Single-channel properties of BK-type calcium-activated potassium channels at a cholinergic presynaptic nerve terminal

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1. A high-conductance calcium-activated potassium channel (BK K_{Ca}) was characterized at a cholinergic presynaptic nerve terminal using the calyx synapse isolated from the chick ciliary ganglion.

2. The channel had a conductance of 210 pS in a 150 mM:150 mM K^+ gradient, was highly selective for K^+ over Na^+, and was sensitive to block by external charybdotoxin or tetraethylammonium (TEA) and by internal Ba^{2+}. At +60 mV it was activated by cytoplasmic calcium $[Ca^{2+}]_i$ with a K_d of ~0·5 μM and a Hill coefficient of ~2·0. At 10 μM $[Ca^{2+}]_i$ the channel was 50% activated ($V_{½}$) at −8·0 mV with a voltage dependence (Boltzmann slope-factor) of 32·7 mV. The $V_{½}$ values hyperpolarized with an increase in $[Ca^{2+}]_i$ while the slope factors decreased. There were no overt differences in conductance or $[Ca^{2+}]_i$ sensitivity between BK channels from the transmitter release face and the non-release face.

3. Open and closed times were fitted by two and three exponentials, respectively. The slow time constants were strongly affected by both $[Ca^{2+}]_i$ and membrane potential changes.

4. In cell-attached patch recordings BK channel opening was enhanced by a prepulse permissive for calcium influx through the patch, suggesting that the channel can be activated by calcium ion influx through neighbouring calcium channels.

5. The properties of the presynaptic BK channel are well suited for rapid activation during the presynaptic depolarization and Ca^{2+} influx that are associated with transmitter release. This channel may play an important role in terminating release by rapid repolarization of the action potential.

Substantial evidence suggests that most, if not all, nerve terminals that secrete neurotransmitters by action potential-dependent mechanisms exhibit a calcium-activated potassium (K_{Ca}) channel. Such terminals include presynaptic nerve terminals at fast-transmitting synapses (Bartschat & Blaustein, 1985; Farley & Rudy, 1988; Anderson *et al.* 1988; Lindgren & Moore, 1989; Schneider *et al.* 1989; Astrand & Stjarne, 1991; Sivaramakrishnan *et al.* 1991; Robitaille & Charlton, 1992; Wangemann & Takeuchi, 1993; Blundon *et al.* 1995; Rahamimoff *et al.* 1995; Katz *et al.* 1997; Sakaba *et al.* 1997; Yazejian *et al.* 1997), hormone-secreting nerve terminals (Bielefeldt *et al.* 1992; Wang & Lemos, 1992; Wang *et al.* 1992; Bielefeldt & Jackson, 1993) and sense-organ cells (such as hair cells, Edgington & Stewart, 1981; Roberts *et al.* 1990, 1991; Issa & Hudspeth, 1994; Art *et al.* 1995) which share many properties with fast-transmitting nerve terminals. It is not clear which specific types of K_{Ca} channel are present at most of these sites but, where tested by specific staining or direct recording, the high-conductance, BK-type has been repeatedly observed (Smith *et al.* 1986; Castle & Strong, 1986; Roberts *et al.* 1991; Robitaille & Charlton, 1992; Bielefeldt & Jackson, 1993; Wangemann & Takeuchi, 1993; Issa & Hudspeth, 1994; Art *et al.* 1995; Knaus *et al.* 1996). Other studies have presented more indirect evidence for the presence of this channel in the nerve terminal on the basis of effects of peptide blockers, primarily charybdotoxin (CTX) or iberiotoxin (IbTX), or intracellular buffers on transmitter release (Kumamoto & Kuba, 1985; Sivaramakrishnan *et al.* 1991; Robitaille & Charlton, 1992; Stretton *et al.* 1992; Takeuchi & Wangemann, 1993; Robitaille *et al.* 1993; Yazejian *et al.* 1997).

Despite the evident broad distribution of BK channels in nerve terminals little is known about their biophysical properties and to what extent these may differ from their relatives in the cell soma. This question has become of particular interest recently with the discovery that the BK

channel can be expressed in many functionally different isoforms (Butler *et al.* 1993; Wei *et al.* 1998). In addition, the channel can associate with a β subunit (Vergara *et al.* 1998) that has marked effects on its biophysical and pharmacological properties. Thus, because of this diversity a full understanding of the role of this channel in the nerve terminal requires direct recording of its properties *in situ*. Furthermore, the complex activation characteristics of the BK channel require analysis at the single-channel level since it is gated by both a ligand and a physical factor, i.e. calcium and voltage. There have been only two nerve terminals at which the single-channel properties of BK channels have been examined (Wang *et al.* 1992; Wangemann & Takeuchi, 1993; Bielefeldt & Jackson, 1993) due, presumably, to the general small size and inaccessibility of these structures. Few nerve terminals are of sufficiently large size to permit direct patch-clamp recording, and in the cases where this is possible obtaining sufficient data requires considerable experimental effort. This study presents the first characterization of single BK channels at the presynaptic nerve terminal of a neuron-to-neuron synapse.

We used the calyx-type synapse of the chick ciliary ganglion (CCG) to record and characterize single presynaptic high-conductance K_{Ca} channels. This experimental preparation was the first in which an intact vertebrate presynaptic terminal could be voltage clamped to record whole-terminal currents (Stanley, 1989) or clamped in the cell-attached configuration (Stanley, 1991) to record single-channel activity. We have recently presented evidence for the presence of K_{Ca} channels on this terminal by direct recording of an outwardly rectifying K^+ current that was sensitive to CTX and IbTX (Tozer *et al.* 1998). In this study we first used the patch-clamp technique in the outside-out and inside-out configurations to test for block with standard K^+ channel blockers. We next used the same techniques to examine the voltage and calcium sensitivity of single BK channels. Finally, we used the cell-attached configuration to test whether presynaptic BK channels could be activated by calcium influx through the same membrane patch.

METHODS

Preparation of calyces

Calyx nerve terminals were prepared as described (Stanley, 1991; Stanley & Goping, 1991). Briefly, 15-day-old chick embryos were killed, in compliance with NIH guidelines, by decerebration immediately upon removal from the egg. Ciliary ganglia were dissected and were incubated in modified Eagle's medium (MEM) with 0·74 mg ml^{-1} collagenase Type IV (Worthington), 12·3 mg ml^{-1} Dispase (Boehringer Mannheim), 800 units ml^{-1} hyaluronidase and 70 μg ml^{-1} trypsin inhibitor Type II (Sigma) for 2–3 h at 37 °C in 5% CO_2 and 95% air. The cells were washed, triturated and allowed to adhere to a glass coverslip in a 0·5 ml chamber for recording. The above treatment resulted in calyx nerve terminals that had been dissociated from the postsynaptic neurones to varying degrees, and included intact, partially dissociated and fully dissociated calyx synapses. Except where specified, only fully isolated calyces (see Haydon *et al.* 1994, Fig. 1*C*) were used for single-channel recording in this study.

