal calf serum, penicillin (50 iu ml^{-1}), streptomycin (50 $\mu g\ ml^{-1}$), chick embryo extract (25 $\mu l\ ml^{-1}$), glucose (39 mM), glutamine (2 mM) and KCl (23 mM) and maintained in 5% CO_2 at 37°C. After 48 h, flurodeoxyuridine (80 μM) was added to the culture medium, in order to reduce non-neuronal cell proliferation; medium was renewed every 3–4 days. Neurones were recorded between 1 and 16 days in culture.

Whole cell currents (Hamill *et al.*, 1981) were recorded by use of the amphotericin B perforated patch clamp technique (Rae *et al.*, 1991) at room temperature (21–23°C). The external solution for all voltage-clamp experiments contained (in mM) NaCl 120, KCl 2.5, $MgCl_2$ 2, $CaCl_2$ 0.5, 4-(2-hydroxyethyl)-1-piperazinethanesulphonic acid (HEPES) 10, glucose 5, and tetrodotoxin 5×10^{-7} M; pH 7.4 with NaOH. The pipette solution for perforated patch recordings comprised (in mM): KCl 125, HEPES 5, $MgCl_2$ 5 and *bis*(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetate (BAPTA) 0.1, supplemented with amphotericin B (240 $\mu g\ ml^{-1}$); pH 7.4 with KOH. Solutions were applied by continuous bath perfusion, at a rate of 4–5 ml min^{-1}, with complete exchange of the bath solution occurring within 20–40 s. Neurones were voltage-clamped with an Axopatch 1-D amplifier (Axon Instruments), and lowpass filtered at 5–10 kHz before sampling and capture online to a Viglen 486 microcomputer with a TL-1 interface. Data acquisition and analysis were performed by use of pClamp software (Axon Instruments), Excel (Microsoft) and Origin (Microcal Inc.). All data are presented without leak subtraction.

Mean values are presented as mean ± s.e.mean, with *n* as the number of neurones. Statistical analyses were performed using Student's *t* test, with paired comparisons where relevant. Probabilities are given for 2-tailed tests. Chord conductances were calculated, using the predicted potassium reversal potential of −99 mV, and the resultant chord conductance-membrane potential relationships were fitted to a Boltzmann function, of the form:

$$g_m = \frac{G_{max}}{1 + e^{\left(\frac{V_m - V_{1/2}}{k}\right)}}$$

where g_m is the chord conductance at a membrane potential V_m, G_{max} the maximal chord conductance, $V_{1/2}$ the membrane potential at which the conductance was half-maximal, and k is a slope factor.

All bulk chemicals were obtained from Sigma. Foetal calf serum, MEM, penicillin, and streptomycin were obtained from Gibco, chick embryo extract from ICN Laboratories, BAPTA-AM and EDTA-AM from Calbiochem. BAPTA-AM and EDTA-AM were made up in dimethylsulphoxide (DMSO) as a 5 mM stock, with the aliquots being stored at −20°C until required. After thawing on day of use, BAPTA-AM and EDTA-AM were diluted in external solution to the required concentration. DMSO was maintained at a constant concentration of 0.5% throughout all experiments, being present to eliminate any artefacts due to possible vehicle effects.

Results

Whole cell K^+ current recordings were made from CG neurones by the amphotericin B perforated patch technique. Under control conditions, the magnitude of I_{Kso} at a membrane potential of −30 mV was 268 ± 42 pA ($n = 21$), but perfusion of 25 μM BAPTA-AM onto CG neurones produced a rapid inhibition of I_{Kso} of 67 ± 1% ($n = 13$), with a full effect being seen within approximately 30 s (see Figure 1). There was a partial recovery to 80 ± 5% of control amplitudes with washing. Since DMSO was present at a constant concentration throughout the experiment (see Methods) the inhibition seen with BAPTA-AM cannot be explained by an effect of the vehicle. The reversibility, albeit incomplete, of this inhibition is strong evidence against the reduction in I_{Kso} being due to chelation of intracellular calcium by BAPTA, since, once hydrolysed, the chelator will remain trapped in the cytosol. The lack of complete recovery may be indicative of the amplitude of I_{Kso} being dependent upon the intracellular Ca^{2+} concentration, with sufficient BAPTA-AM having crossed the plasma membrane (and been hydrolysed to active BAPTA) at the end of the 10 min application to attenuate the current via chelation of intracellular Ca^{2+}.

