Small conductance Ca\(^{2+}\)-activated K\(^+\) channels modulate action potential-induced Ca\(^{2+}\) transients in hippocampal neurons.

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Author Contributions: MS, CDR and PP conceived the study, designed and supervised the experiments; RT, TF, MS-C, AC, CDR and PP performed the experiments and analyzed the data; RT, MS, CDR and PP wrote the manuscript.

Running Head: SK channel modulate spike-induced Ca\(^{2+}\) transients
Abstract

In hippocampal pyramidal neurons, voltage-gated Ca\(^{2+}\) channels open in response to action potentials. This results in elevations in the intracellular concentration of Ca\(^{2+}\) that are maximal in the proximal apical dendrites and decrease rapidly with distance from the soma. The control of these action potential-evoked Ca\(^{2+}\) elevations is critical for the regulation of hippocampal neuronal activity. As part of Ca\(^{2+}\) signaling microdomains, small conductance Ca\(^{2+}\)-activated K\(^{+}\) channels (SK channels) have been shown to modulate the amplitude and duration of intracellular Ca\(^{2+}\) signals by feedback regulation of synaptically activated Ca\(^{2+}\) sources in small distal dendrites and dendritic spines, thus affecting synaptic plasticity in the hippocampus. In this study, we have investigated the effect of the activation of SK channels on Ca\(^{2+}\) transients specifically induced by action potentials in the proximal processes of hippocampal pyramidal neurons. Our results, obtained by using selective SK channel blockers and enhancers, show that SK channels act in a feedback loop, in which their activation by Ca\(^{2+}\) entering mainly through L-type voltage-gated Ca\(^{2+}\) channels leads to a reduction in the subsequent dendritic influx of Ca\(^{2+}\). This underscores a new role of SK channels in the proximal apical dendrite of hippocampal pyramidal neurons.

Keywords: SK channel; afterhyperpolarization; apamin; pyramidal neuron; calcium imaging.
Introduction

The afterhyperpolarizing current $I_{\text{AHP}}$ is mediated by apamin sensitive, small conductance $Ca^{2+}$-activated $K^+$ channels (SK channels) that are voltage-independent and activated by increases in intracellular $Ca^{2+}$, thereby linking intracellular $Ca^{2+}$ elevations to changes in the membrane potential in a variety of neurons (reviewed by Adelman et al. 2012; Pedarzani and Stocker 2008). The constitutive binding of calmodulin to SK channels is responsible for their high sensitivity to $Ca^{2+}$ (Xia et al. 1998). However, the sources of $Ca^{2+}$ leading to the activation of SK channels vary in different types of neurons (reviewed by Pedarzani and Stocker 2008; Stocker 2004).

SK channels are part of $Ca^{2+}$ microdomains (Fakler and Adelman 2008; Lujan et al. 2009; Marrion and Tavalin 1998; Oliver et al. 2000) created by the functional coupling of $Ca^{2+}$ permeable channels and $Ca^{2+}$-sensitive channels, and may serve diverse roles depending on their subcellular localization. In the soma of CA1 neurons $Ca^{2+}$ channels and small conductance $Ca^{2+}$-activated $K^+$ channels are found within 50-150 nm of each other (Marrion and Tavalin 1998).

Synaptic NMDA receptors and SK channels are functionally coupled in the dendritic spines of hippocampal, amygdala and striatal neurons (Bloodgood and Sabatini 2007; Faber et al. 2005; Higley and Sabatini 2010; Lujan et al. 2009; Ngo-Anh et al. 2005). The activation of SK channels in dendritic spines limits the influx of $Ca^{2+}$ through NMDA receptors and decreases glutamatergic excitatory postsynaptic potentials (Bloodgood and Sabatini 2007; Faber et al. 2005; Ngo-Anh et al. 2005). Moreover, in distal apical dendrites of hippocampal neurons SK channel activation controls the duration of glutamate-induced $Ca^{2+}$ plateau potentials (Cai et al. 2004). Thus, SK channels are part of a negative feedback loop that limits $Ca^{2+}$ influx through those $Ca^{2+}$ sources that initially activated them, shaping the amplitude and duration of synaptically evoked $Ca^{2+}$ transients and modulating glutamatergic synaptic responses.
The regulation by SK channels of Ca\textsuperscript{2+} sources that are not dependent on synaptic activation, however, has not been explored so far. This may be of particular relevance in the proximal apical dendrite of hippocampal neurons, where SK channels have also been localized (Lin et al. 2008; Lujan et al. 2009; Sailer et al. 2002) and elevations of intracellular Ca\textsuperscript{2+} induced by the activation of voltage-gated Ca\textsuperscript{2+} channels by somatic action potentials (APs), which back propagate to the dendrites, are largest (Callaway and Ross 1995; Christie et al. 1995; Jaffe et al. 1992; Regehr et al. 1989; Regehr and Tank 1994; Spruston et al. 1995). The proximal dendritic compartment is different from the distal one in CA1 neurons also because it receives more effective GABAergic innervation (Papp et al. 2001), while most glutamatergic excitatory inputs converge on the distal portion. Indeed, proximal and distal compartments of apical dendrites have different synaptic plasticity thresholds, which may also reflect a different contribution of voltage-gated Ca\textsuperscript{2+} channels to plasticity induction mechanisms (Parvez et al. 2010).

In view of the feedback regulation of synaptically evoked Ca\textsuperscript{2+} entry by SK channels shown in distal dendrites and spines, the present study addresses the question as to whether SK channels can modulate AP-induced Ca\textsuperscript{2+} transients in the proximal apical dendrites of hippocampal pyramidal neurons. We demonstrate that pharmacological modulation of SK channel activity regulates the amplitude and duration of AP-induced intracellular Ca\textsuperscript{2+} elevations mainly triggered by L-type voltage-gated Ca\textsuperscript{2+} channels in the proximal neurites of hippocampal neurons.

**Methods**

**Chemicals**

Tetrodotoxin was obtained from Alomone Laboratories (Jerusalem, Israel); apamin from Laxotan (Rosans, France); DL-AP5, NBQX, picrotoxin and 1-Ethyl-2-benzimidazolinone (1-EBIO) from Tocris Cookson (Bristol, UK) or Ascent Scientific (Weston-super-Mare, UK); tetraethylammonium (TEA), Na\textsubscript{2}-ATP, Na\textsubscript{3}-GTP and 8-(4-chlorophenylthio)adenosine 3’,5’-cyclic
monophosphate (8CPT-cAMP) from Sigma-Aldrich (Dorset, UK); all other salts and chemicals were obtained from Fluka (Sigma-Aldrich, Dorset, UK).

Cell culture

Rats were handled in accordance to the UK Home Office Animal Procedures Act (1986) and University College London Animal Ethical Committee guidelines. Primary hippocampal neurons were cultured from 0-1 day old rats (Banker and Goslin 1991) following a modified protocol. Briefly, after dissection, the hippocampi were treated with 2.5% trypsin (Invitrogen) and mechanically dissociated with a flame-polished Pasteur pipette. Cells were plated onto poly-D-lysine-coated (0.1 mg/ml) glass or plastic (Nalgene) coverslips at a density of 35000 cells/cm² (for the electrophysiology and imaging recordings) and of 21000 cells/cm² (for immunofluorescence staining) in Minimum Essential Medium (Invitrogen, Paisley), supplemented with 10% horse serum, 1 mM pyruvic acid and 0.59% glucose. After 4-14 hours, the medium was substituted with Neurobasal medium supplemented with Penicillin (100 u/ml), Streptomycin (100 µg/ml), 0.59% glucose and B27 supplement (Invitrogen, Paisley). Neurons were kept at 5% CO₂, 37°C and 95% humidity for a variable number of days before the experiments.