Single-channel recording

The patch clamp was used in several configurations including cell-attached, outside-out and inside-out. Patch pipettes (WPI, TW150F) were fire polished and coated with beeswax. For cell-attached recording patch electrodes were filled with standard extracellular solution (SES) containing (mM): 140 NaCl, 2 $CaCl_2$, 2 $MgCl_2$, 5 KCl, 0·001 TTX and 10 Hepes; pH = 7·3 (adjusted with NaOH). 4-Aminopyridine (4-AP; 10 mM) was added to block calcium-insensitive K^+ channels. The bath contained SES in which the NaCl had been replaced with KCl (K^+-SES) to depolarize the membrane potential to ~0 mV. For outside-out recordings the bath contained SES while the pipette was filled with standard internal solution (SIS) containing (mM): 150 KCl or 150 potassium aspartate, 1 $MgCl_2$, 2 $CaCl_2$, 2 EGTA and 10 Hepes; pH 7·3 (adjusted with KOH). The calculated free internal Ca^{2+} ($[Ca^{2+}]_i$) is 7 μM (see below for method). Inside-out patches were recorded with K^+-SES in the pipette and SIS in the bath. Free-calcium levels on the cytoplasmic face of the membrane were set by adding the calculated ratio of $CaCl_2$ and EGTA (using Chelator 1.0 software, Schoenmakers, Nijmen, The Netherlands). Patches were excised initially in low free-calcium of 0·01–0·1 μM. Channels that exhibited detectable inactivation (Fig. 7) were not used in analysis of steady-state open probablity (P_o) and kinetics. The dish was continually perfused and drugs and salts were added directly to the perfusate, allowing 15 min for complete solution exchange. Experiments were carried out at room temperature (~22 °C).

Channel blockers

CTX, tetraethylammonium (TEA), and 4-aminopyridine (4-AP) were from RBI.

Data acquisition

Single-channel currents were acquired, filtered at 5 kHz, and sampled at 10–50 kHz with an Axopatch 1C amplifier (Axon Instruments) controlled using pCLAMP (v. 5.5 to 6.03; Axon Instruments) software. Data were stored on disc and were analysed with pCLAMP (v. 6.03) software.

Analysis

All membrane potentials are presented as the potential inside the cell relative to that outside. The number of channels in the patch was determined by holding the membrane at +60 mV in the presence of a high $[Ca^{2+}]_i$ and recording for > 30 s. Since the maximum open probability ($P_{o(max)}$) is high (> 0·7, see Fig. 4) multi-channel patches could be readily identified. All patches used for lifetime distributions contained only one channel and were recorded for a minimum of 30 s under all conditions. Longer durations were used when the open probability was low. The permeability ratio ($\alpha = P_K/P_{Na}$) was calculated using a simplification of the Goldman–Hodgkin–Katz equation for monovalent ions:

$$E_{rev} = \frac{RT}{F} \ln\left(\frac{\alpha[K^+]_o + [Na^+]_o}{\alpha[K^+]_i + [Na^+]_i}\right), \quad (1)$$

where R, T and F have their usual meanings.

Single-channel current–voltage curves were fitted with the predicted K^+ current using the Goldman current equation:

$$I_K = P_K \frac{EF^2}{RT}\left(\frac{[K^+]_o - [K^+]_i e^{EF/RT}}{1 - e^{EF/RT}}\right).$$

To make this calculation we assumed that K^+ and Na^+ are the only permeant ions and subtracted the small contribution of Na^+ current. That is, the measured current $(I_{BK}) = I_K + I_{Na}$:

$$I_{BK} = P_K \frac{EF^2}{RT}\left(\frac{[K^+]_o - [K^+]_i e^{EF/RT}}{1 - e^{EF/RT}}\right) + \frac{P_K}{\alpha}\frac{EF^2}{RT}\left(\frac{[Na^+]_o - [Na^+]_i e^{EF/RT}}{1 - e^{EF/RT}}\right). \quad (2)$$

Open–closed transitions were detected as current transitions beyond a threshold level set at 50% of the fully open channel. Although subconductance states were occasionally seen in these channels (see Fig. 2), they were too infrequent to significantly affect the channel-state analysis. Dwell times were binned using a log-scale bin size, plotted on linear bin amplitude co-ordinates and were fitted to multiexponential functions with a Levenberg–Marquardt curve fitting algorithm in the pCLAMP (v. 6.03) analysis suite. The mean open and closed durations were obtained as the sum of the relative area of each exponential component multiplied by the time constant of the component. Data are expressed as means ± S.E.M, with n indicating the number of recordings.

RESULTS

Characterization of the steady-state behaviour of the K_{Ca} channel

Identification of the K_{Ca} channel

We used single-channel recordings to characterize the presynaptic K_{Ca} channels in detail. Cell-attached recordings from the calyx nerve terminals exhibited little or no channel activity at membrane potentials up to +20 mV. However, after excision into high $[Ca^{2+}]_i$, 145/181 membrane patches exhibited active K^+ channels. Of the remaining patches, 21 exhibited no channel activity and 15 exhibited a smaller-conductance channel that was not examined further. Channel conductance was 210 ± 7 pS ($n = 20$) in symmetrical 150 mM K^+ solutions, as calculated from the slope of the I–V curve over the −60 to +60 mV range. These single-channel currents were blocked by external TEA or the peptide toxin CTX or by internal Ba^{2+} ions, confirming their identity as large-conductance K_{Ca} channels (BK channel; Fig. 1). The block by all three agents was reversible, with TEA or Ba^{2+} block relieved immediately on removal, whereas CTX block reversed only slowly.

Ion selectivity

Channels were examined in excised patches in order to completely control the ions on both sides of the membrane. The BK channel had a large conductance and usually opened to a single current level (e.g. Fig. 1). However, infrequent subconductance states were also observed and these could be resolved to defined levels in selected traces (Fig. 2*A* and *B*).

The conductance of the main open state was compared at different K^+:Na^+ ratios (Fig. 2*C*). In all gradients the current reversal was close to the calculated K^+ equilibrium potential, E_K, confirming the high selectivity for K^+. P_K/P_{Na} was ~32, calculated using eqn (1) and the difference between the calculated E_K and the reversal potential, E_{REV}, estimated by eye from the data with 150 mM K^+ and 5 mM Na^+ in the extracellular solution and 20 mM K^+ and 135 mM Na^+ in the internal solution. From a least-squares fit of each of the curves in Fig. 2*C* to eqn (2), P_K was estimated to be $4{\cdot}04 \pm 0{\cdot}15 \times 10^{-13}$ cm s^{-1} (range, 3·62–4·39; $n = 5$), a

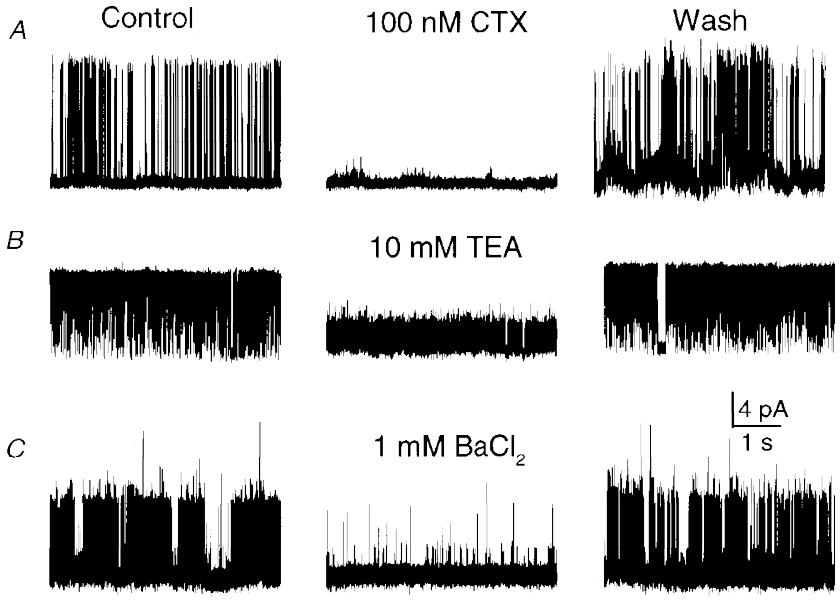

Figure 1. Effect of blockers on single K_{Ca} channels in isolated patches

Block by external CTX (*A*) or TEA (*B*), recorded in outside-out patches, or by internal Ba^{2+} (*C*), recorded from an inside-out patch, were all reversible. Channel openings are upward. Symmetrical 150 mM K^+ was used with 100 μM $[Ca^{2+}]_i$ on the cytoplasmic face. Membrane potential: +60 mV in *A*, +40 mV in *B* and *C*.

