Presynaptic Kv1.2 channels suppress synaptic terminal hyperexcitability following action potential invasion

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Summary

Voltage-gated K^+ channels activating close to resting membrane potentials are widely expressed and differentially located in axons, presynaptic terminals and cell bodies. There is extensive evidence for localisation of Kv1 subunits at many central synaptic terminals but few clues to their presynaptic function. We have used the calyx of Held to investigate the role of presynaptic Kv1 channels in the rat by selectively blocking Kv1.1 and Kv1.2 containing channels with dendrotoxin-K (DTX-K) and tityustoxin-Kα (TsTX-Kα) respectively. We show that Kv1.2 homomers are responsible for two-thirds of presynaptic low threshold current, whilst Kv1.1/1.2 heteromers contribute the remaining current. These channels are located in the transition zone between the axon and synaptic terminal, contrasting with the high threshold K^+ channels Kv3.1, which are located on the synaptic terminal itself. Kv1 homomers were absent from bushy cell somata (from which the calyx axons arise); instead somatic low threshold channels consisted of heteromers containing Kv1.1, Kv1.2 and Kv1.6 subunits. Current-clamp recording from the calyx showed that each presynaptic action potential (AP) was followed by a depolarising after-potential (DAP) lasting around 100ms. Kv1.1/1.2 heteromers had little influence on terminal excitability, since DTX-K did not alter AP firing. However TsTX-Kα increased DAP amplitude, bringing the terminal closer to threshold for generating an additional AP. Paired pre- and postsynaptic recording confirmed that this aberrant AP evoked an excitatory postsynaptic current (EPSC). We conclude that Kv1.2 channels have a general presynaptic function in suppressing terminal hyperexcitability during the depolarising after-potential.

Voltage-gated K^+ conductances play multiple roles in regulating neuronal excitability. High threshold channels (such as Kv3) shape the action potential waveform and facilitate rapid repolarisation (Rudy & McBain, 2001). Low threshold K^+ channels which activate close to resting potentials (such as Kv1) regulate firing threshold and excitability (Brew & Forsythe, 1995) and their mutation or deletion may result in epilepsy and ataxia (Smart *et al.*, 1998). Six of the seven *Shaker*-related Kv1 family members (Kv1.1-1.6) are expressed throughout the mammalian brain and there is extensive evidence for Kv1 localisation adjacent to nodes of Ranvier and at many central synaptic terminals (Wang *et al.*, 1994).

Owing to the difficulties in directly recording from nerve terminals, the function of presynaptic Kv1 channels remains elusive. Blockade of channels containing Kv1.1, Kv1.2 or Kv1.6 subunits by dendrotoxin-I caused spontaneous firing at the neuromuscular junction (Anderson & Harvey, 1988). Similar observations have been made at central synapses such as inhibitory cerebellar basket cell terminals where blockade of channels containing Kv1.1 and Kv1.2 subunits increased the rate and amplitude of spontaneous inhibitory postsynaptic currents (sIPSCs) (Southan & Robertson, 1998; Zhang *et al.*, 1999; Southan & Robertson, 2000). However the sensitivity of these sIPSCs to tetrodotoxin and the absence of a rise in presynaptic $[Ca^{2+}]_i$ (Tan & Llano, 1999), suggest that the firing rate in basket cell terminals was increased rather than APs broadened. Similarly, in MNTB neurones DTX-I has no effect on the amplitude of evoked EPSCs (Brew & Forsythe, 1995) suggesting that the low threshold currents at the calyx of Held do not contribute to AP broadening.

We have used the calyx of Held to examine the role of Kv1 channels at central excitatory synapses by making direct pre- and postsynaptic recordings, combined with subunit specific pharmacology and immunohistochemistry. We show that Kv1 channels do not influence terminal AP waveform but instead suppress axonal excitability ensuring that aberrant APs are not generated.