Electrophysiology

For recordings, coverslips were mounted in a custom-built recording chamber placed on the stage of a Nikon E600FN upright microscope. Cells were continuously superfused by a flow of 2.5 ml/min extracellular solution containing (in mM): 140 NaCl, 3.5 KCl, 10 HEPES, 20 Glucose, 2.5 CaCl₂ and 1.5 MgCl₂ (pH 7.4, 300-305 mOsm/kg) (20°C). CaCl₂ was reduced to 1.5 mM for current clamp recordings with trains of four APs. Borosilicate patch pipettes (5-6 MΩ; TW100F-4 glass, World Precision Instruments, USA) were filled with a solution containing (in mM): 135 KMeSO₄ (voltage clamp and current clamp experiments) or 135 K-gluconate (voltage clamp experiments), 10 KCl, 10 HEPES, 1 MgCl₂, 2 Na₂-ATP, 0.4 Na₃-GTP (pH 7.2-7.3, 280-290
mOsm/kg). 8-(4-chlorophenylthio)adenosine 3’,5’-cyclic monophosphate (8CPT-cAMP; 50 µM) was included in the majority of the voltage clamp experiments to inhibit the slow I_{AHP} (sI_{AHP}).

Voltage clamp experiments were performed on pyramidal cells at 10-18 days in vitro (DIV). Neurons were clamped at a membrane holding potential of –50 mV, and repetitively depolarized to +30 mV for 100-200 ms at a frequency of 0.033 Hz to activate voltage-gated Ca^{2+} channels. After each depolarization the membrane potential was stepped back to –50 mV, where the apamin-sensitive I_{AHP} was observed as an outward current. The voltage-clamp experiments were conducted in the presence of 0.5 µM tetrodotoxin and 1 mM TEA to block voltage-gated Na^{+} channels and some voltage-gated K^{+} channels. Series resistance (range 15-25 MOhm) was monitored at regular intervals throughout the recording and presented minimal variations (≤15%) in the analyzed cells. Data are reported without corrections for liquid junction potentials.

Data were acquired using an Axopatch 1-D amplifier (Axon Instruments, Foster City, California) controlled by the Strathclyde Electrophysiology Software WinWCP V.3.2.9 (John Dempster, University of Strathclyde, UK). Data were filtered at 1 kHz and sampled at 3 kHz with a Micro 1401 interface (Cambridge Electronic Design, UK). Data were analyzed by pClamp9 Clampfit routine (Axon Instruments, Foster City, California) and Origin 7.0 (Microcal software).

Action potentials were elicited in the presence of glutamate receptor blockers (DL-AP5 25 µM; NBQX 5 µM) in the whole-cell current-clamp mode, and data were filtered at 10 kHz and digitized at 20 kHz. Somatic current injections of 10 ms, which evoked single action potentials, were delivered at a frequency of 20 Hz. In between stimulations the membrane potential of the cells was kept at –60 mV by DC current injection. The resting membrane potential was frequently checked, and only cells with a stable resting potential more hyperpolarized than -50 mV were included in the analysis.

Ca^{2+} transients were imaged with 20 µM Fluo-4 (Molecular Probes, Eugene, Oregon) dissolved in the intracellular recording solution. 1 mM Fluo-4 stock solution was prepared in
purified H₂O (Super Purity Reagent, Romil) and stored at -20°C (Yasuda et al. 2004). Neurons were filled with Fluo-4 via the patch electrode for 10-15 minutes before imaging to allow dye equilibration in the proximal neurites (Helmchen et al. 1996; Maravall et al. 2000). Drugs were bath applied for 5-10 minutes. Due to the pharmacological accessibility of cultured neurons, drug effect was maximal already at 5 minutes.

**Two-photon imaging**

Two photon Ca²⁺ imaging was performed with a Bio-Rad multiphoton microscope based on a 1024 scan head mounted on a Nikon E600FN upright microscope equipped with a Nikon 60x NA 1.0 water-immersion objective. A Millennia V pump laser coupled to a mode-locked Ti:sapphire infra-red laser (Tsunami, Spectra Physics) was used for fluorescence excitation, tuned to 790 nm. For most experiments the laser power at the sample was 3 mW, but for those cells with a weak dye loading a higher power (up to 7 mW) was used to obtain a clear image. The fluorescence emission was collected with an external photomultiplier detector and was not descanned.

Since two dimensional scans are too slow for accurate determination of the time course and amplitude of the calcium transients, line scans were used. These consisted of successive sweeps at 6 ms intervals across a single line in the field of view. Images were collected using Lasersharp software (Bio-Rad, UK) and analyzed using ImageJ (NIH) and Origin 7.0 (Microcal software). For each recording, background fluorescence was determined from a cell free area of comparable size to the one of the line scan image. After subtracting the averaged background signal, fluorescence values were taken 300 ms before the triggering of action potentials, and averaged to measure the basal fluorescence (F_{basal}). The amplitude of the fluorescence transients at the recording sites was expressed as the fractional change in basal fluorescence, (F-F_{basal})/F_{basal} = (ΔF/F), which is approximately proportional to the changes in intracellular Ca²⁺ (Maravall et al. 2000). F_{basal} did not change by more than 2 times the standard deviation of F_{basal} measured under control conditions over the course of the experiment. For data analysis, transients were digitally filtered off-line (adjacent-
averaging routine, smoothing factor $n = 5$, Origin 7). Peak fluorescence was calculated averaging data points 30-60 ms around the maximum. The decay time course of Ca$^{2+}$ transients was fitted by a single exponential function.

**Statistical analysis**

Data were analyzed with Prism (GraphPad Software, La Jolla, California), Student's t-test, paired or unpaired as appropriate, or the non-parametric Mann-Whitney test were used for statistical comparisons between two groups ($\alpha = 0.05$), with * indicating $P<0.05$. For comparisons between more than two groups one-way ANOVA or one way ANOVA repeated measures followed by the Bonferroni post-hoc test was used. All values are expressed as mean ± SEM.

**Immunofluorescence**

Hippocampal neurons were fixed in phosphate saline buffer (PBS: 10 mM sodium phosphate, 130 mM NaCl, pH 7.2) containing 4% paraformaldehyde and 4% sucrose for 10 minutes at room temperature, rinsed twice in PBS, permeabilized in 0.3% Triton X-100 for 15 minutes, followed by two more washes in PBS. After a 1-hour incubation in 2% $\text{H}_2\text{O}_2$ to block the activity of the endogenous peroxidase, the immunodetection was performed using the tyramide signal amplification method (Invitrogen, Paisley). In short, the fixed, permeabilized and peroxide-treated neurons were incubated overnight at 4°C with the affinity purified anti-NSK2 antibody (0.25 µg/ml, see also Cingolani et al. 2002) diluted in blocking buffer. Controls were performed in parallel either by omitting the purified anti-NSK2 or by using the anti-NSK2 pre-adsorbed to a SK2 fusion protein (TRX-NSK2, 20 µg/ml). After repeated washes with PBS to remove the unbound primary antibodies, a 1-hour incubation with the HRP-conjugated anti-rabbit secondary antibody (1:200) in blocking buffer was performed. After washing the cultures with PBS, the Tyramide-Alexa Fluor 488 reagent was added and incubated in the dark for 5 minutes. Slides were washed, mounted using ProLong Antifade (Invitrogen, Paisley) and examined with a fluorescence microscope (Axiophot, Zeiss). Pictures were taken with a MicroPublisher camera (QImaging).
Results

The apamin-sensitive $I_{\text{AHP}}$ in cultured hippocampal neurons.