In contrast to the inhibitory effect of 25 μM BAPTA-AM, 25 μM BAPTA (free acid) did not significantly affect I_{Kso} (mean reduction 3 ± 2%, $n = 4$, $P > 0.2$), demonstrating that the inhibition seen with BAPTA-AM was not due to the active chelator BAPTA acting from an extracellular site. Another acetoxymethyl ester EDTA-AM, the membrane-permeant form of the magnesium chelator EDTA was tested to ascertain whether other acetoxymethyl esters also inhibited I_{Kso}. However, application of 25 μM EDTA-AM had no significant effect on the magnitude of I_{Kso} at −30 mV (mean reduction 2 ± 2%, $n = 4$, $P > 0.1$).

The use of appropriate voltage protocols can evoke 'A-' (I_{KA}) and delayed rectifier-type (I_{Kv}) K^+ currents in these neurones (Bardoni & Belluzzi, 1993; Watkins & Mathie, 1994), in addition to I_{Kso}. In order to measure the effects of 25 μM BAPTA-AM on other voltage-activated K currents, CG neurones that had been in culture for 7–15 days were held at a membrane potential of −30 mV, hyperpolarized to −140 mV for 164.5 ms before stepping to a test potential of +50 mV. This voltage protocol allows the recording of I_{Kso} at the holding potential of −30 mV, whilst the peak current at +50 mV will be predominantly I_{KA}, and the current at the end of the test step will be composed of I_{Kv} and I_{Kso}, since I_{KA} will have inactivated over the duration of the step (see Figure 2). The peak current evoked by this voltage protocol was not significantly affected by 25 μM BAPTA-AM, with only a 1 ± 2% ($n = 8$; $P > 0.5$, paired *t* test) reduction in magnitude being seen. In contrast to its apparent lack of effect on I_{KA},

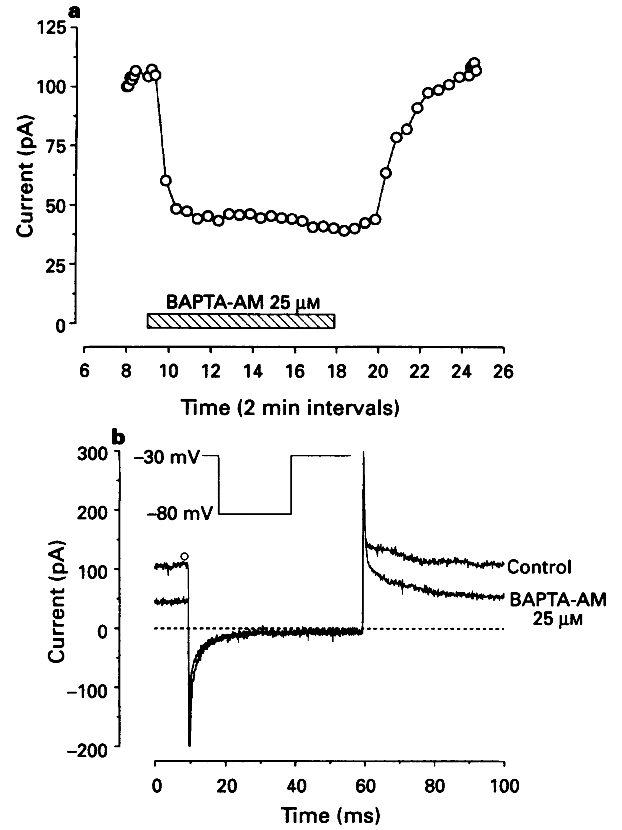

Figure 1 Effect of BAPTA-AM on I_{Kso} in CG neurones: a representative example of the effects of 25 µM BAPTA-AM on the standing outward K$^+$ current I_{Kso} of a cerebellar granule neurone 15 days in culture. Membrane currents were evoked by voltage steps from a holding potential of −30 mV to −80 mV for 50 ms, once every 5 s, before returning to the holding potential. The magnitude of the current immediately prior to the hyperpolarizing step (○) is plotted as a function of time in (a). Application of 25 µM BAPTA-AM is shown by the bar. Example data traces in control and in the presence of 25 µM BAPTA-AM are shown in (b).