Methods

Electrophysiology

Cochlear nucleus and brainstem slices (100-150μm thick) were prepared as previously described (Rusznak *et al.*, 1997; Dodson *et al.*, 2002). 8-14 day old Wistar and Lister Hooded rats were killed by decapitation in accordance with UK Home Office regulations. Whole-cell patch recordings were made from visually identified presynaptic terminals using an Optopatch amplifier (Cairn, Faversham, UK). Bushy cells were identified by location and morphology when filled with Lucifer yellow. During recording, slices were perfused with artificial cerebrospinal fluid (aCSF, ~1 ml min^{-1}, 25°C). Patch pipettes were pulled from thick-walled borosilicate glass (GC150F-7.5, Harvard Apparatus, UK). Presynaptic series resistances were 19.1±2.2MΩ (n=11) and compensated by 70% with 10μs lag; recordings with a change exceeding ±2MΩ were excluded from analysis. Input resistances were 529±100MΩ (n=11) and did not correlate with axon length. Trapezoid axons were stimulated (4-10V, 0.2ms, DS2A isolated stimulator, Digitimer, Welwyn Garden City, UK) at the midline using a bipolar platinum electrode. For simultaneous pre- and postsynaptic recordings, synaptic connections were detected using postsynaptic

calcium imaging (Billups *et al.*, 2002). Postsynaptic EPSCs were recorded using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), series resistances were <10MΩ and 5mM QX-314 was included in the intracellular patch solution. IPSCs were blocked by 10µM bicuculline and 1µM strychnine. Data were acquired with a CED1401 interface using Patch v6.39 software (Cambridge Electronic Design Ltd., Cambridge, UK), filtered at 2 kHz and digitized at 5 to 40 kHz. The current amplitude for current-voltage (I/V) relationships was measured 10ms into the test step for the presynaptic recordings but delayed until 180ms for bushy cells, to avoid contamination by the transient outward K^+ current (I_A). Leak currents, estimated from a linear fit between -90mV to -75 mV, were subtracted from the I/V curve. Example I/V curves from single cells are presented but similar results were observed in at least 4 cells, averaged data for which are presented in the text as mean ± S.E.M. Stated voltages exclude a -7 mV junction potential.

Immunohistochemistry

Sections were prepared from P9 Lister Hooded rats as described previously (Dodson *et al.*, 2002). Primary antibodies were applied overnight at 4°C at a dilution of 1:100 and incubated with a FITC secondary antibody (1:1000) at 20°C or for co-localisation FITC and Texas Red (both 1:500) applied together. Slides were prepared using ProLong Antifade (Molecular Probes, Eugene, OR) and examined on an Olympus Fluoview confocal microscope (IX70) with a 60X (NA 1.4) objective.

Solutions and Toxins

aCSF contained (in mM): 125 NaCl, 2.5 KCl, 10 glucose, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 sodium pyruvate, 3 myo-inositol, 0.5 ascorbic acid, 2 $CaCl_2$, and 1 $MgCl_2$, gassed with 95% O_2 5% CO_2 (pH 7.4). Except for synaptic studies, 0.5 mM $[Ca^{2+}]_o$ and 2.5 mM $[Mg^{2+}]_o$ were used to minimize Ca^{2+} dependent currents, ZD7288 (10µM, Tocris Cookson, Bristol, UK) to block I_H and tetrodotoxin (1µM TTX, Latoxan, Valence, France) to block I_{Na}. Intracellular patch solution contained (in mM): 97.5 K-gluconate, 32.5 KCl, 10 HEPES, 5 EGTA, 1 $MgCl_2$ (pH 7.2 with KOH) and Lucifer yellow or sulforhodamine 101 (1mg/ml Molecular Probes, Eugene, OR). Chemicals were obtained from Sigma (Poole, UK) except: DTX-I, Kv1.1, 1.2, 1.4, 1.6 and Kv3.1b antibodies, Alomone Labs (Jerusalem, Israel); Texas Red and FITC (Goat anti-rabbit), Jackson ImmunoResearch Laboratories (West Grove, PA); Kv1.1, 1.2 and 1.6 antibodies from Upstate Biotechnology (NY) were used for co-localisation; TsTX-Kα, Peptide Institute (Osaka, Japan) and DTX-K was a kind gift from Brian Robertson, University of Strathclyde, Glasgow.