Whole-cell recordings were performed on morphologically identified hippocampal pyramidal neurons in primary culture. After 10 to 18 days in vitro, neurons showed a mean resting potential of $-59 \pm 1 \text{ mV (n = 38)}$. 100-200 ms long somatic depolarizations to $+30 \text{ mV}$ from a holding potential of $-50 \text{ mV}$, delivered in the presence of 0.5 $\mu$M tetrodotoxin and 1 mM tetraethylammonium (TEA), activated voltage-gated $\text{Ca}^{2+}$ currents, followed by an outward current. The observed currents decayed with either a time constant ($\tau$) of $222 \pm 17 \text{ ms}$ when elicited by a 100 ms-long depolarization (n = 8), or a $\tau$ of $485 \pm 46 \text{ ms}$ for 200 ms-long depolarizations (n = 17). The mean amplitude of the outward current was $83 \pm 20 \text{ pA (n = 8)}$ in response to 100 ms-long depolarizing pulses, and $140 \pm 23 \text{ pA (n = 17)}$ following 200 ms-long pulses.

d-Tubocurarine (dTC; 100 $\mu$M), which blocks SK channels in a reversible manner, inhibited the outward current (Fig. 1A and B). Similarly, the specific SK channel blocker apamin (5 nM) produced a strong suppression of the outward current (Fig. 1A and B), demonstrating that it is mediated by SK channels.

To further validate the molecular identity of the outward current and complete its pharmacological characterization, we tested the SK channel enhancer 1-EBIO (250 $\mu$M) (Pedarzani et al. 2001), which increased the $I_{\text{AHP}}$ peak amplitude (Fig. 1C and E). A similar increase in $I_{\text{AHP}}$ amplitude was observed with 5 $\mu$M of the more specific and potent SK channel enhancer 6,7-dichloro-1H-indole-2,3-dione3-oxime (NS309, Fig 1D and F) (Pedarzani et al. 2005). In addition, both 1-EBIO (Fig. 1C and E) and NS309 (Fig 1D and F) prolonged the decay time constant ($\tau$) of the $I_{\text{AHP}}$. 

[Figure 1 near here]
Taken together, these results demonstrate that the $I_{\text{AHP}}$ is expressed in cultured primary hippocampal neurons and has similar properties to the SK-mediated $I_{\text{AHP}}$ recorded in pyramidal neurons from acute hippocampal slices (Sailer et al. 2002; Stocker et al. 1999).

**SK2 channel expression in hippocampal neurons.**

Evidence obtained in pharmacological and biochemical studies, and work performed on genetically modified animals, point to the SK2 ($K_{\text{Ca}2.2}$) subunit as a main contributor to the formation of SK channels mediating the $I_{\text{AHP}}$ in hippocampal neurons (Bond et al. 2004; Sailer et al. 2002; Stocker et al. 1999).

Therefore, the distribution of SK2 channel subunits in post-natal hippocampal neurons was investigated using a specific antibody (anti-NSK2) raised against the NH$_2$-terminal region of the SK2 protein (Cingolani et al. 2002). Clear SK2 immunostaining was observed in the soma of the neurons from 2-13 days *in vitro* (DIV; Fig. 2B-E). The staining increased progressively from DIV 2. Maximal expression was seen at DIV 10-13 (Fig. 2E). For this reason neurons between DIV 10-18 were used for electrophysiological and imaging experiments. The SK2 immunoreactivity was visible in the soma of the hippocampal pyramidal neurons and in the proximal and distal portions of neurites (Fig. 2E). Pre-adsorption of the anti-NSK2 antibodies (Fig. 2F at DIV 13) and the omission of the primary antibody (data not shown) resulted in the lack of fluorescent staining at all developmental stages, confirming the specificity of the detected signal.

*Imaging and modulation of Ca$^{2+}$ transients elicited by action potentials back-propagating to the proximal dendrite of hippocampal neurons.*

In hippocampal pyramidal neurons there is plentiful evidence that SK channels can be activated by Ca$^{2+}$ entering through voltage-gated Ca$^{2+}$ channels opening during trains of action potentials (Cai et al. 2007; Empson and Jefferys 2001; Fernandez de Sevilla et al. 2006;
Kaczorowski et al. 2007; Kramar et al. 2004; Oh et al. 2000; Shah et al. 2006; Stocker et al. 1999; but see Gu et al. 2005). This opens the possibility that the activation of SK channels regulates local, voltage-gated Ca$^{2+}$ channel-mediated calcium signals.

Simultaneous two-photon Ca$^{2+}$ imaging and whole-cell current clamp recordings allow the testing of this hypothesis. Recent measurements of Ca$^{2+}$ transients evoked by single action potentials (APs) in small secondary to quaternary dendritic branches of CA1 pyramidal neurons demonstrated that the calcium sensitive fluorophore Fluo-4, with a $K_d$ ranging from 340 nM (34$^\circ$C) to 800 nM (24$^\circ$C) (Yasuda et al. 2004) and its large dynamic range, is the dye of choice, when used at low concentrations (Sabatini et al. 2002). A general concern with Ca$^{2+}$-sensitive processes is the potential interference exerted by Ca$^{2+}$ sensitive dyes due to their concentration and binding properties. In the case of SK channels activated by Ca$^{2+}$ entering the cell via voltage-gated Ca$^{2+}$ channels, the dye might act as an exogenous Ca$^{2+}$ buffer that captures the incoming Ca$^{2+}$, thereby reducing the activation of the SK channels.

The amplitude and the time course of decay of the I$_{AHP}$ in the absence and presence of Fluo-4 were first tested in a subset of voltage-clamp experiments. Fluo-4 at 20 µM did not significantly alter the amplitude (Control: 140 ± 23 pA, n = 17; Fluo-4: 98 ± 13 pA, n = 14) and the time constant of decay (Control: 458 ± 46 ms, n = 17; Fluo-4: 574 ± 50 ms, n = 14) of the I$_{AHP}$ elicited by a 200 ms-long pulse ($P = 0.1$).

Consequently, this concentration of Fluo-4 was used to study the effect of SK channels on the dynamics of intracellular free Ca$^{2+}$ concentration in response to APs in the proximal dendrite. A typical filled pyramidal cell with two neuronal processes (p1, p2) in the field of vision is shown in Fig. 3A. For visualization, cells were subjected to a prolonged depolarization at the end of the experiment to increase the intracellular [Ca$^{2+}$]. This was necessary because under resting conditions the low level of Fluo-4 was too dim to reliably detect neuronal processes. Action potentials were elicited by somatic current injections of 10 ms-long depolarizing pulses at 20 Hz (Fig. 3B, upper
panel), a firing frequency in the range observed in CA1 place cells when the animals are in proximity of their place field centre (Dragoi and Buzsaki 2006; O'Keefe and Dostrovsky 1971). The two APs induced an increase in fluorescence, indicative of Ca\(^{2+}\) entry, in the proximal neurites of the cell (<50 µm from soma; Fig. 3B). The fluorescence signal was collected by line scans along the dashed line (Fig. 3A) using a two photon imaging system with a photomultiplier as a detector to measure the dynamics of intracellular free Ca\(^{2+}\) concentration (Fig. 3B, middle panel). The time course of decay of the evoked fluorescence transients (Fig. 3B, lower panels) showed a cell to cell variability with an average time constant of decay ($\tau$) of 443 ± 34 ms (range: 221 to 987 ms in 28 processes of 25 cells). To test whether increasing the action potential duration (Fig. 3C) saturates the Fluo-4 fluorescence signal elicited by two APs at 20 Hz, the K\(^+\) channel blocker TEA was applied by bath perfusion. TEA at 10 mM reversibly prolonged the duration of the action potentials (Fig. 3C) and doubled the amplitude of the fluorescence transients (198 ± 46 %, n = 4, Mann-Whitney test $P<0.04$, Fig. 3D). TEA also prolonged the decay times of the transients. The prolongation was quite variable, and in the presence of TEA one cell displayed a fluorescence transient that developed into a plateau and did not decay back to baseline values over the duration of the recording. In the cells where the fluorescence transient returned to baseline the decay time of the transients was increased ($\tau_{\text{Control}}$: 316 – 674 ms (n=4); $\tau_{\text{TEA}}$: 464 – 4800 ms (n=3)). The changes in the amplitude and duration of the Ca\(^{2+}\) transients in the proximal dendrite of hippocampal pyramidal neurons are comparable with the effect of TEA on the action potential induced Ca\(^{2+}\) transients in the dendrites of neocortical neurons (Markram et al. 1995).