25 µM BAPTA-AM significantly inhibited the current at the end of the test step (I_{Kv} and I_{Kso}) in these cultured neurones, by 45±3% ($n=8$; $P<0.0001$), with complete recovery on washing.

Cerebellar granule neurones in culture for only 1−2 days possess I_{KA} and I_{Kv}, but not I_{Kso} (Watkins & Mathie, 1996). When recording from these short-term culture neurones, 25 µM BAPTA-AM reversibly inhibited the peak current at +50 mV by 11±10% ($n=4$; $P>0.2$) and the end current by 65±5% ($n=4$; $P<0.05$) using the same voltage protocol described above. This disparity in terms of the degree of inhibition of peak current with the results obtained with neurones that had been cultured for >7 days may be due to the fact that, in these short-term culture neurones, the predominant K$^+$ conductance is I_{Kv}, with I_{KA} being relatively small in comparison. However, after 2 days *in vitro*, I_{KA} becomes much larger in size than I_{Kv} (data not shown). It is likely that the inhibition of the peak current seen with BAPTA-AM in these neurones is due to I_{Kv} contributing a major portion of the peak current, in contrast to cultured CG neurones where I_{KA} predominates. In any case, these data indicate that BAPTA-AM blocks more than one type of K$^+$ current in CG neurones.

A hyperpolarizing prepulse will relieve the steady-state inactivation of I_{KA}, whereas a depolarizing prepulse to −50 mV will evoke only I_{Kv}. Thus, if a depolarizing prepulse to −50 mV is used in these short-term cultured neurones, the delayed rectifier current I_{Kv} can be studied in isolation. We utilized this relatively simple technique to study the inhibitory effect of BAPTA-AM on a single class of K$^+$ current in more detail, and to compare this inhibition with that of TEA, a well characterized selective blocker of I_{Kv}.

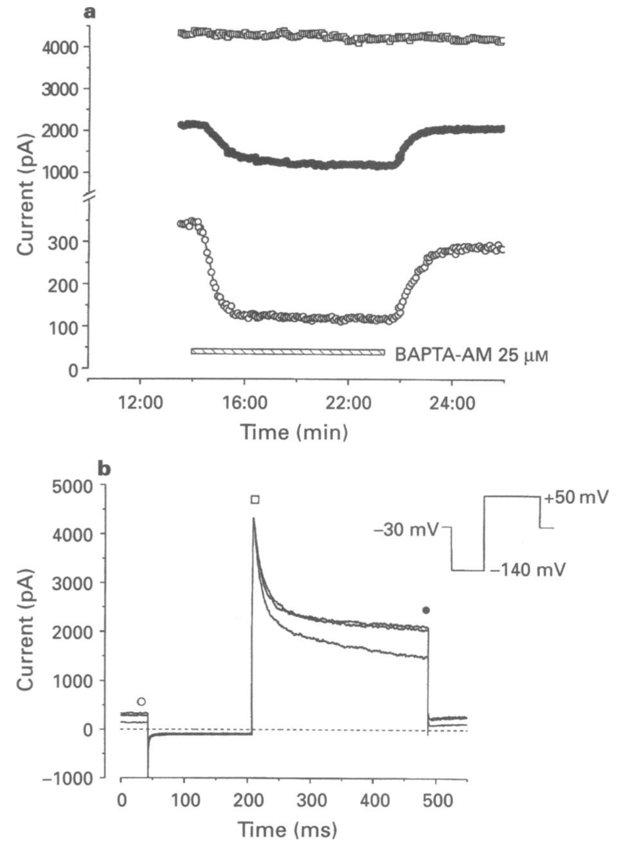

Figure 2 Effects of BAPTA-AM on voltage-activated K$^+$ currents in cultured CG neurones: an example of the effects of 25 µM BAPTA-AM on I_{KA}, I_{Kv} and I_{Kso} in a cerebellar granule neurone that had been in culture for 15 days. Currents were evoked by a depolarizing test step to +50 mV of 180 ms duration from a holding potential of −30 mV, following a 164.5 ms hyperpolarizing prepulse to −140 mV, before returning to the holding potential. The magnitude of the currents immediately prior to the hyperpolarizing step (corresponding to I_{Kso}; ○), the peak current (I_{KA}, I_{Kv} and I_{Kso}; □), and the current at the end of the test step (I_{Kv} and I_{Kso}; ●) are plotted as a function of time (a). Application of 25 µM BAPTA-AM is shown by the bar. Examples of data traces in control, in the presence of 25 µM BAPTA-AM, and after wash are shown in (b).