Results

Presynaptic low threshold current is dominated by Kv1.2 homomers

Presynaptic outward K^+ currents were found to consist of both high and low threshold components (Fig. 1*A1*). TsTX-Kα (100nM), which blocks channels containing Kv1.2 subunits (Hopkins, 1998), blocked 87±4.8% of the current evoked at -45mV (Fig. 1*C3* n=7). The remaining current (13%) was high threshold K^+ current which activates at around -45mV, is blocked by tetraethylammonium (TEA) and is DTX-I insensitive (Dodson and Forsythe, unpublished observations). DTX-K (100nM), which blocks

channels containing Kv1.1 subunits (Robertson *et al.*, 1996) blocked 32±4.1% of the current (Fig. 1*C3* n=6). Since only one toxin-sensitive subunit is required for these toxins to block and Kv1.1 and Kv1.2 are the only Kv1 subunits present in presynaptic axons in the MNTB (Dodson *et al.*, 2002), these data suggest that two-thirds of presynaptic low threshold current is mediated by Kv1.2 homomers and the remaining third by Kv1.1/Kv1.2 heteromers.

Kv1.1 and Kv1.2 are concentrated in the axonal region just preceding the terminal

The distribution of Kv1.1 and Kv1.2 containing channels at the calyx was investigated by co-localisation with Kv3.1b, which is expressed in the calyceal fingers and proximal region of the axon (Fig. 2*A1*). Kv3.1 is also expressed at the postsynaptic MNTB soma (Elezgarai *et al.*, 2003) but clearly distinguishable from presynaptic immunofluorescence (Fig. 2*A1*). Kv1.1 and Kv1.2 are concentrated in a 20µm region of the presynaptic axon adjacent to the terminal, but not in the calyx itself (Fig. 2*A3* & B). Kv1.6 was not expressed in the presynaptic axon or terminal (data not shown).

Heteromeric channels containing Kv1.1, Kv1.2 and Kv1.6 are expressed at the Bushy cell soma.

To examine whether K^+ currents expressed at the terminal were similar to somatic currents, we compared the calyx data with somatic recordings from bushy cells, which give rise to the calyx of Held. Immunostaining for Kv1.1, Kv1.2 and Kv1.6 but not Kv1.4

was detected in aVCN neurones (Fig. 3*A*). In whole-cell recordings from bushy neurones, DTX-K (100nM) blocked 83.7±13% of the somatic current at -45mV (Fig. 3*B*, n=4). TsTX-Kα (100nM) blocked 69.3±15.2% of the low threshold current in 4 neurones, (Fig. 3*C*); whereas in the remaining 3 neurones TsTX-Kα had no effect (<5% block). This disparity in TsTX-Kα sensitivity is consistent with two bushy cell populations (spherical and globular cells) expressing different complements of Kv1 channels. Taken together, these data suggest that low threshold somatic currents are mediated by heteromers including Kv1.1, Kv1.2 and Kv1.6 subunits. In contrast to the calyx (Fig. 1), bushy cell somata possess a transient outward current (I_A), which is insensitive to TsTX-Kα and DTX-K (Fig. 3*B2 & C2*), activates at around -45mV, rapidly inactivates and is TEA-insensitive (Rusznak and Szucs unpublished observations). I_A currents are either mediated by Kv4 channels, Kv3.4, Kv1.4 or other Kv1 channels with inactivating Kvβ subunits (Coetzee *et al.*, 1999). As this I_A is a high threshold TEA-insensitive current, we can exclude Kv3 and Kv1 channels. Since Kv4.2 is the only Kv4 subunit expressed in bushy cells (Fitzakerley *et al.*, 2000), we conclude that the bushy cell I_A is most likely comprised of Kv4.2 homomers.

Kv1.2 containing channels regulate presynaptic AP firing

To investigate the role of Kv1 channels at the calyx we applied subunit specific toxins whilst recording from terminals under current-clamp. In brain slices calyces with less than 20μm of intact axon fire a single AP in response to sustained depolarisation (Fig. 4*A1* n=10). Axon length was assessed under epifluorescent illumination by inclusion of sulphorhodamine in the intracellular solution. DTX-K (100nM) had no effect on AP