The effect of TEA on the action potential duration (Fig. 3C) and the amplitude and time course of the fluorescence transients was reversible (Fig. 3D). The reversibility of the effect of TEA demonstrates the stability of the signals observed under our recording and imaging conditions.

[Figure 3 near here]
SK channels regulate action-potential induced Ca$^{2+}$ influx.

TEA at 10 mM blocks several voltage-gated potassium channels, including members of the Kv1, Kv2, Kv3 and Kv7 families, voltage and Ca$^{2+}$-activated large-conductance K$^{+}$ (BK) channels (Coetzee et al. 1999) and, to some extent, SK channels (reviewed in Pedarzani and Stocker 2008). Consequently, the effect observed in the presence of TEA on the Ca$^{2+}$ transient (Fig. 3C and D) is due to the block of different types of potassium currents that contribute to the action potential repolarization and afterhyperpolarization phases in hippocampal pyramidal neurons. SK channels have been shown to terminate glutamate-evoked Ca$^{2+}$ plateau potentials in distal apical dendrites (Cai et al. 2004) and regulate the Ca$^{2+}$ influx through NMDA receptors through a negative feedback mechanism in spines of hippocampal pyramidal neurons (Bloodgood and Sabatini 2007; Ngo-Anh et al. 2005). This makes SK channels good candidates to regulate AP-induced Ca$^{2+}$ signals in proximal dendritic regions. To investigate whether and to what extent SK channels specifically contribute to the regulation of Ca$^{2+}$ transients in the proximal dendrites of hippocampal neurons, SK channels were inhibited by apamin, a selective inhibitor. Apamin increased the amplitude of the fluorescence transient (Fig. 4A and B) elicited by two APs at 20 Hz in the proximal dendrite to 115 ± 4 % (n = 5, P<0.05, Fig. 4C). Apamin also slowed the decay of the fluorescence transients by 18 ± 5% (Fig. 4B and D; control, $\tau$ = 298 ± 11 ms; apamin, $\tau$ = 353 ± 18 ms; n = 5, P<0.05). As a result of the increase in amplitude and prolongation of $\tau$, the amount of Ca$^{2+}$ entering the cell, characterized by the area under the curve of the fluorescence transient, was significantly increased in the presence of apamin (Fig. 4B and E; 138 ± 13 %; n = 5, P<0.05).

To test whether the observed effects were a consequence of apamin acting specifically on Ca$^{2+}$ influx triggered by APs rather than caused by the direct action of the depolarizing current injection, we measured the impact of the SK channel inhibitor following TTX application. TTX strongly attenuated the fluorescent transients (Fig. 4F, G and H; n=4), confirming that, under our experimental conditions, Ca$^{2+}$ signals in the proximal dendrite mainly arise from the back-
propagation of APs. In the presence of TTX, apamin failed to increase Ca\(^{2+}\) influx (Fig. 4F, G and H; \(F_{3,2} = 27, \ P=0.001\); TTX vs control \(P<0.05\); TTX+apamin vs control \(P<0.05\); TTX+apamin vs TTX \(P>0.05\); one-way repeated measures ANOVA with Bonferroni post-hoc test). This indicates that AP-induced activation of calcium channels is necessary for the SK-mediated modulation of Ca\(^{2+}\) signals.

To corroborate the result obtained with apamin, we tested the effect of a structurally unrelated, small organic SK channel blocker, d-tubocurarine, on the Ca\(^{2+}\) transients in the proximal dendrite of hippocampal pyramidal neurons. Although less specific than apamin, dTC has the advantage that it blocks SK channels in a reversible manner. Application of 100 µM dTC led to an increase in the fluorescence transient amplitude to 113 ± 3% (Fig. 5A and B), similar to the change observed with apamin. The effect of dTC on the amplitude of the fluorescence transients was reversed in 5 out of 6 cells (Fig. 5A and B). The reversibility of the dTC effect on the amplitude of the Ca\(^{2+}\) transients rules out the possibility that the observed increase might be a consequence of dye loading. Blocking SK channels with dTC also resulted in an increase of the area of the fluorescence transients to 120 ± 4% (Fig. 5A and C) and in a prolongation of the time course of decay, which is reflected by an increase of \(\tau\) by 20 ± 5%.

Several SK channel enhancers have been recently characterized (reviewed in Pedarzani and Stocker 2008), and act by increasing the apparent Ca\(^{2+}\)-sensitivity of SK channels (Pedarzani et al. 2001). If the increase in the Ca\(^{2+}\) transients observed upon application of apamin and dTC is due to the inhibition of SK channels acting as negative feedback regulators of Ca\(^{2+}\) influx triggered by APs, then enhancement of SK channel activity should lead to a reduction in the Ca\(^{2+}\) transients. To test this hypothesis the SK channel enhancer NS309 was used (Pedarzani et al. 2005). NS309 (5 µM) reduced both the amplitude of the fluorescence transients by 25 ± 5% (Fig. 5D and E) and their area by 30 ± 6 % (Fig. 5D and F) in all cells tested (n = 6). In the presence of NS309 the time constant of
decay of the fluorescence transients was also shortened by 21 ± 6% (n = 6, Fig. 5D). The overall reduction of the Ca\(^{2+}\) transients observed in the presence of NS309 and the observed opposite effect obtained with SK channel blockers further support the hypothesis that SK channels regulate Ca\(^{2+}\) transients elicited by APs in the proximal dendrite of hippocampal pyramidal neurons by a negative feedback mechanism.