Effects of tetraethylammonium ions and BAPTA-AM on I_{Kv}

TEA has been widely used in the study of I_{Kv} in a number of tissues (see Cook & Quast, 1990), and the mechanism by which it inhibits K channels has been studied intensively (see Hille, 1992). In CG neurones, it is a relatively selective blocking agent for I_{Kv} (Cull-Candy *et al.*, 1989; Bardoni & Belluzzi, 1993; Watkins & Mathie, 1994). At a concentration of 10 mM, TEA inhibited the average current over the last 2 ms of a test step to +50 mV (end current) by 82±4% (681±53 pA to 124±28 pA, $n=4$; see Figure 3); this inhibition was fully reversible. BAPTA-AM 25 µM reduced the current evoked by an identical voltage protocol by 43±5% (668±38 pA to 380±36 pA; $n=10$). Even at a ten-fold lower concentration of 2.5 µM, BAPTA-AM significantly reduced the current at the end of the test step by 14±3% ($P=0.0012$; $n=8$), with further inhibition being seen when the perfusate was changed to one containing 25 µM BAPTA-AM (see Figure 3). It is noteworthy that, as is illustrated in Figure 3b, the rate of block of I_{Kv} by BAPTA-AM is similar to that by TEA.

It can be seen from the data traces in Figure 3a that there is a difference in the degree of inhibition of I_{Kv} by BAPTA-AM,

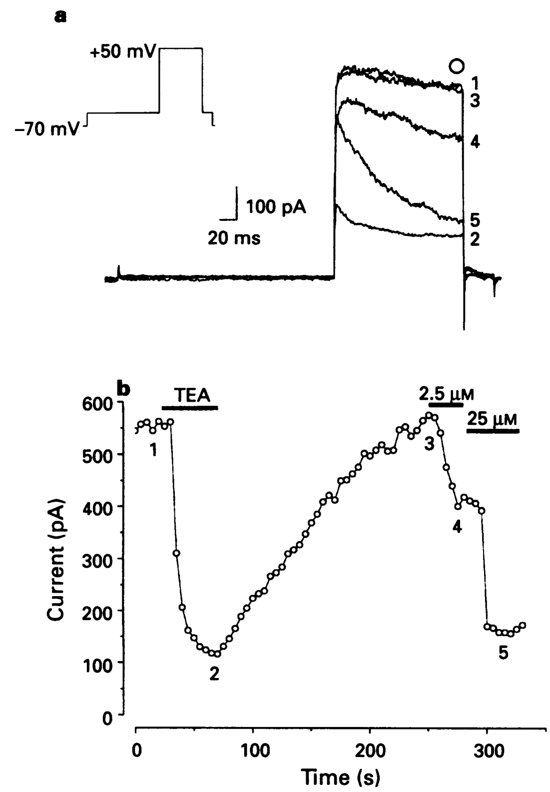

Figure 3 Concentration-dependent inhibition of I_{Kv} by BAPTA-AM in acutely isolated CG neurones. Currents were evoked by stepping from a holding potential of −70 mV to a test potential of +50 mV for 150 ms, following a 250 ms prepulse to −50 mV. Examples of data traces in control (1), in the presence of 10 mM TEA (2), after wash-off of TEA (3), in the presence of 2.5 μM BAPTA-AM (4), and in 25 μM BAPTA-AM (5) are shown in (a). The magnitude of the current at the end of the depolarizing step to +50 mV (○) is plotted as a function of time in (b), with drug application denoted by bars. Application of 10 mM TEA inhibited I_{Kv}, with a return to control levels with washing. Subsequent application of 2.5 μM BAPTA-AM blocked I_{Kv}; with a further reduction being seen when the perfusate was changed to one containing 25 μM BAPTA-AM.

such that the current at the end of the test step was blocked to a greater degree than the current at the start of the step. Thus, 25 μM BAPTA-AM reduced the current 9 ms after the onset of the depolarizing step (peak current) by 18 ± 3% (787 ± 37 pA to 640 ± 33 pA; $n = 4$), whilst the end current was reduced by 43 ± 5% (see above). This difference in the degree of block of the current at the two time points by 25 μM BAPTA-AM was significant ($P < 0.0001$, $n = 10$; paired t test). In contrast to the time-dependent block by 25 μM BAPTA-AM, there was no significant difference between the inhibition of the peak and end currents by 10 mM TEA (82 ± 4% for peak current vs. 82 ± 4% for end current; $P > 0.5$; $n = 4$).