firing (Fig. 4*A1* inset, n=3), suggesting that Kv1.1/1.2 heteromers make little or no contribution to terminal excitability. In contrast 100nM TsTX-Kα caused AP firing throughout the current injection (Fig. 4*A2*, n=4) suggesting that Kv1.2 homomers regulate AP firing at the terminal. Calyces with more than 20μm of intact axon will typically fire multiple APs during sustained current injection (Forsythe, 1994), however such long depolarisations are not physiological in axons. To produce a more physiological stimulus we triggered orthodromic APs at the midline. Simultaneous dual recordings were made of presynaptic APs under current-clamp along with the resulting MNTB neurone EPSCs under voltage-clamp. In control conditions each electrical stimulus evoked a single presynaptic AP which generated one EPSC in the postsynaptic neurone (Fig. 4*B1*). From a membrane potential of -75mV, evoked APs peaked at 23±6.8mV, had a half-width of 414±30μs and repolarised to -71.2±2mV (n=10). APs were followed by a depolarising after-potential (DAP), which peaked at -62.5±1.6mV, had a half-width of 15.1±2.4ms and is attributable to passive discharge from the myelin sheath (Barrett & Barrett, 1982). Single presynaptic APs were maintained when the terminal membrane potential was changed over a range of -90 to -25mV (data not shown). Following TsTX-Kα however, two APs were fired after a single stimulus (Fig. 4*B2*, 100nM, 10 of 10 terminals), resulting in two EPSCs in the postsynaptic neurone. Similarly aberrant firing was observed during trains of stimuli in the presence of TsTX-Kα (Fig. 4*C2*). Multiple firing in response to orthodromic AP propagation has never been observed under control conditions, but following TsTX-Kα multiple APs were observed from at all potentials of -90 to -25mV. TsTX-Kα had no effect on EPSC amplitude in the postsynaptic neurone (P=0.93, 2 tailed paired t-test, n=5), confirming

that low threshold K^+ currents do not influence AP waveform or release probability (Brew & Forsythe, 1995). The AP half-width in the presence of TsTX-Kα was unchanged from control (P=0.59 2 tailed, paired t-test); but the DAP was significantly larger (P=0.005) peaking at -55.4±1.5mV (n=7). This suggests that the presynaptic low threshold K^+ conductance serves to shunt and reduce the DAP; hence when Kv1 currents are blocked, threshold is exceeded and a second AP is generated during the DAP. These data suggest that presynaptic Kv1.2 channels prevent terminal hyperexcitability following AP invasion and thus preserve the timing and pattern of AP firing.

Discussion

Kv1 channels are present in axons and presynaptic terminals throughout the brain (Wang *et al.*, 1994; Rhodes *et al.*, 1997; Southan & Robertson, 2000). We show that presynaptic Kv1 channels make little contribution to action potential repolarisation at the calyx of Held, but promote transmission fidelity by preventing aberrant firing following an action potential. All presynaptic Kv1 channels contain Kv1.2 subunits; Kv1.2 homomers contribute two-thirds of the current and Kv1.1/1.2 heteromers the remaining third. This contrasts with somatic Kv1 currents that are exclusively mediated by heteromeric channels, composed of Kv1.1, Kv1.2 and Kv1.6 subunits. Our results suggest that presynaptic Kv1 channels play a pivotal role in preventing nerve terminal hyperexcitability.

The presynaptic low threshold current is dominated by Kv1.2 homomers; these channels are largely responsible for ensuring that each AP propagating from the bushy cell results in a single AP arriving at the terminal. Kv1.1/1.2 heteromers are also present presynaptically, but blocking these channels does not influence AP firing (Fig. 4*A1*). This contrasts with the postsynaptic MNTB neurones, which have no homomeric channels but where Kv1.1/1.2 heteromers, located at the initial segment of the axon, are dominant in regulating AP firing (Dodson *et al.*, 2002). Immunohistochemical localisation shows bushy cell bodies express Kv1.1/1.2/1.6 subunits similar to MNTB somata (Dodson *et al.*, 2002; Brew *et al.*, 2003). Subunit composition can influence localisation of channels by association of certain subunits with Kvβ or Caspr (Poliak *et al.*, 1999; Manganas *et al.*, 2001). A general hypothesis consistent with our observations is that Kv1.6 containing channels are restricted to somatic regions whereas channels containing Kv1.1 and Kv1.2 are permitted access to axonal regions and contribute to initial segment, juxtaparanodal and terminal low threshold K^+ conductances. Heteromers contribute at all sites, with Kv1.2 homomers located exclusively at the transition zone between the axon and terminal. This observation may have therapeutic implications for selective regulation of presynaptic versus postsynaptic excitability.