[Figure 5 near here]

If SK channels, activated by Ca\(^{2+}\) entering through voltage-gated Ca\(^{2+}\) channels, modulate the Ca\(^{2+}\) transients generated by APs, then increasing the number of action potentials should lead to an enhanced Ca\(^{2+}\) influx, and therefore a stronger SK channel recruitment and a greater effect on the Ca\(^{2+}\) transients. The fluorescence transients measured in the proximal dendrites of pyramidal neurons in response to four APs were 21% larger than those observed in response to two APs at 20 Hz (four APs: Fig. 6A and B; $\Delta F/F = 1.72 \pm 0.11$; n = 18; two APs: Fig. 3-5; $\Delta F/F = 1.42 \pm 0.12$; n = 28; P=0.02, Mann-Whitney test). The inhibition of SK channels by dTC (100 µM) resulted in an increase of amplitude of the fluorescence transients to 130 ± 8% (Fig 6A-C), and area to 144 ± 9% (Fig. 6B and E, n = 4; P<0.05). Additionally, dTC caused a prolongation of the fluorescence transients by 21 ± 5% (Fig. 6D, n = 4, P<0.05). The relative increases in amplitude and area of the fluorescence transients were significantly larger than those observed with two APs after applying dTC (compare: Fig. 5B, C with Fig. 6C, E; P<0.05). To test if the relative increase in the amplitude of Ca\(^{2+}\) transients observed in response to four APs in the presence of dTC was limited by dye saturation, we applied 10 mM TEA in the presence of dTC. As expected (see also Fig. 3C and D), TEA substantially increased the influx of Ca\(^{2+}\) by prolonging the duration of the APs (Fig. 6A, lower panel), thereby causing a further large increase of the fluorescence transients (Fig 6B; amplitude, 149 ± 15 %; area, 213 ± 22%; n = 3). The effect of TEA in this context confirms that the increase of the fluorescence transients observed after dTC application was not limited by Fluo-4 saturation following four APs at 20 Hz.
In the proximal dendrites of CA1 neurons, different subtypes of voltage-gated Ca\(^{2+}\) channels are activated by the back-propagation of APs and contribute to local Ca\(^{2+}\) elevations, with a predominant role played by Ca\(_v\)L (L-type) channels (Christie et al. 1995). Moreover, L-type Ca\(^{2+}\) channels have been shown to be physically co-localized and selectively coupled to the activation of small-conductance Ca\(^{2+}\)-activated K\(^+\) channels in somatic patches from acutely dissociated CA1 pyramidal neurons (Marrion and Tavalin 1998). L-type Ca\(^{2+}\) channels were shown to be the main contributors to the activation of the SK-mediated I\(_{AHP}\) also in whole-cell recordings from CA1 neurons, with a reduction of I\(_{AHP}\) by ~48% upon application of the L-type Ca\(^{2+}\) channel blocker nifedipine (Bosurgi and Pedarzani 2006). We therefore tested whether the regulatory effect of SK channels on AP-induced Ca\(^{2+}\) transients is triggered by Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels. First the contribution of L-type Ca\(^{2+}\) channels to the Ca\(^{2+}\) transients in the proximal dendrite was assessed. In all cells tested, application of the L-type channel blocker nifedipine at 10 µM markedly decreased the amplitude and the area of the fluorescence transients induced by four APs at 20 Hz (Fig. 7A and B; n = 5). As expected, the time constant of decay of the fluorescence transients was not affected by nifedipine (Fig. 7A, \(\tau_{\text{control}} = 414 \pm 39\) ms, \(\tau_{\text{Nifedipine}} = 423 \pm 64\) ms, n = 5), because \(\tau\) is mainly determined by Ca\(^{2+}\) extrusion. The subsequent application of dTC in the presence of nifedipine did not increase the amplitude of the fluorescence transients significantly (Fig. 7A and B; \(F_{4,2} = 61, P<0.0001;\) nifedipine vs control 69.5±1.2%, P<0.05; nifedipine+dTC vs control 77.9±1%, P<0.05; nifedipine vs nifedipine+dTC, P>0.05; one-way repeated measures ANOVA with Bonferroni post-hoc test), and similar results were obtained for the area of the Ca\(^{2+}\) transients (\(F_{4,2} = 18.47, P<0.001;\) nifedipine vs control 81±2 %, P<0.01, nifedipine + dTC vs control 85.2±4.1 %, P<0.01; nifedipine vs nifedipine+dTC, P>0.05; one-way repeated measures ANOVA with Bonferroni post-hoc test). Similarly, no significant difference for the time constant of decay of the fluorescence transients was observed when comparing controls, nifedipine alone and the dTC-
nifedipine co-application (Fig. 7A; F_{4,2} = 0.14, P = 0.9; one-way repeated measures ANOVA).
When applied to neurons in the presence of nifedipine, the effect of dTC on both the amplitude and
the duration of the fluorescence transients was therefore strongly attenuated compared with the
results obtained in the absence of the L-type Ca\textsuperscript{2+} channel blocker (Fig. 6B-D and Fig. 7A). We
performed additional experiments by first applying dTC, followed by the addition of nifedipine.
When applied in the presence of dTC, nifedipine reduced the amplitude (F_{14,2} = 22, P<0.0001; dTC
vs control 123±5%, P<0.01; dTC+nifedipine vs control 78±7%, P<0.05; dTC vs dTC+nifedipine,
P<0.001; one-way ANOVA with Bonferroni post-hoc test) and area (F_{14,2} = 21, P<0.0001; dTC vs
control 133±7%, P<0.01; dTC+nifedipine vs control 76±6%, P>0.05; dTC vs dTC+nifedipine,
P<0.001; one-way ANOVA with Bonferroni post-hoc test) of the Ca\textsuperscript{2+} transients to values similar or
below the control ones. These results suggest that the activation of SK channels by APs is at least in
part due to the activation of L-type Ca\textsuperscript{2+} channels and in turn regulates Ca\textsuperscript{2+} influx in the proximal
dendrite.

Discussion

The dynamic response of pyramidal neurons in the hippocampus is modulated by Ca\textsuperscript{2+}
transients that result from influx through voltage-gated Ca\textsuperscript{2+} channels. In particular, Ca\textsuperscript{2+} elevations
triggered by back-propagating APs show a maximal amplitude in the proximal dendrites and
decrease rapidly with distance from the soma (Callaway and Ross 1995; Christie et al. 1995; Jaffe et
al. 1992; Regehr et al. 1989; Regehr and Tank 1994; Spruston et al. 1995). In this study, we have
investigated how the activation of SK channels affects AP-induced changes in intracellular Ca\textsuperscript{2+}
levels in proximal processes of hippocampal pyramidal neurons. We have found that in this cellular
compartment SK channels limit the amplitude and duration of AP-induced Ca\textsuperscript{2+} transients.

SK channels have been shown to modulate the amplitude and duration of intracellular Ca\textsuperscript{2+}
signals by feedback regulation of the relevant Ca\textsuperscript{2+} sources in different dendritic compartments, thus affecting dendritic signal integration and synaptic plasticity. In organotypic hippocampal cultures, SK channels are responsible for the repolarization of local dendritic plateau potentials triggered by focal glutamate application to distal apical dendrites of CA1 pyramidal neurons (Cai et al. 2004). In acute hippocampal slices, synaptic stimulation activates glutamate receptors leading to the activation of SK channels located on the spine heads, which in turn reduces Ca\textsuperscript{2+} influx through the NMDA receptors (Bloodgood and Sabatini 2007; Lujan et al. 2009; Ngo-Anh et al. 2005). Both types of feedback regulation were shown to occur in response to synaptically evoked processes and on dendritic compartments receiving primarily glutamatergic excitatory synaptic inputs. The physiological role of the second and higher order dendrites, where the excitatory inputs predominate, is fundamentally different from that of the proximal apical dendrite, which mainly receives inhibitory synaptic inputs from GABAergic interneurons (Papp et al. 2001). In the proximal dendrite our results show feedback regulation of the amplitude and duration of AP-induced Ca\textsuperscript{2+} transients by SK channels. This modulation of transient Ca\textsuperscript{2+} elevations by SK channels could affect the communication between the soma and the distal apical dendritic tree at the single cell level and shift the balance between excitation and inhibition at the network level.

Given their high sensitivity to Ca\textsuperscript{2+} (EC\textsubscript{50} ~ 300 nM, Xia et al. 1998) and relatively fast time constant of activation (~5 ms at saturating Ca\textsuperscript{2+} concentrations, Pedarzani et al. 2001; Xia et al. 1998), SK channels are well suited to take part in a feedback loop to regulate Ca\textsuperscript{2+} influx in proximal apical dendrites of CA1 neurons, where a single AP leads to Ca\textsuperscript{2+} elevations in the order of ~300 nM lasting 70-90 ms, while higher and longer-lasting free Ca\textsuperscript{2+} concentrations are reached in response to trains of APs (Helmchen et al. 1996; Maravall et al. 2000).