Table 1. Effect of calcium on the voltage dependence of single BK channel openings

	$[Ca^{2+}]_i$ (μM)				
	0·2	0·5	1	10	100
$V_{½}$ (mV)	41·1	34·0	20·5	−8·0	−12·5
Slope factor (mV)	11·9	15·8	21·5	32·7	41·8
n	3	4	6	6	7

Pooled data from n individual single-channel patch recordings were fitted to a Boltzmann curve for each $[Ca^{2+}]_i$.

value that is very close to that reported previously (Carl & Sanders, 1989).

Calcium and voltage dependence of the BK channel

The steady-state open probability as a function of $[Ca^{2+}]_i$ was examined using inside-out patches held at a constant voltage of +60 mV. At this potential P_o increased steeply with increasing $[Ca^{2+}]_i$ and channel openings were observed at a cytoplasmic calcium level as low as 0·1 μM (Fig. 3), reaching a plateau at 5–10 μM. The average data from three to eight single-channel recordings were well fitted with a dose–response curve, to give a dissociation constant (K_D) of $\sim 5 \times 10^{-7}$ and an estimated $P_{o(max)}$ at +60 mV of ~0·7. A Hill plot gave a slope of ~2, suggesting strong co-operative binding of at least two calcium ions, or weaker co-operative

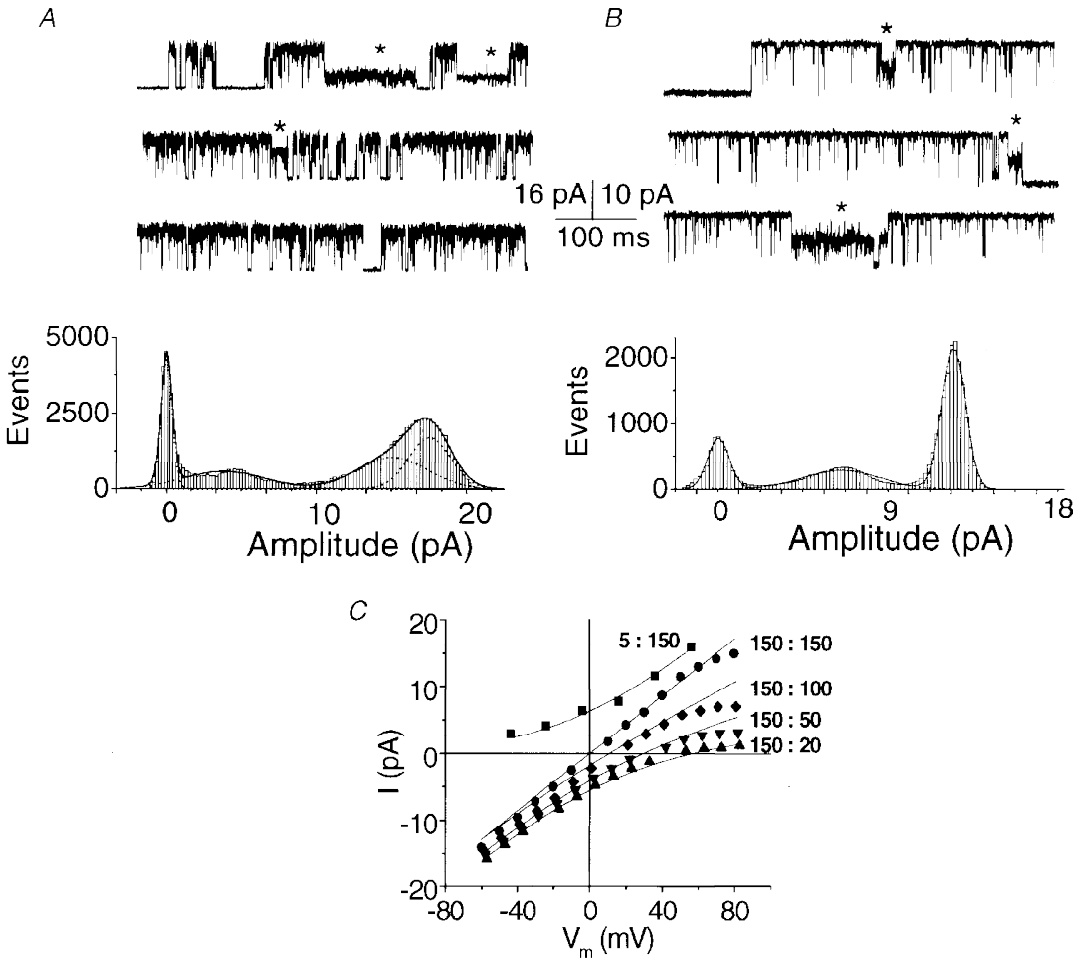

Figure 2. Single-channel K_{Ca} currents, subconductance states and cation selectivity

A and *B*, two examples of current fluctuations (upper panels) and all-points histograms with fitted Gaussian distributions (lower panels) in inside-out membrane patches held at +60 mV with 10 μM $[Ca^{2+}]_i$. Channel openings are upward. Traces were selected to illustrate current amplitude substates (*). *A*, the internal solution contained 5 mM Na^+ and 150 mM K^+ and the external solution contained 155 mM Na^+ and 5 mM K^+. *B*, internal and external solutions were symmetrical, with 5 mM Na^+ and 150 mM K^+. *C*, current–membrane potential (V_m) curves from two inside-out patches in symmetrical 5 mM $[Na^+]_o$ plus different K^+ gradients (substituting Na^+ for K^+) with 10 μM $[Ca^{2+}]_i$. Ratios indicated are for $K_o^+ : K_i^+$ (both mM). Current–voltage relations were fitted to the theoretical potassium current using a modified Goldman current equation that included a term used to subtract the sodium component (Methods, eqn (2)).