Voltage-dependence of activation of I_{Kv}

The voltage-dependence of activation of I_{Kv} in CG neurones was determined by performing current-voltage relationships at test potentials between −50 mV and +60 mV (Figure 4). Under control conditions, the conductance-voltage relationship of I_{Kv} could be well described by a single Boltzmann function, whether considering either the peak or the end conductances (Figures 4d and e). Thus the peak I_{Kv} had a half-maximal activation at a potential of +12 ± 3 mV and a slope factor of 14 ± 1 mV, and the end current was half-maximally activated at a potential of +6 ± 3 mV with a slope factor of 13 ± 1 mV ($n = 6$). In the presence of 25 μM BAPTA-AM, the peak I_{Kv} had half-maximal activation at a potential of +9 ± 3 mV and a slope factor of 13 ± 1 mV, while the end I_{Kv} had a half-maximal activation potential of −1 ± 2 mV and a slope factor of 11 ± 1 mV ($n = 6$). Only the end half-maximal activation potential was significantly ($P < 0.05$, paired t test) shifted in the presence of 25 μM BAPTA-AM.

Voltage-dependence of BAPTA-AM inhibition of I_{Kv}

The voltage-dependence of block of I_{Kv} by BAPTA-AM is illustrated in Figure 5, where the degree of inhibition of the current at the peak and end time points of the test step have been plotted as a function of membrane potential. As can be seen from Figure 5, the degree of block of I_{Kv} by 25 μM BAPTA-AM increased with the strength of the depolarizing test step for both the peak current and the current at the end of the test step. The voltage-dependence of inhibition by BAPTA-AM was stronger for the end current than for the peak of the current. Thus, at a test potential of +10 mV, the peak current was inhibited by 7 ± 4% ($n = 8$) and the end current by 23 ± 4% ($n = 8$), whilst at a more depolarized test potential of +60 mV, the inhibition of the peak current was 18 ± 3% ($n = 8$), and the end current was inhibited to a significantly greater extent, namely 40 ± 5% ($P = 0.0008$; $n = 8$). This increase is in contrast to the inhibition of I_{Kv} by 10 mM TEA, which was barely voltage-dependent (Figure 5). Thus, at a test potential of +10 mV, the peak current was inhibited by 84 ± 4% ($n = 4$) and the end current by 85 ± 4% ($n = 4$), whilst at a test potential of +60 mV, TEA inhibited the peak current by 80 ± 4% ($n = 4$), and the end current by 81 ± 4% ($n = 4$).

Discussion

We have shown that bath application of BAPTA-AM, a membrane-permeant form of the Ca^{2+} chelator BAPTA, to cerebellar granule neurones reduces the magnitudes of two voltage-dependent K^+ conductances, I_{Kso} and the delayed rectifier current I_{Kv}. This inhibition is concentration-dependent and reversible, and is not a vehicle effect. The free acid BAPTA does not inhibit K^+ currents in these neurones, nor does another acetoxymethyl ester, EDTA-AM. The block of I_{Kv} by BAPTA-AM is time-dependent, such that the current is inhibited to a greater extent at the end of the test step than at the beginning, and the inhibition is also voltage-dependent.

The observation that outward currents of cerebellar granule neurones were inhibited by application of the membrane-permeant BAPTA-AM would, at first sight, imply that chelation of intracellular Ca^{2+} by BAPTA has reduced one or more Ca^{2+}-dependent K^+ conductances. However, there are a number of reasons why we believe that this interpretation is erroneous, and that a more likely explanation is that BAPTA-AM directly inhibits K channels.