Our results suggest that SK channels act in a negative feedback loop by reducing Ca\textsuperscript{2+} influx through the Ca\textsuperscript{2+} channels that activate them following APs. This role of SK channels is supported by the effects of specific SK channel blockers (apamin, dTC) and enhancers (NS309) on the
magnitude of Ca\(^{2+}\) transients. The presence of apamin or dTC boosted the Ca\(^{2+}\) influx induced by a train of two APs. Consistent with a negative feedback mechanism, application of the SK channel enhancer NS309 resulted in a reduction of the Ca\(^{2+}\) transient.

The regulatory effect of SK channels was particularly evident when a train of four APs was used to elicit a larger Ca\(^{2+}\) influx, leading to a stronger recruitment of SK channels. The increases in the amplitude of Ca\(^{2+}\) transients following application of SK channel blockers were larger compared to the stimulation with two APs and consistently observed in every cell tested.

Ca\(^{2+}\) buffering by Ca\(^{2+}\)-sensitive dyes could in principle mimic the effect of SK channel inhibition. However, this is unlikely in our case because we used a low concentration of Ca\(^{2+}\)-sensitive dye and did not observe significant changes in the baseline fluorescence over the course of our experiments. If anything, the added buffer capacity would result in gradual decrease in the Ca\(^{2+}\) transients (Helmchen et al. 1996; Maravall et al. 2000) and lead to an under-estimation of the effects of SK channel inhibitors on the amplitude of Ca\(^{2+}\) transients. The validity of the increase of the Ca\(^{2+}\) transients induced by SK blockers is further supported by the reversibility achieved upon wash out of dTC.

In CA1 pyramidal neurons, APs induce Ca\(^{2+}\) transients that are largest in the proximal dendrites (Callaway and Ross 1995; Christie et al. 1995; Spruston et al. 1995), where they are mediated by different subtypes of high voltage-activated Ca\(^{2+}\) channels (Bloodgood and Sabatini 2007; Christie et al. 1995; Christie et al. 1996). In particular, L-type Ca\(^{2+}\) channels are highly expressed in the somato-dendritic compartment of pyramidal cells in sections (Leitch et al. 2009; Tippens et al. 2008; Westenbroek et al. 1990) and primary cultures (Pravettoni et al. 2000). Additionally, experiments on the specific high voltage-activated Ca\(^{2+}\) channel subtypes coupled to the activation of the SK-mediated I\(_{\text{AHP}}\) in hippocampal pyramidal neurons have revealed that L-type Ca\(^{2+}\) channels are important contributors to the activation of I\(_{\text{AHP}}\), which was reduced by \(\sim\)48% by the L-type Ca\(^{2+}\) channel blocker nifedipine (Bosurgi and Pedarzani 2006). Application of nifedipine
showed a contribution of ~30% by L-type Ca\(^{2+}\) channels to the total AP-induced Ca\(^{2+}\) elevation in the proximal dendrites of cultured hippocampal pyramidal neurons (Fig. 7). This is in line with a previous report on the relative contributions of different voltage-gated Ca\(^{2+}\) channel subtypes to spike-induced Ca\(^{2+}\) influx in hippocampal pyramidal neurons in brain slices (Christie et al. 1995). L-type Ca\(^{2+}\) channel inhibition prevents the increase in the amplitude of the Ca\(^{2+}\) transients by SK channel blockers (Fig. 7) or reverses it back to control values or below when nifedipine is applied after dTC. This is evidence that L-type Ca\(^{2+}\) channels are implicated in the AP-induced Ca\(^{2+}\) influx leading to SK channel activation in the proximal dendrite of hippocampal pyramidal neurons. We cannot however exclude the contribution of other Ca\(^{2+}\) channel subtypes (see also Jones and Stuart 2012).

How do SK channels regulate transient Ca\(^{2+}\) elevations triggered by APs in dendrites? Voltage-gated Ca\(^{2+}\) channels open during the repolarizing phase of action potentials. While inhibition of BK and voltage-dependent K\(^+\) channels by TEA leads to broader APs and increased Ca\(^{2+}\) influx (Fig. 3C-D), SK channel inhibition does not affect the duration of somatic APs (Fig. 6A). However, we cannot exclude the possibility that SK channels might contribute to shaping the waveform of dendritic APs, which have a lower amplitude and longer duration in CA1 dendrites (Johnston et al. 2000; Spruston et al. 1995). While the waveform of the somatic AP is not directly affected by SK channel activation, the SK channel effect on AP-induced Ca\(^{2+}\) entry in the proximal dendrite might result from the functional interaction of these channels with other dendritic conductances. Thus, two K\(^+\) currents, I\(_A\) and I\(_D\), are expressed in CA1 dendrites (Golding et al. 1999; Hoffman et al. 1997), and are inactivated at depolarized potentials. The voltage-dependent inactivation properties of A-type K\(^+\) channels enable modest levels of membrane depolarization to decrease the size of the available A channel population and likewise increase dendritic AP amplitude and duration (Hoffman et al. 1997). By hyperpolarizing the membrane potential, SK channels could affect the availability and activation state of these conductances in AP trains, with
SK channel inhibition and corresponding membrane depolarization favoring the inactivation of $I_A$ and $I_D$. Upon inhibition of $I_A$ and $I_D$, large-amplitude, back-propagating APs have been shown to activate dendritic Ca$^{2+}$ channels or favor the dendritic initiation of Ca$^{2+}$-dependent potentials, resulting in a massive influx of Ca$^{2+}$ into the dendrites (Golding et al. 1999; Hoffman et al. 1997; Magee and Carruth 1999). This supports the possibility of a potential interaction between SK channels and A- and/or D-type K$^+$ channels that may underlie the increase in Ca$^{2+}$ influx observed upon SK channel inhibitions in our recordings. The relatively small, albeit significant, effect exerted by SK channel inhibition on Ca$^{2+}$ influx in proximal hippocampal pyramidal cell dendrites might well match the gradient of A-type K$^+$ channel density, with less channels in the proximal compared with the distal dendritic compartment.

A second potential mechanism for the SK-mediated enhancement of Ca$^{2+}$ transients in proximal dendritic compartment is linked to the co-existence of L-type Ca$^{2+}$ channels with different gating behaviors in neurons, which are thought to give rise to distinct intracellular calcium signals in response to neuronal activity (Forti and Pietrobon 1993; Kavalali and Plummer 1994; Koschak et al. 2007). Thus, in addition to cardiac-like L-type channels, hippocampal neurons display L-type Ca$^{2+}$ channels with anomalous gating properties, characterized by long channel reopenings after repolarization following strong depolarizations, such as bursts or trains of action potentials (Kavalali and Plummer 1994; Schjott and Plummer 2000). One potential mechanism by which SK channels might modulate Ca$^{2+}$ influx would therefore be by reducing the activity of L-type Ca$^{2+}$ channels in their “anomalous gating” phase. This hypothesis is supported by our results showing that strong depolarization caused by APs is essential to generate the SK-mediated feedback on Ca$^{2+}$ influx, because this was absent in response to pure electrotonic spread when Na$^+$ channels were blocked by TTX (Fig. 4F-H).