Immunohistochemical studies demonstrate that most Kv1 channels isolated from brain synaptic membranes contain Kv1.2 subunits with Kv1.2 homomers being prevalent (Shamotienko *et al.*, 1997). Our results show that Kv1.2 channels are located at the transition zone between the axon and terminal (the last 20μm) but are excluded from the terminal itself (Fig. 2). Kv1 channels are also localised at transition zones at the

neuromuscular junction (Zhou *et al.*, 1998) thus allowing greatest influence over AP invasion of the terminal and minimizing antidromic reflection by raising threshold following an AP. Kv1 channels also exert control over axonal APs in the initial segment (observed in recordings from MNTB neurones, Dodson *et al.*, 2002).

In some nerve terminals such as hippocampal mossy fibre boutons (Geiger & Jonas, 2000) and neurohypophysis terminals of the pituitary (Jackson *et al.*, 1991) inactivating K^+ currents are responsible for activity-dependent AP broadening and facilitation of transmitter release. For example in hippocampal mossy fibre boutons, low threshold DTX-sensitive channels, presumed to contain Kv1.1, Kv1.4 and Kvβ1 subunits, inactivate during repetitive stimulation causing AP broadening (Rhodes *et al.*, 1997; Geiger & Jonas, 2000).

In other terminals however Kv1 channels are involved in preventing hyperexcitability. In the frontal cortex, blockade of putative Kv1.2 homomers causes local spiking in thalamocortical axon terminals (Lambe & Aghajanian, 2001) and in the entorhinal cortex, α-DTX block of Kv1.2 containing channels increases the frequency of sIPSCs (Cunningham & Jones, 2001). Kv1.1 and Kv1.2 are highly concentrated in cerebellar basket cell terminals (Wang *et al.*, 1994; Rhodes *et al.*, 1997) and block of the presynaptic Kv1 current by α-DTX increases both frequency and amplitude of sIPSCs in Purkinje cells (Southan & Robertson, 1998, 2000). Kv1.1 null mice also exhibit increased spontaneous activity at cerebellar basket cell synapses (Zhang *et al.*, 1999), hyperexcitability of peripheral axons (Zhou *et al.*, 1998) and epilepsy (Smart *et al.*,

1998). We have demonstrated that Kv1.2 channels prevent hyperexcitability at the calyx of Held, preserving AP fidelity which is critical for sound source localisation (Trussell, 1999). Our results also highlight the general importance of Kv1 channels in preventing errors during repetitive firing. The widespread distribution of presynaptic Kv1 channels, combined with their importance in preventing aberrant firing suggests a general role for presynaptic Kv1 channels in preventing hyperexcitability in nerve terminals throughout the central and peripheral nervous system.

Acknowledgements

Figure Legends

Figure 1. Kv1.2 homomers dominate the presynaptic low threshold current.

Outward currents were evoked by voltage step from -70 mV to 0mV. A 750ms pre-pulse to -100mV was followed by 5 ms at -70mV, before the 200ms test step. The filled bar (left of traces) represents the magnitude of the current at -40mV. *A1*, Control traces after leak subtraction. *A2*, DTX-K (100nM) partially blocked the low threshold current. *A3*, Current-voltage relationship (I/V) of the data in *A1*, (closed circles) and *A2*, (open circles). *B1*, Control traces from a different presynaptic terminal. *B2*, TsTX-Kα, (100nM) blocked the low threshold current, leaving only the high threshold current. *B3*, I/V of the data in *B1* (closed circles) and *B2* (open circles). *C*, Superimposed traces showing the current during a step to -45mV in control and DTX-K (C1) or TsTX-Kα (C2). *C3*, Bar

chart showing the percentage block of the current evoked at -45mV. The terminals in *A*, and *B*, had axons cut within 20μm of the terminal during slicing; identical currents were measured in terminals with long axons.

Figure 2. Kv1.1 and Kv1.2 are localised to the final 20μm of the presynaptic axon but are excluded from the terminal.