The rate at which BAPTA-AM inhibits I_{Kv} is similar to that seen for TEA (Figure 3b) and this reduction in current is reversible. The rationale behind the use of the AM analogue is that the compound itself is not capable of chelating Ca^{2+}, but once it crosses the plasma membrane, it is hydrolysed by cytosolic esterases to give active BAPTA, which remains trapped within the cell (Tsien, 1981). Consequently, any effects mediated by chelation of intracellular Ca^{2+} should remain even when the cell is no longer being perfused by BAPTA-AM. Clearly this is not the case with the inhibition that we describe herein. We have not determined the rate at which BAPTA-AM crosses the plasma membrane, nor at which BAPTA is generated. However, studies on the loading of Fura 2-AM of cultured spinal neurones have found that the rate was dependent on the temperature and external concentration (Tymianski *et al.*, 1994). Tymianski *et al.* (1994) found that, at 37°C, the time to half-maximal loading with 1 μM extracellular Fura 2-AM was 5–6 min, falling to 2–4 min at a concentration of 10 μM. These authors proposed that BAPTA-AM would load at approximately the same rate, given the similarity in structure, polarity and molecular weight between Fura 2-AM and BAPTA-AM. We performed our experiments at room temperature (21–23°C), and yet the reduction in current with BAPTA-AM had reached a steady-state within approximately 30 s (Figures 1 and 3). This rapidity of effect and the reversibility argue strongly against the inhibition seen with BAPTA-AM being mediated by intracellular BAPTA. It is unclear what confers the inhibitory nature of BAPTA-AM, but it is unlikely to be the BAPTA portion of the molecule, since we have found that the free acid itself does not inhibit K^+ currents when applied from the outside (see above). In addition, there are numerous examples where relatively high concentrations (> 5 mM) of BAPTA have been included in the pipette solution for conventional whole cell recording, without any appreciable inhibition of I_{Kv} being evident (e.g. Fagni *et al.*, 1991; Watkins & Mathie, 1994; Wooltorton & Mathie, 1995). Since EDTA-AM did not block K^+ currents, the inhibition seen with BAPTA-AM is unlikely to be due to formaldehyde liberated when these AM compounds are hydrolysed.

The data we have presented here (see Figure 2) shows that more than one type of voltage-activated K^+ current is reduced by BAPTA-AM, with I_{Kso} being more sensitive than I_{Kv}. The fact that the channels that underlie I_{KA} do not seem to be blocked by BAPTA-AM is interesting, given the high degree of sequence homology that exists in the pore region of the α subunits that comprise voltage-activated K channels (Pongs, 1992). BAPTA-AM may block K channels in the open state, in a similar manner to that described for the inhibition of a human cardiac K channel by bupivacaine. This local anaesthetic blocks both transient (Castle, 1990) and delayed rectifier K^+ currents (Valenzuela *et al.*, 1995) by an open channel block mechanism, but at an intracellular site (Valenzuela *et al.*, 1995). BAPTA-AM, being membrane-permeable, could act at a site on the K channel that is intracellular, extracellular, or even in the membrane. However, the rapid rate of onset of block of I_{Kv} by BAPTA-AM (and its wash-off) might suggest