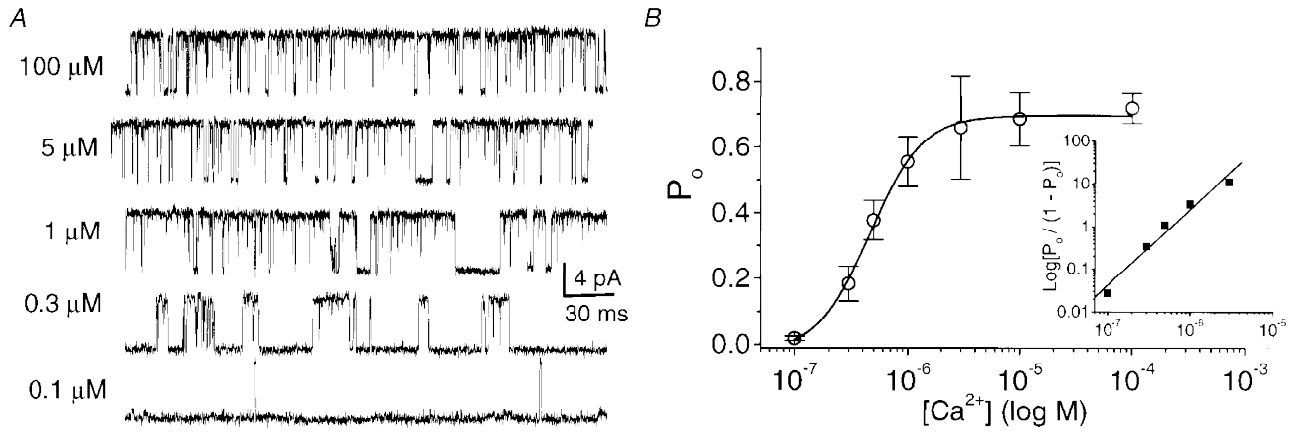

Figure 3. Effects of $[Ca^{2+}]_i$ on BK open probability

A, representative unitary K_{Ca} current in an inside-out patch recorded at +60 mV in symmetrical K^+ (150 mM) and in various $[Ca^{2+}]_i$. *B*, the effect of $[Ca^{2+}]_i$ on open probability (P_o). Each point represents the average of 3–8 single-channel patch recordings at a holding potential (V_h) of +60 mV. The data are fitted to a dose–response curve with a $P_{o(max)}$ of 0·72 and a K_D of $4{\cdot}8 \times 10^{-7}$. Inset: Hill plot derived from the first 5 points after normalizing to $P_{o(max)}$. A Hill coefficient of 2·0 was estimated as the slope of a straight line fit to the log–log plot.

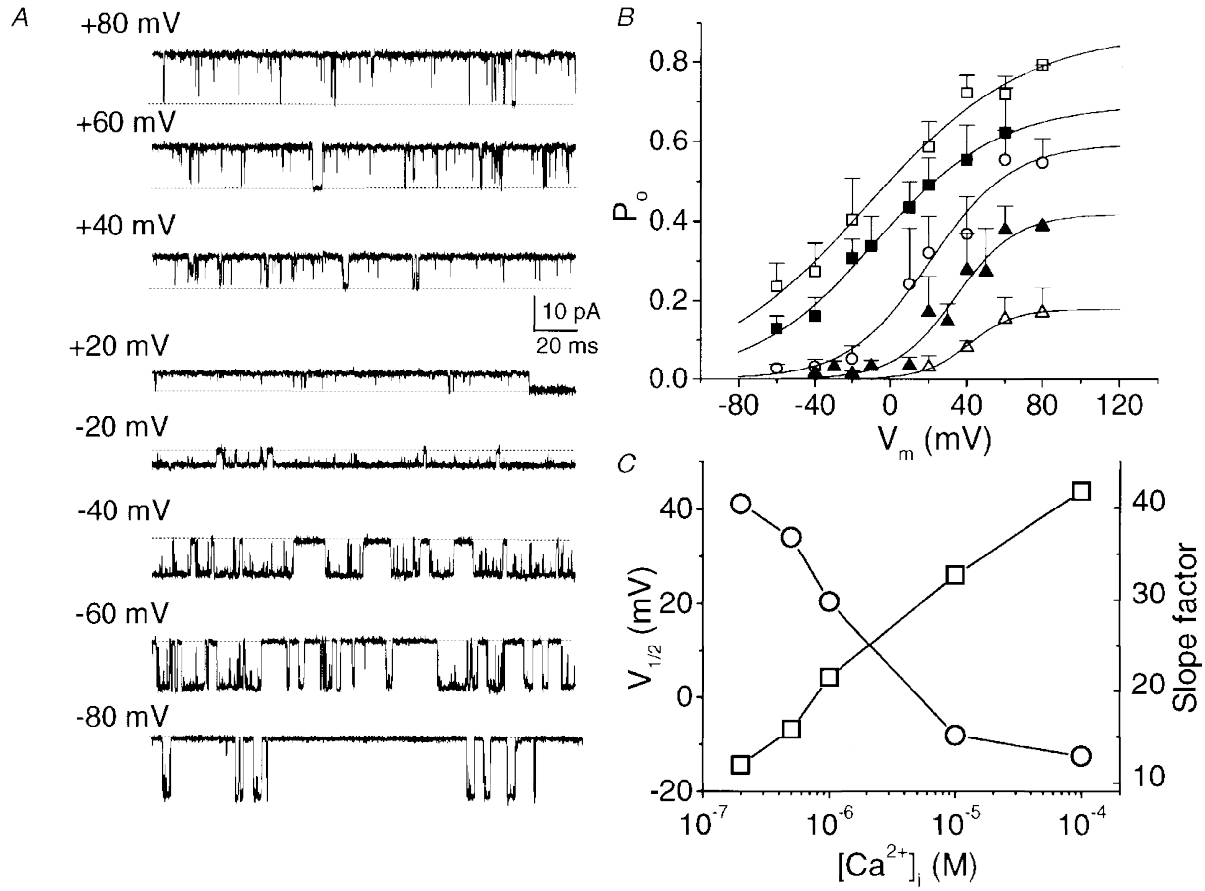

Figure 4. Voltage dependence of BK channels at different $[Ca^{2+}]_i$

A, BK channel activity recorded at a range of membrane potentials in symmetrical K^+ (150 mM) with 100 μM $[Ca^{2+}]_i$. *B*, P_o as a function of membrane potential at various $[Ca^{2+}]_i$ (μM): □, 100; ■, 10; ○, 1; ▲, 0·5; △, 0·2. Each point represents the mean of single channels in 3–8 different patches. Smooth curves are fitted with Boltzmann relations which were set to $P_{o(min)} \rightarrow 0$. *C*, plot of $V_{½}$ (○) and slope factors (□) derived from the Boltzmann fits to the data in *B* against $[Ca^{2+}]_i$.

binding of more than two ions, to maximally activate the channel.

We next examined the effect of membrane potential on steady-state channel open probability at different $[Ca^{2+}]_i$ levels (Fig. 4*A*). At each $[Ca^{2+}]_i$ the voltage dependence could be fitted by a single Boltzmann relation (Fig. 4*B*). The effect of increasing $[Ca^{2+}]_i$ on the relation between voltage and P_o is particularly interesting with respect to three channel properties. First, there was a hyperpolarizing shift in the voltage range over which the channel was active, as indicated by a shift of $V_{½}$ (Table 1, Fig. 4*C*). Second, the slope factor of the Boltzmann fit, a reflection of the voltage dependence of the channel, increased at higher $[Ca^{2+}]_i$ (Art *et al.* 1995; Table 1), indicating a reduced voltage dependence when the $[Ca^{2+}]_i$ binding sites are occupied. Finally, there was a progressive increase in $P_{o(max)}$ but we did not examine this further.

BK channel kinetics

Patches with single K_{Ca} channels (e.g. Fig. 4*A*) were used to analyse open–closed kinetics at different $[Ca^{2+}]_i$ levels.