In addition to anomalous gating properties, L-type Ca$^{2+}$ channels are subject to various mechanisms of channel inactivation that contribute to the control of Ca$^{2+}$ entry during ongoing
neuronal electrical activity. These include Ca\(^{2+}\)-dependent inactivation, and fast and slow voltage-dependent inactivation (Budde et al. 2002). The inactivation kinetics of L-type Ca\(^{2+}\) channels are generally described as slow, but vary in different cell types, possibly due to molecular diversity of the channels (splice variants of the pore-forming subunit; interaction with other Ca\(^{2+}\) channel subunits and modulatory proteins) (Budde et al. 2002). The inactivation profile of L-type Ca\(^{2+}\) channels in hippocampal pyramidal neurons has not been specifically characterized. We cannot exclude that SK channels could interfere in some indirect manner with the inactivation process of Ca\(^{2+}\) channels in proximal dendritic processes, contributing to the increase in Ca\(^{2+}\) influx we have observed upon SK channel inhibition.

The pharmacological manipulation of SK channel activity not only increased or decreased the amplitude but also affected the area under the curve of the Ca\(^{2+}\) transients elicited by APs in the proximal dendrite of hippocampal pyramidal neurons (Fig. 4-6). SK channel inhibition led also to a significant prolongation of the Ca\(^{2+}\) transients (Fig. 4-6). Interestingly, the L-type Ca\(^{2+}\) channel blocker nifedipine prevented the effect of SK channel inhibitors on the time constant of decay of Ca\(^{2+}\) transients (Fig. 7). The time course of decay of AP-induced Ca\(^{2+}\) transients in dendrites directly reflects the rate of Ca\(^{2+}\) clearance (Scheuss et al. 2006). Sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPases (Mainen et al. 1999; Majewska et al. 2000; Sabatini et al. 2002), plasma membrane Ca\(^{2+}\) ATPases and Na\(^+\)/Ca\(^{2+}\) exchangers (Lorincz et al. 2007; Scheuss et al. 2006) are responsible for the Ca\(^{2+}\) clearance from the cytosol of dendrites and spines in CA1 pyramidal neurons. Notably, both plasma membrane Ca\(^{2+}\) ATPases and Na\(^+\)/Ca\(^{2+}\) exchangers are expressed in the dendrites of primary hippocampal neurons (Kiedrowski 2004; Kip et al. 2006). The increased duration of Ca\(^{2+}\) signals might simply reflect the longer time needed to clear the augmented Ca\(^{2+}\) following SK channel inhibition (Regehr and Tank 1992). Alternatively, SK channels might affect extrusion mechanisms in different ways. In the thalamus, for example, SK channels and sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPases compete for available Ca\(^{2+}\) and shape Ca\(^{2+}\) transients in an interactive manner (Cueni et al.
Another possibility is that the larger Ca\textsuperscript{2+} accumulations due to SK channel inhibition attenuated Ca\textsuperscript{2+} extrusion by plasma membrane Ca\textsuperscript{2+} ATPases and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers, whose function is reduced in a Ca\textsuperscript{2+}-dependent manner (Scheuss et al. 2006), thereby leading to the observed prolongation of the duration of Ca\textsuperscript{2+} signals.

Our results demonstrate for the first time that the activity of SK channels can regulate the duration of Ca\textsuperscript{2+} transient decays in the proximal dendrite of hippocampal neurons. This may affect temporal summation of Ca\textsuperscript{2+} signals, potentially leading to changes in spike-timing-dependent plasticity (Caporale and Dan 2008), as we have recently shown in another brain region, the striatum (Nazzaro et al. 2012). Here SK channels take part in the regulation of Ca\textsuperscript{2+}-dependent release of endocannabinoids and plasticity, through a functional coupling with L-type voltage-gated Ca\textsuperscript{2+} channels activated by trains of APs (Nazzaro et al. 2012). SK-mediated modulation of intracellular Ca\textsuperscript{2+} dynamics may similarly be relevant for the activation of Ca\textsuperscript{2+}-dependent signaling cascades to induce different forms of plasticity also in the hippocampal region (Cummings et al. 1996).
References


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Acknowledgements

The authors gratefully acknowledge J. Dempster for supplying the Strathclyde Electrophysiology Software and Dr D. DiGregorio for useful advice and discussion.

Grants

This work was supported by an EMBO short-term fellowship (EMBO ASTF 137.00-03) and a Human Frontier Science Program (HFSP) short-term fellowship (ST00323/2002-C) to R.T.; a Career Establishment Grant from the UK Medical Research Council to P.P. (CEG G0100066); a Wellcome Trust Student Prize fellowship to T. F. (068583/Z/02/Z); an MRC-DTA PhD fellowship to M. S.-C.; and a Wellcome Trust Senior Research fellowship to M. S. (061198/Z/00A). P. P. and M. S. acknowledge support by the ENI-Net. P. P. acknowledges support by the HFSP (RGP0013/2010).
Figure Legends

FIGURE 1. Electrophysiological characterization of $I_{AHP}$ in cultured hippocampal neurons. (A) Inhibition of the $I_{AHP}$, seen as an outward tail current, by d-tubocurarine (dTC; 100 µM; upper panel) and the specific SK channel blocker apamin (5 nM; lower panel). (B) Box and whisker diagrams summarizing the effects of the two blockers on the $I_{AHP}$. The filled circle symbols represent the mean values of current inhibited by apamin (81 ± 6%; n = 5) and dTC (89 ± 3%; n = 18). (C) and (D) Effect of 250 µM 1-EBIO (C) and 5 µM NS309 (D) on the amplitude and time course of decay of the $I_{AHP}$. The increase in current amplitude in the presence of the drug is clearly visible (middle panels). In the superimposition (right panels) the $I_{AHP}$ traces have been normalized to peak to show the prolongation of the time course. (E) and (F) Box and whiskers diagrams summarizing the effects of 1-EBIO and NS309 on the peak amplitude (1-EBIO: 47 ± 3% increase, P<0.001; NS309: 56 ± 18% increase, P=0.01) and time constant of decay (τ; 1-EBIO: 122 ± 18% increase, P=0.03; NS309: 317 ± 55% increase, P=0.01) of $I_{AHP}$ measured from 5 cells.

FIGURE 2. Expression of SK2 in cultured postnatal hippocampal neurons at different stages of in vitro development. (A) Immunohistochemistry performed with a specific anti-NSK2 antibody, at 1 day in culture shows a fluorescence signal indistinguishable from the background signal. The inset shows a bright field picture of the neurons stained in (A). (B)-(D) By day 2 in culture SK2 can be clearly detected (B) and its expression increases at days 4 (C) and 6 (D). (E) Distribution of the SK2 subunit at day 13, showing a clear somato-dendritic localization of the channel. (F) Non-specific staining with pre-adsorbed antibodies (+Trx-NSK2) shows a very weak signal in soma but not in neurites. The inset shows a bright field picture of the neurons in (F). Scale bars: (A)-(D) 20 µm (shown in (B)); (E) and (F) 10 µm (shown in (E)); insets 20 µm in (A) and 10 µm in (F).
FIGURE 3. $\text{Ca}^{2+}$ transients induced by APs backpropagating to the proximal processes of hippocampal neurons. (A) Image of a cultured hippocampal neuron filled with Fluo-4 (20 µM) and stimulated with a prolonged depolarization showing the $\text{Ca}^{2+}$ accumulation within the cell. Two neuronal processes (p1 and p2) are indicated by arrows. Scale bar: 10 µm. (B) Short (10 ms) somatic depolarizing current injections elicited two action potentials (upper panel) at 20 Hz. Line scans (middle panel) were recorded from the two processes (p1 and p2), at the position indicated by the dashed line in (A). The line scans show an increase in fluorescence as a consequence of intracellular $\text{Ca}^{2+}$ elevation. Scale bar: 250 ms. The corresponding relative changes in fluorescence (lower panel) show a faster transient for the thinner process (p1) simply due to the different surface to volume ratio. For display purposes transients were digitally filtered off-line (adjacent-averaging routine, smoothing factor n=10, Origin 7). (C) Effect of the $\text{K}^+$ channel blocker TEA (10 mM) on the repolarization of the action potentials that induced the fluorescence transients displayed in (D). Black line: action potentials before TEA application (Control); red line: action potentials in the presence of TEA; blue line: action potentials after wash out of TEA. (D) Line scans recorded under control conditions, during application of 10 mM TEA and after wash out (upper panel) and the corresponding relative changes in fluorescence (lower panel). Scale bar: 250 ms.