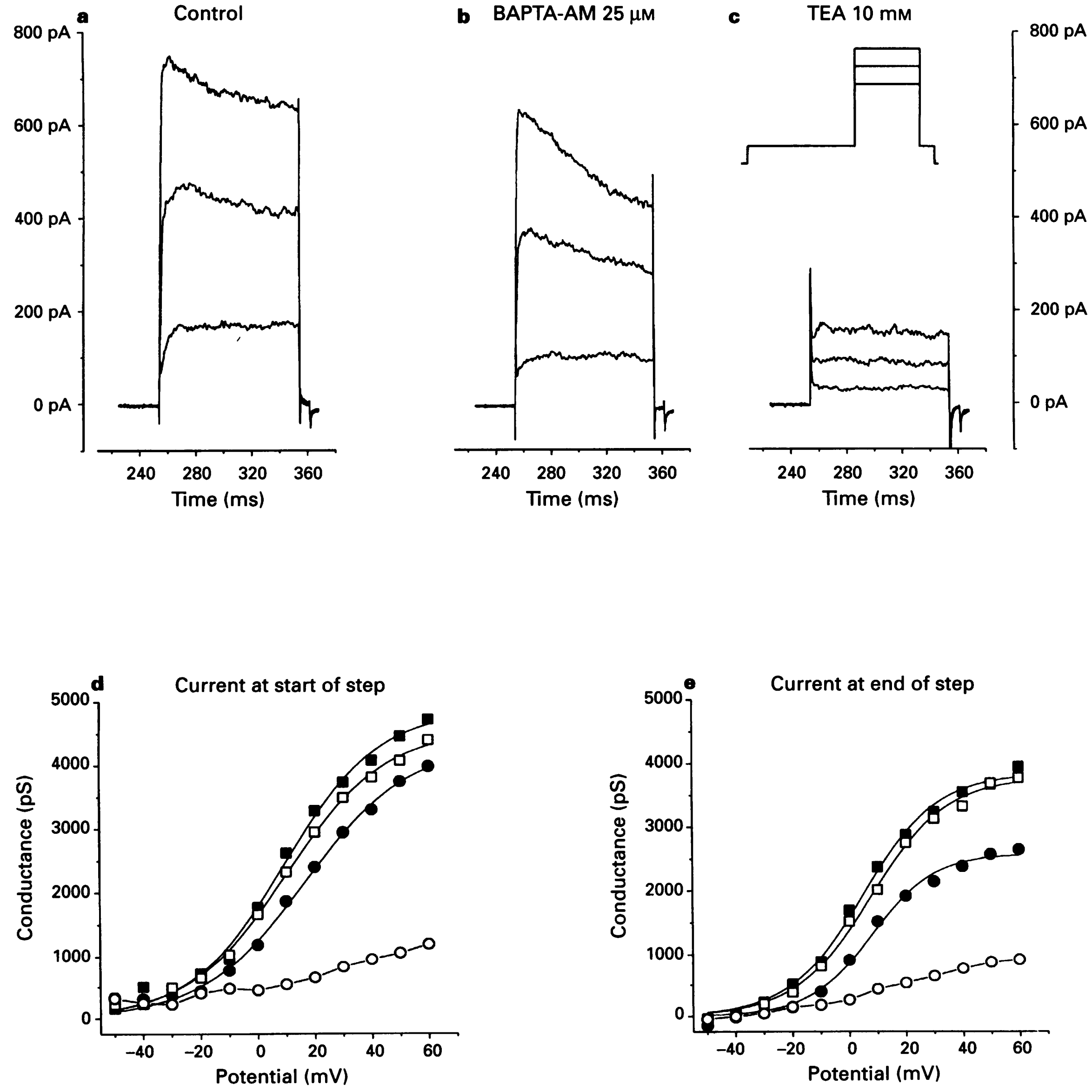

Figure 4 Conductance-voltage relationships for I_{Kv}: an example of the voltage-dependence for I_{Kv} under control conditions (a), in the presence of 25 μM BAPTA-AM (b) and in the presence of 10 mM TEA (c), after recovery from BAPTA-AM inhibition. Currents were evoked by stepping from a holding potential of −70 mV to various test potentials between −50 mV and +60 mV for 100 ms, following a 250 ms prepulse to −50 mV (see inset voltage protocol); the currents evoked by the test steps to +20 mV, +40 mV and +60 mV only are shown for clarity. The magnitude of the chord conductances at the various test potentials were plotted as a function of test potential for control data (■), in the presence of 25 μM BAPTA-AM (●), after wash (□), and in the presence of 10 mM TEA (○), for the peak (d), and end currents (e). The solid lines are fits of the chord conductance versus potential data to a Boltzmann function, as described in Methods. Note that the chord conductance data in the presence of 10 mM TEA are not fitted to a Boltzmann function.

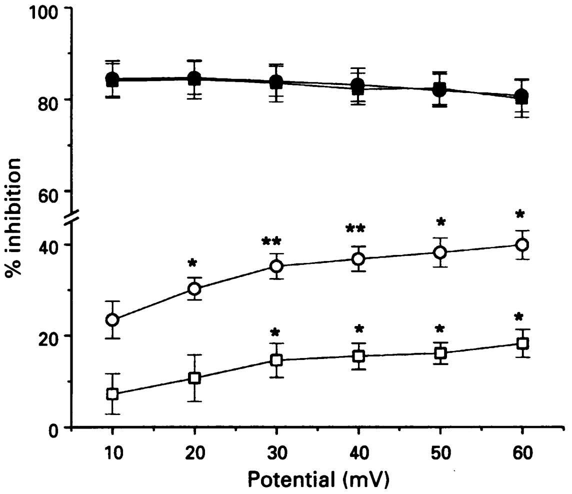

Figure 5 Contrast between the voltage-dependence of BAPTA-AM inhibition of I_{Kv} and the voltage-independent block of this current by TEA. The magnitude of the inhibitions of the peak (□) and end (○) currents by 25 μM BAPTA-AM and of the peak (■) and end (●) currents by 10 mM TEA are plotted as a function of test potential. Values are the mean ± s.e.mean of 8 neurones for BAPTA-AM and 4 neurones for TEA. *$P<0.05$, **$P<0.01$; paired t test.