A1, Kv3.1b is concentrated in the calyceal fingers of the terminal and the proximal region of the presynaptic axon (arrow) and is also expressed at lower levels in MNTB neurones. *A2,* Kv1.2 is located in a 20μm region of presynaptic axons in the same section as *A1* but is absent from the calyceal fingers (arrow). *A3,* Co-localisation of Kv3.1b (red) and Kv1.2 (green), confirming that Kv1.2 is localised to presynaptic (†) as well as postsynaptic (*) axons. *B,* Co-localisation of Kv3.1b and Kv1.1 shows that Kv1.1 is located in an axonal region similar to Kv1.2 (arrow). Images in *A* and *B* are single confocal optical sections n=3 animals. Scale bar in *A1* is 20μm and applies to all parts.

Figure 3. Heteromers containing Kv1.1, Kv1.2 and Kv1.6 subunits comprise bushy cell low threshold somatic currents.

A, Neurones of the aVCN express Kv1.1 (*A1*), Kv1.2 (*A2*) and Kv1.6 (*A5*) at their soma. Kv1.4 is not expressed (*A4*) since immunofluorescence is similar to the background level (*A3*). Immunofluorescence was visualised using a X10 objective on an epifluorescent microscope (n=3 animals). Scale bar represents 200μm. *B1,* Voltage-clamp recordings from bushy cells. Voltage protocols were as in Fig. 1 without leak subtraction. *B2,* DTX-K (100nM) blocked 83.7±13% of the low threshold current, leaving the high threshold

current and a rapidly inactivating transient current, both of which activate around -45mV. *B3*, I/V for the data in *B1* (filled circles) and *B2* (open circles). *C1*, Control currents recorded in a different bushy cell. *C2,* TsTX-Kα (100nM) blocked 69.3±15.2% of the low threshold current in 4 neurones. In the remaining 3 neurones TsTX-Kα had no effect (<5% block). *C3,* I/V for the currents in *C1* (filled circles) and *C2* (open circles).

Figure 4. Kv1.2 containing channels prevent aberrant presynaptic action potential firing.

A1, Control response to 150pA current injection recorded from a presynaptic terminal under current-clamp. DTX-K (100nM) had no effect on AP firing (inset; n=3). *A2,* TsTX-Kα (100nM) caused firing throughout the current injection (n=3). Scale bars in *A* represent 50mV and 50ms. *B1*, Simultaneous recording of the presynaptic AP (under current-clamp) and the postsynaptic EPSC (under voltage-clamp). APs were evoked by electrical stimulation at the midline (arrows). In control conditions, a single AP and corresponding EPSC are generated by each stimulus (n=13; upper portion of the postsynaptic stimulus artefact was removed). *B2*, Following Kv1.2 block with 100nM TsTX-Kα, an aberrant AP is fired in the terminal during the DAP which triggered a second EPSC in the MNTB neurone (n=10). *C1*, 20 Hz train under control conditions. *B3*, In TsTX-Kα aberrant APs are fired during the DAPs at 20 Hz stimulation (n=3).

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A1
0
-20
-40
Control

B1
Control

0.5nA
50ms

1nA
50ms

A2
DTX-K

B2
TsTX-K

A3
Control
DTX-K
Current (nA)
2
1
0
Voltage (mV)
-70 -50 -30 -10

B3
Control
TsTX-K
Current (nA)
3
2
1
0
Voltage (mV)
-70 -50 -30 -10

C1
Control
0.25nA
50ms
-45
DTX-K

C2
Control
0.5nA
50ms
TsTX-K

C3
% Block at -45mV
100
80
60
40
20
0
87
32
TsTX-K   DTX-K

A1 Post ← Pre Kv3.1b

A2 ← Kv1.2

A3 † * * † Kv3.1b & Kv1.2

B ↑ Kv3.1b & Kv1.1

A1 Kv1.1
A2 Kv1.2
A3 aVCN Control
A4 Kv1.4
A5 Kv1.6

B1 Control
−5
−25
−45

C1 Control

0.5nA
50ms

1nA
50ms

B2 DTX-K

C2 TsTX-K

B3
Control
DTX-K
Current (nA)
1.5
1
0.5
0
−70 −50 −30 −10
Voltage (mV)

C3
Control
TsTX-K
Current (nA)
3
2
1
0
−70 −50 −30 −10
Voltage (mV)

A1

DTX-K

Control

150pA

50mV
50ms

A2

TsTX-K

B1

Control

50mV
1nA
10ms

B2

TsTX-K

C1

Control

50mV
40ms

C2

TsTX-K