FIGURE 4. Effect of apamin on the $\text{Ca}^{2+}$ transients recorded from proximal neuronal processes. (A) Line scans showing the fluorescence changes triggered by two APs at 20 Hz in the proximal process of a hippocampal pyramidal neuron in the absence (Control) and in the presence (Apamin) of the SK channel blocker apamin (5 nM). (B) Plot of the relative change in fluorescence obtained from the line scans in (A). The transients were fitted with mono-exponential functions and the time constant of decay ($\tau$) is indicated. The fluorescence transients are superimposed (right panel) to show the increase in amplitude and decay. (C) A mean increase of $115 \pm 4 \%$ (n = 5, P<0.05) in the amplitude of the relative change in fluorescence was observed in the presence of apamin. (D) Mean
time constant of decay before ($\tau = 298 \pm 11 \text{ ms}$) and after application of apamin ($\tau = 353 \pm 18 \text{ ms}$, n = 5). (E) Relative increase in the area under the fluorescence transients in the absence (Control) and presence of apamin ($138 \pm 13 \%$; n = 5, P<0.05). (C)-(E) Symbols represent individual cells. Bar diagrams, mean ± SEM. (F) Effect of the Na$^+$ channel blocker TTX (1 µM) on the action potentials that induced the fluorescence transient displayed in (G). Black line: action potentials before TTX application (Control); grey line: membrane depolarization in the presence of TTX; dashed line: membrane depolarization in the presence of TTX and apamin. (G) Fluorescence transients elicited by two depolarizing pulses under control conditions (i.e. in the presence of two action potentials, see panel (F); black trace), in the presence of TTX (grey), and TTX + apamin (black trace). (H) Summary bar diagram showing a mean decrease to $22 \pm 8 \%$ (n = 4) in the amplitude of the relative change in fluorescence in the presence of TTX. Apamin, when applied in the presence of TTX, did not cause any increase in the fluorescence transient ($F_{3,2} = 27$, P=0.001; TTX vs control P<0.05; TTX+apamin vs control P<0.05; TTX+apamin vs TTX P>0.05; one-way repeated measures ANOVA with Bonferroni post-hoc test).

**FIGURE 5.** Modulation of AP-induced Ca$^{2+}$ transients by d-tubocurarine (dTC) and NS309. (A) Representative fluorescence transients elicited by two APs at 20 Hz in the proximal dendrite, showing an increase in amplitude and area under the curve caused by the SK channel blocker dTC (100 µM). This effect was partially reversed (wash). (B) Bar diagram showing the increase of mean, relative amplitude of fluorescence transients in the presence of dTC to $113 \pm 3 \%$ of control (n=13; P<0.01). The effect of dTC on the amplitude of the fluorescence transients was reversed in 5 out of 6 cells (wash, $107 \pm 5 \%$ of control; n=5). Only cells with a current amplitude >90% after wash out were analyzed and included. (C) The normalized area under the curve of the fluorescence transients after dTC application increased to $120 \pm 4 \%$ of the control value (n=13; P<0.01). (D) The SK channel enhancer NS309 (5 µM) caused a reduction of the fluorescence transient under the same
recording conditions. (E) Bar diagram showing a reduction by $25 \pm 5\%$ (n=6; P = 0.03) of the mean, relative amplitude of fluorescence transients in the presence of NS309. (F) With respect to the control, the normalized area under the curve of the fluorescence transients was reduced by $30 \pm 6\%$ (n=6; P = 0.02) after application of NS309 (5 µM). Symbols in (B), (C), (E) and (F) represent individual cells. Bar diagrams, mean ± SEM.

FIGURE 6. Effects of dTC and TEA on the $\text{Ca}^{2+}$ transients induced by a train of four APs. (A) Line scans (upper panel) and action potentials (20 Hz; lower panel) triggered by somatic current injections in control, after the application of dTC (100 µM) and further addition of TEA (10 mM). (B) Blocking SK channels by dTC resulted in an increase of the relative fluorescence signal in the proximal dendritic process. This effect was further enhanced by TEA application. (C) Bar diagram showing the increase of the mean, relative amplitude of fluorescence transients in the presence of dTC to $130 \pm 8\%$ of control (n = 4; P<0.05). (D) The mean time constant of decay, obtained from mono-exponential fits to the fluorescent transients, increased from $\tau = 486 \pm 99$ ms before to $\tau = 593 \pm 123$ ms after application of dTC (n = 4, P<0.05). (E) The application of dTC resulted in a mean increase in the area under the curve of the fluorescence transients to $144 \pm 9\%$ (n = 4; P<0.05) with respect to the control. Symbols in (B), (C), (E) and (F) represent individual cells. Bar diagrams, mean ± SEM.

FIGURE 7. Inhibition of L-type voltage-gated $\text{Ca}^{2+}$ channels prevents the dTC-mediated effect on $\text{Ca}^{2+}$ transients induced by a train of four APs. (A) Application of the L-type $\text{Ca}^{2+}$ channel blocker nifedipine (Nif, 10 µM) significantly decreased the fluorescent transient induced by a train of four APs. Subsequent co-application of dTC (100 µM) caused only a small, non-significant increase in the fluorescence transient (Nif + dTC). (B) Bar diagram showing the decrease in the mean amplitude of the relative changes in fluorescence to $69.5 \pm 1.2\%$ (n=5) in the presence of nifedipine (Nif) with
respect to control. When dTC was subsequently applied in the presence of nifedipine, the amplitude of the fluorescence transients was not significantly changed ($F_{4,2} = 61$, $P<0.0001$; nifedipine vs control $P<0.05$; nifedipine+dTC vs control $P<0.05$; nifedipine vs nifedipine+dTC $P>0.05$; one-way repeated measures ANOVA with Bonferroni post-hoc test). Symbols represent individual cells. Bar diagram, mean ± SEM.
Figure 1

A

washes dTC

Apamin

25 pA

200 ms

B

% I_{AHP} Inhibited

(18)

(5)

dTC Apamin

C

+ 1-EBIO

1-EBIO

40 pA

200 ms

D

+ NS309

NS309

100 pA

200 ms

E

1-EBIO

(5)

(5)

F

NS309

(5)

(5)

Amp τ

Increase %
Figure 3

A

B

C

D

Control

TEA

Wash

- Control
- TEA
- Wash

1.0 ΔF/F

250 ms

20 ms

20 mV
Figure 5

A

B

C

D

E

F

Amplitude (% of control)

Area (% of control)

Control

dTC

Wash

Control

dTC

Wash

Control

dTC

Control

NS309

Control

NS309

Control

NS309

**

*

*
Figure 6
Figure 7

A

Control
Nif + dTC
Nif

B

Amplitude (% of control)

Control
Nif
Nif + dTC

0.5 ΔF/F
250 ms

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