that this compound acts at an extracellular site. If BAPTA-AM preferentially binds to the open channel, an explanation for the apparent lack of effect on I_{KA} may be that transient channels have inactivated before BAPTA-AM binds. The voltage-dependence of the inhibition of I_{Kv} by BAPTA-AM may also be explained in terms of the open probability of the underlying channels increasing with the degree of depolarization, as has been proposed for bupivacaine block (Valenzuela *et al.*, 1995). Thus, at more depolarized test potentials, more channels will be in the open state and thus BAPTA-AM will block I_{Kv} to a greater extent. An alternative, more complicated, interpretation is that I_{Kv} is composed of multiple components, with BAPTA-AM selectively blocking one slowly activating constituent, such that an inactivating component of I_{Kv} is revealed. This second interpretation is unlikely, since I_{Kv} in cerebellar granule neurones has not been previously reported to have such a complex nature, and both components would be required to have an identical TEA sensitivity.

It should be noted that loading of cerebellar granule neurones with BAPTA has occurred by the end of a 10 min incubation with 25 μM BAPTA-AM. This is demonstrated by the muscarinic modulation of I_{Kso} being significantly attenuated by BAPTA-AM treatment (Watkins, Amos & Mathie, unpublished observations). In addition, we have found that a 10 min application of 25 μM BAPTA-AM dramatically reduced the rise in intracellular Ca^{2+} produced by 10 μM muscarine (Watkins, Amos & Mathie, unpublished observations), as determined by ratiometric measurements using Fura-2.

The fact that BAPTA-AM inhibits K^+ currents would imply that, during incubation with BAPTA-AM, cells may be depolarized, since inhibition of only I_{Kso} by muscarine depolarizes these neurones by 15 mV from approximately −90 mV to −75 mV and increases their input resistance (Watkins & Mathie, 1996). The degree of depolarization will depend upon the precise nature of ion channels that determine the excitability of the particular cell, and also on whether BAPTA-AM inhibits other ion channels; we have not studied the effects of BAPTA-AM on any other ion channels. The depolarization produced by BAPTA-AM inhibition of I_{Kso} alone may not be sufficient for voltage-activated Ca channels to open, and Ca^{2+} influx to occur. However, if other channels are also blocked, as we have shown for I_{Kv}, or depolarizing stimuli were present, this may be sufficient for a significant Ca^{2+} influx to occur, which will have any of a number of consequences (e.g. stimulation of second messenger pathways, ion channel regulation, Ca^{2+} overloading; see Tsien & Tsien, 1990; Hille, 1992), or at the very least may compromise studies where intracellular Ca^{2+} levels are being investigated. This depolarization would not persist if cells were washed after incubation in BAPTA-AM prior to their experimental use, but its consequences may remain. Obviously, if slice preparations are incubated in BAPTA-AM, depolarization may lead to release of neurotransmitters and other neurochemicals, which again may profoundly affect the interpretation of experimental results.

There are no previous reports, to our knowledge, describing ion channel blockade by BAPTA-AM. This may be due, at least in part, to the fact that many workers preincubate tissues in BAPTA-AM before recording (e.g. Fredholm & Hu, 1993; Tymianski *et al.*, 1994), and thus will not see the effects that we describe here. It has been shown very recently that BAPTA-AM could block synaptically evoked field potentials in a fully reversible manner, without affecting the presynaptic volley or antidromically evoked responses (Ouanounou *et al.*, 1995). These data might be explained in terms of inhibition of postsynaptic ion channels.

Thus we have demonstrated that BAPTA-AM reversibly inhibits I_{Kv} and I_{Kso} in cerebellar granule neurones, in a time- and voltage-dependent manner. These effects cannot be attributed to intracellular calcium chelation by hydrolysed BAPTA, and are due to a direct action of this BAPTA analogue on the channels which underlie these K^+ currents. The inhibition of the currents may have significant repercussions in the interpretation of data obtained in experiments using BAPTA-AM.

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