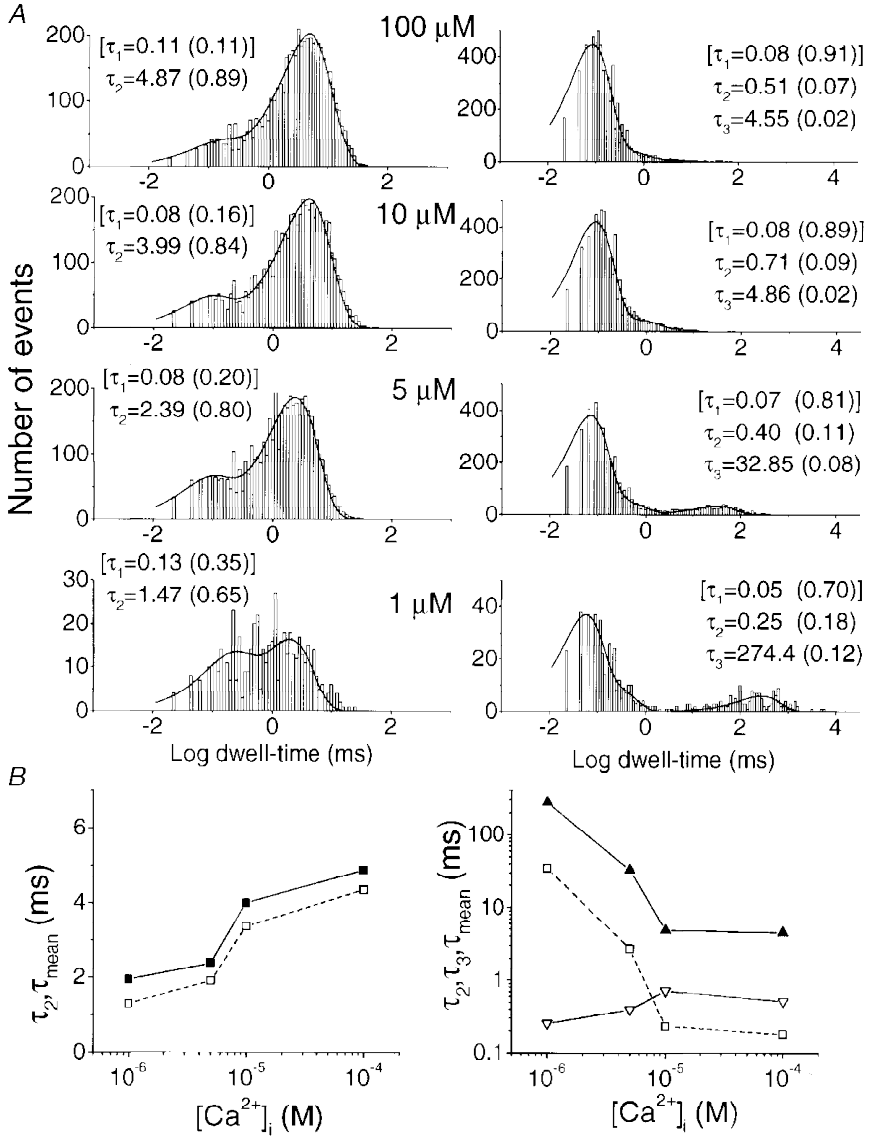

Figure 5. Intracellular Ca^{2+} modulates the steady-state kinetics of the BK channel

Single-channel current fluctuations were recorded at various $[Ca^{2+}]_i$ levels in inside-out patches held at +60 mV in symmetrical 150 mM K^+ solutions. *A*, fitted dwell-time histograms are shown for a typical single-channel recording (e.g. Fig. 3*A*). Open times (left panels) were fitted with double exponentials and closed times (right panels) with three exponentials, with the time constants indicated on each panel. The fastest time constants (τ_1, square brackets) in each case were too short to be measured reliably and were used for fitting purposes only. The numbers in parentheses show the fractional contribution of each component to the area under the curve. *B*, open (left) and closed (right) time constants for the reliably measured slow (τ_2, ■, left; τ_3, ▲, right), intermediate (τ_2, ▽, right) and mean time constants (□). Results are from the data in Fig. 5*A*.

Channel behaviour could be adequately described by two open and three closed states (Fig. 5*A*). The fastest (τ_1) open and closed states were used for fitting purposes only since they were too brief to be resolved reliably under our recording conditions. Increasing $[Ca^{2+}]_i$ increased the duration of the slow open state and markedly decreased the duration of the slowest closed state (Fig. 5*B*).

We also examined the effect of voltage on steady-state single-channel kinetics (Fig. 6). Data were analysed as in Fig. 5 but at different holding potentials and at a fixed $[Ca^{2+}]_i$ of 100 μM. Again, the slowest time constants were the most sensitive to voltage shifts for both open and closed states (Fig. 6*B*).

Channel inactivation

In general, the channels did not inactivate during sustained depolarizations, as is evident from the observed high $P_{o(max)}$ (Figs 3 and 5). However, inactivation was observed during a depolarizing voltage pulse in about 4% of the patch recordings (8/181 patches; Fig. 7*A*). Openings were clustered at the beginning of the trace and ensemble averages demonstrated an inactivating current. The rate of inactivation was weakly voltage dependent and the time

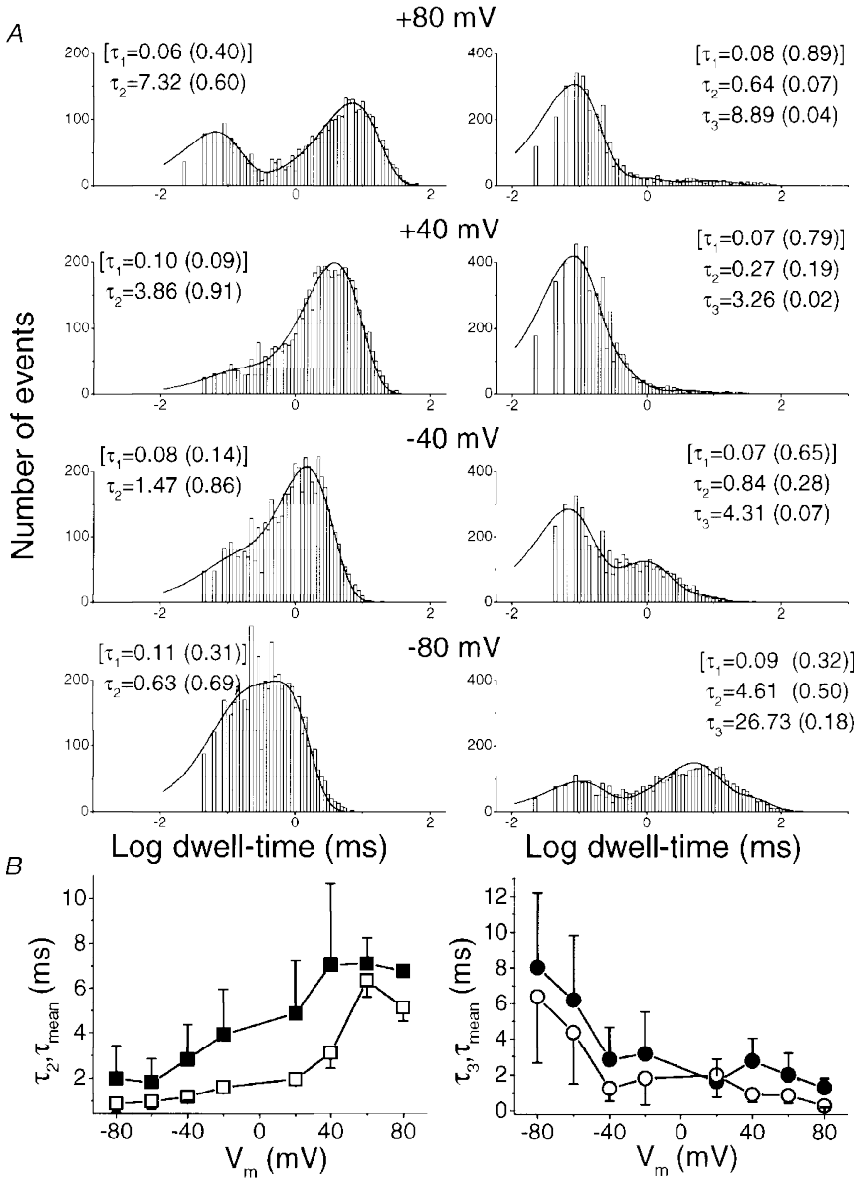

Figure 6. Voltage modulates the steady-state kinetics of the K_{Ca} channel. Inside-out patches, exposed to symmetrical 150 mM K^+ solutions with 100 μM $[Ca^{2+}]_i$, were held at various membrane potentials

A, fitted dwell-time histograms for a representative single-channel patch held at four membrane potentials. Data were analysed and are presented as in Fig. 5. *B*, open (left) and closed (right) time constants for the reliably measured slow (τ_2, ■, left; τ_3, ●, right) and mean time constants (□, ○) averaged for 4 single-channel patches.

Table 2. Comparison of the properties of BK channels recorded from the calyx nerve terminal transmitter release face with those on the back face

Location	Frequency*	Single-channel conductance† (pS)	K_D‡
Release face	100% (6/6)	217·0 ± 10·4 (4)	$7{\cdot}7 \times 10^{-6}$ (2)
Back face	80% (24/30)	200·3 ± 8·2 (5)§	$8{\cdot}0 \times 10^{-6}$ (2)

* Percentage of patches with BK channel activity (positive patches/number of patches examined). † Mean ± S.E.M. (number of trials). ‡ Ca^{2+} dissociation constant calculated at +40 mV (number of single-channel patches). § $P > 0{\cdot}05$ (Student's t test) *vs.* release face.

constant decreased with the degree of depolarization (Fig. 7*A* and *B*).

Relation of the K_{Ca} channel to the nerve terminal

BK channel properties and location

We carried out a limited comparison of BK channel properties at two main regions of the calyx: on the back, Schwann cell-facing aspect, or on the front, transmitter-release face aspect (Table 2). Channels were noted in most patches at both locations and there were no obvious differences in channel conductance nor in calcium sensitivity.

Activation by calcium domains

We recorded BK channels *in situ* in the cell-attached configuration to test whether these channels could be activated by calcium channels in the same membrane region. This was tested by first examining BK channel activation during a sufficiently large depolarization (to +80 mV) to inhibit calcium influx (i.e. to well beyond the reversal potential, E_{rev}, ~+65 mV; see Stanley, 1991). The amount of BK current activated at +80 mV was then compared after a short pre-pulse to a potential within the range that allows calcium influx (−20 or 0 mV, $n = 7$). We selected patches that were likely to be located on the transmitter-release face of the nerve terminal, the location of the calcium channels (Stanley, 1991). Representative experiments are shown in Fig. 8, from a multi-channel patch, and Fig. 9, from a single-channel patch. Some openings were observed in the absence of a prepulse (Figs 8*A* and 9*A*), as expected from their voltage dependence, but these were enhanced by the short conditioning pulse to above threshold for opening calcium channels (Fig. 8*B* and 9*B*). A small inward current, consistent with a calcium current, is evident during the prepulse in Fig. 9*B* but in Fig. 8*B* this was too small to detect. The presence and activation of a calcium current in

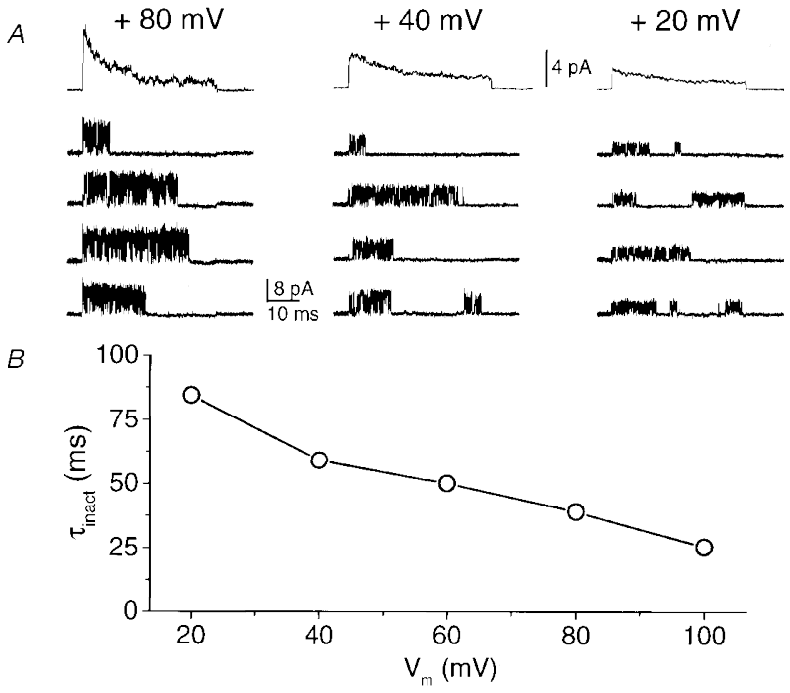

Figure 7. Inactivation of single-channel BK currents

A, each panel shows recordings of a single K_{Ca} channel in an inside-out patch during voltage steps from $V_h = -70$ mV to the indicated potentials. The bottom four traces are representative recordings whilst the top trace is the ensemble average of 100 traces. This behaviour was only observed in a few patches. *B*, the decay of each ensemble average was fitted to a single exponential and the time constant (τ_{inact}) plotted as a function of the membrane potential. Symmetrical 150 mM K^+ solutions were used, with 10 μM $[Ca^{2+}]_i$.

the patch in Fig. 9*B* is evident from the prominent inward tail current at the end of the +80 mV test pulse. The lack of effect of a +80 mV prepulse (Fig. 8*C*) demonstrates that the recruitment of BK channel activity is not simply due to a preceding depolarization. Further, no increase in P_o was observed with a prepulse to −20 mV when Ca^{2+} channels were blocked by ω-conotoxin GVIA in the external solution in the patch electrode ($n = 2$; data not shown). By subtracting the ensemble averaged currents in the single-channel recording, a prepulse-induced increase in BK ensemble current (and thus P_o) was revealed (Fig. 9*D*). This observation, together with the decreased time to first channel opening after prepulses to −20 or 0 mV (Figs 8*C* and 9*E*) demonstrated that BK channel activity resulted from changes in channel kinetics, and did not require recruitment of new channels. The simplest interpretation of these findings is that Ca^{2+} influx through the calcium channels in the membrane patch activated the neighbouring K_{Ca} channels.

BK channel density

In over 180 cell-attached patches the highest density of BK channels was six channels, as shown in Fig. 8. The bulk of patches exhibited one to three channels.

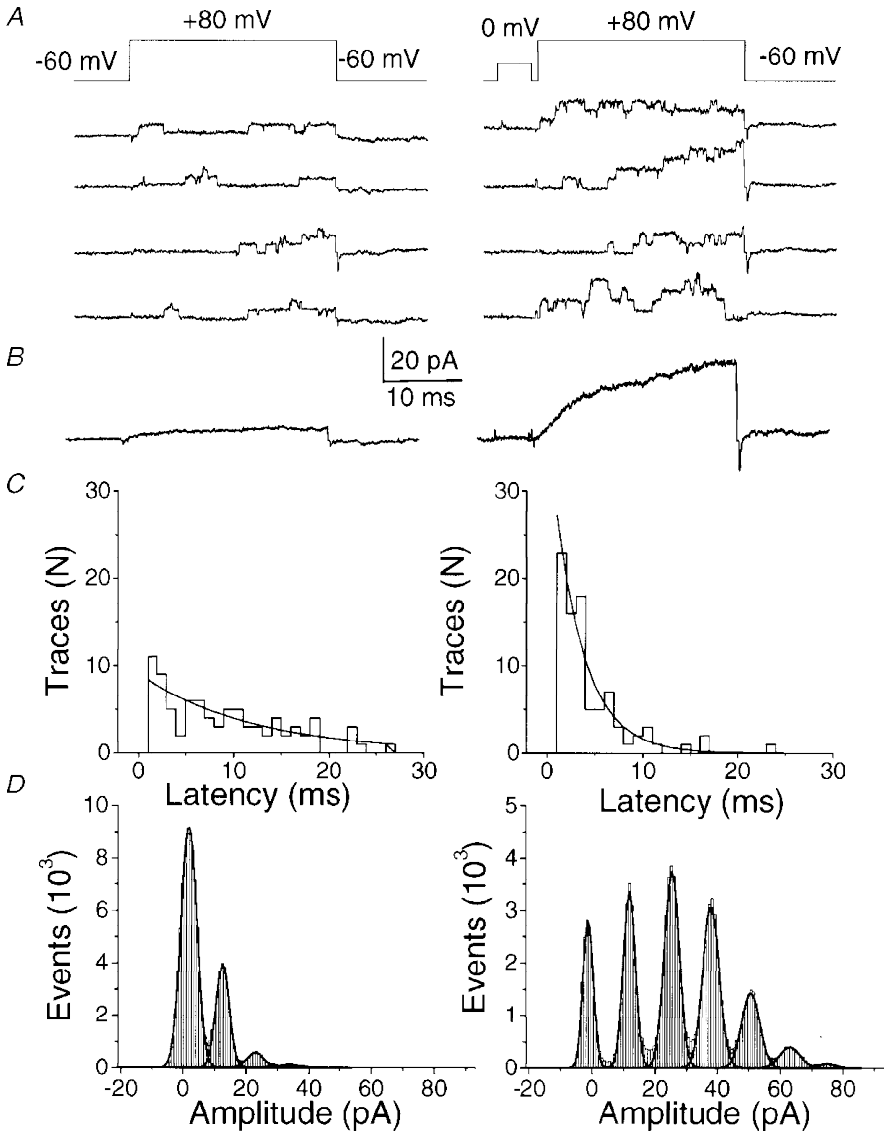

Figure 8. BK single-channel current is recruited by prepulses favouring Ca^{2+} entry

Depolarizing pulses to +80 mV (above, E_{rev}) were applied from a holding potential of −60 mV to the same cell-attached patch containing multiple BK channels either without (all left panels) or following (all right panels) a 5 ms prepulse to 0 mV. *A*, examples of individual sweeps. *B*, currents calculated as the average of 100 traces. *C*, histograms of latency to first opening after the pulse to +80 mV. *D*, all-points amplitude histograms with (left panel) and without (right panel) a prepulse. Each peak above 0 pA corresponds to one open channel. The pipette contained SES with 10 mM $CaCl_2$ while the bath contained 150 mM K^+ solution.

DISCUSSION

The object of this study was to identify and characterize a BK channel on a vertebrate presynaptic nerve terminal at a fast-transmitting synapse. Nerve terminal BK channels have previously been characterized at the single-channel level in only two preparations, the neurosecretory terminal of the posterior pituitary (Bielefeldt *et al.* 1992), which secretes hormones, and the efferent terminals in the organ of Corti (Wangemann & Takeuchi, 1993), which modulates a sensory hair cell. The present results represent the first examination of the single-channel properties of BK channels in the presynaptic terminal of a neuron-to-neuron synapse.

We find that the presynaptic K_{Ca} channel has many properties in common with a subset of BK channels recorded in a variety of cell types (see Vergara *et al.* 1998, for a recent review). Four properties are of particular interest with regard to nerve terminal function. First, block by CTX is reversible, second, calcium affinity is in the low to mid range for this channel type, third, the channels exhibit a strong voltage dependence particularly at low $[Ca^{2+}]_i$ and,

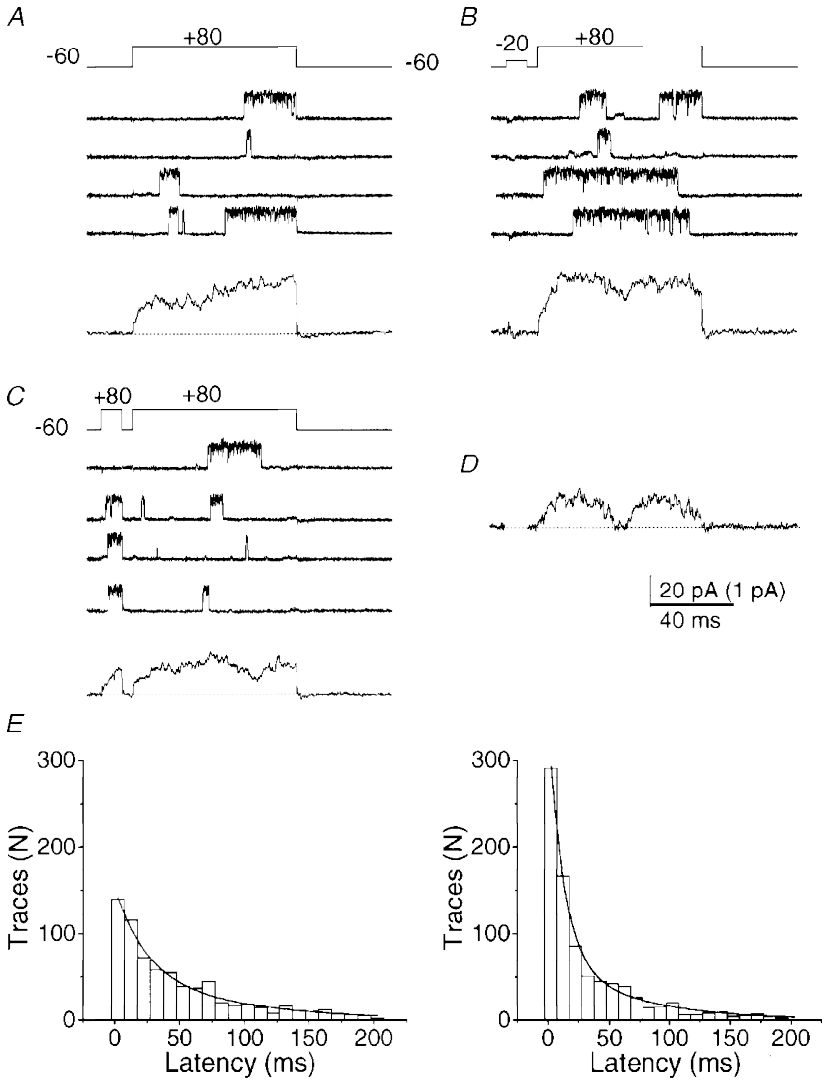

Figure 9. A depolarizing prepulse can increase the open probability of a single BK channel in a cell-attached patch

Test pulses were given to +80 mV (above, E_{rev}) from a holding potential of −60 mV. Four individual current traces and their respective ensemble average of 200 traces (below) are shown in the absence of a prepulse (*A*) or following a 10 ms prepulse to −20 mV (*B*) or +80 mV (*C*) in the same patch. Recording solutions were as in Fig. 8. *D*, subtraction of ensemble current in *C* from that in *B* shows the current activated by the −20 mV prepulse (ensemble average current). For clarity, the current during the prepulse has been blanked. The scale bar for the ensemble currents is 1 pA. *E*, time to first opening of BK channels after the onset of the test pulse to +80 mV without (left) or following (right) a prepulse to −20 mV. Data are pooled from 1200 sweeps from 6 calyces. The smooth curves were fitted to double exponential functions with time constants of 23·5 and 81·6 ms in the control (no prepulse) and 12·0 and 62·4 ms in the prepulse group.

finally, we present evidence that these channels can be activated by $[Ca^{2+}]_i$ influx through neighbouring calcium channels.

The calyx BK channel was blocked by the peptide toxin, CTX, as is typical for channels of this type. We did not determine the affinity of CTX binding but did note that after repeated washing the block was reversible. Previous studies report that K_{Ca} currents in other presynaptic terminals range from those that are blocked by CTX (Schneider *et al.* 1989; Robitaille *et al.* 1993) to those that are totally insensitive (Bielefeldt *et al.* 1992; Blundon *et al.* 1995).

The BK channel was highly selective for potassium over sodium and exhibited an outward current over the entire physiological range (−75 to +50 mV), as is typical of this channel type. At +60 mV the BK channel was essentially closed at 100 nM $[Ca^{2+}]_i$ but reached maximal activation at ∼3 μM. Thus, these presynaptic BK channels are of moderate calcium sensitivity, similar to those at the organ of Corti synapse (Wangemann & Takeuchi, 1993) and only slightly less sensitive than those at the posterior pituitary neurosecretory terminal (Bielefeldt *et al.* 1992). Again, in common with many other reports on BK channels and with data from the posterior pituitary neurosecretory terminal we obtained a Hill coefficient of ∼2. Although this has been taken to suggest that two calcium ions are necessary to activate this channel it is safer to conclude that a minimum of two are required. Furthermore, Hill coefficients have been determined only for steady-state behaviour: the dependence may be steeper when the channel is activated, as it almost certainly is *in vivo*, by acute increases in $[Ca^{2+}]_i$.

The open and closed times determined from single-channel recordings in different $[Ca^{2+}]_i$ could be fitted by two open and three closed states, consistent with many previous studies on this channel type (Vergara *et al.* 1998). However, the shortest of the open and closed times were too rapid to be recorded reliably under our conditions. Of the kinetic states that could be examined, the slowest closed state was the most sensitive to $[Ca^{2+}]_i$.

The voltage dependence of the channel open probability was examined at steady state across a wide range of calcium concentrations. The open probability curve shifted to more hyperpolarized potentials with increases in $[Ca^{2+}]_i$. As observed in BK channels in cell bodies (Vergara *et al.* 1998) as well as in the posterior pituitary neurosecretory terminal (Bielefeldt *et al.* 1992), there was a reduction in the steepness of the voltage dependence of the relation at higher $[Ca^{2+}]_i$. This finding has been interpreted to indicate an independence of the calcium and voltage effects on channel gating (Cui *et al.* 1997; Vergara *et al.* 1998). It is of particular interest that the observed shift in slope can be virtually superimposed on results reported recently for expressed *hslo* channels in frog oocytes (DiChiara & Reinhart, 1995). Analysis of the single-channel kinetics demonstrated that voltage affected both the channel open and closed states, with the primary effect on the slowest observed rate constants. In all, gating of BK channels is highly controlled, depending as it does on both a $[Ca^{2+}]_i$ increase and membrane depolarization. This is in contrast to small-conductance K_{Ca} (SK) channels that are gated by $[Ca^{2+}]_i$ alone. A dual control mechanism for high-conductance K^+ channels, with its greater control over random openings, may be necessary at presynaptic terminals where channel openings are likely to markedly affect the input impedance. This is of particular importance in the much smaller bouton-type presynaptic nerve terminal in which a single BK channel opening might severely attenuate action potential invasion and, hence, transmitter release.

In a few cases the calyx BK channels exhibited inactivation during sustained depolarizations but the inactivation rate was only weakly voltage sensitive and was slow at even the most depolarized potentials. Thus, as in the posterior pituitary neurosecretory terminal, inactivation is not a prominent feature of the presynaptic BK channel, unlike that observed in adrenal chromaffin cells (Solaro *et al.* 1995). Nonetheless, the presence of inactivation is of interest since it may indicate either a different variant (Chang *et al.* 1997; Farley & Rudy, 1998) or that the channels can be modulated by a second-messenger system (e.g. Egan *et al.* 1993).

The BK channels detected in this, as well as in the two other nerve terminal preparations examined, exhibit characteristics consistent with those reported for expressed *hslo* K_{Ca} channels (DiChiara & Reinhart, 1995). Expressed BK channels can be modified by a β subunit which has been reported to markedly increase both sensitivity to block by CTX (Hanner *et al.* 1997) and activation by calcium (McManus *et al.* 1995). This subunit is not essential and the channel has been reported to exist and function in its absence (McManus *et al.* 1995; Chang *et al.* 1997). It is interesting to note that the BK channel expressed in the absence of the β subunit exhibits a $[Ca^{2+}]_i$ dependence and CTX sensitivity that are similar to those observed here and to those reported previously in the organ of Corti (Wang *et al.* 1992) and the posterior pituitary neurosecretory terminal (Bielefeldt & Jackson, 1993). This raises the possibility that nerve terminal BK channels exist in the β subunit-free state. Whether this is in fact the case, and whether this represents a general property for nerve terminal BK channels will require further study. Even so, the reported range of sensitivities to CTX indicate that there is some additional variability in presynaptic BK channel characteristics.

Several lines of evidence suggest that BK channels can be activated by the plume, or domain, of ions entering through closely associated calcium channels (Roberts *et al.* 1990; Gola & Crest, 1993; Thompson, 1994; Protti & Uchitel, 1997; Marrion & Tavalin, 1998). Since the calcium channels and the BK channels are physically co-localized at the nerve terminal (Robitaille *et al.* 1993) domain-based activation might be expected, as appears also to be the case for transmitter release (Stanley, 1997). Evidence for a close association of these channel types in presynaptic structures

has been presented at the hair cell (Roberts *et al.* 1990), and the neuromuscular junction (Yazejian *et al.* 1997; Protti & Uchitel, 1997) and our results are consistent with these findings.

We did not note any obvious differences between transmitter release face and non-release face BK channels. In over 180 recordings from membrane patches we never observed more than six BK channels in a single patch, a channel density that is consistent with estimates from whole-terminal recordings at the frog neuromuscular junction in culture (Yazejian *et al.* 1997). This contrasts markedly with the density of single calcium channels recorded at the calyx transmitter release face which can exceed 200 channels in similar sized patches (Stanley, 1991; Haydon *et al.* 1994). If both the BK and calcium channels are located along the secretory zones, as suggested by immunofluorescent staining (Robitaille *et al.* 1993), the BK channels are presumably dispersed amongst the much more numerous calcium channels.

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