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BK channels control glutamate release at CA3-CA3 synapses in the rat hippocampus

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TRIESTE

"Io...fottuna che ho un cevvello eccezziunalo...veramente eccezziunalo
ma...la gente mi ritiene un animalo...un gran bell'animalo
pepputè campà...papparapà...m'hanno fatto emigràààà"

D. Abatantuono, *Eccezziunale veramente*

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NOTES

The work described in this dissertation was carried out at the International School forAdvanced Studies, Trieste, between November 2001 and January 2003. All of the work comes from my own experiments with the exceptions of Cresyl violet staining a nd some experiments on action potential broadening, carried out by Chiara Saviane.

Part of this work has been submitted in the article:

BK channels control transmitter release at CA3-CA3 synapses in the rat hippocampus. Giacomo Raffaelli, Chiara Saviane, Paola Pedarzani and Enrico Cherubini (2003).

ABSTRACT

Modifications of synaptic efficacy play a crucial role in information processing in the brain. In particular, they are thought to be very important for the refinement of neural circuitry, information storage, learning and memory. Therefore, investigating the mechanisms that modulate synaptic transmission is of fundamental importance for understanding brain functions. In the present study, patch-clamp recordings were performed in order to investigate synaptic transmission in the hippocampus, focusing on a particular presynaptic mechanism that may affect synaptic efficacy. In particular we have explored the possibility that a particular type of calcium-dependent potassium channel, the BK channel, may affect the probability of transmitter release modifying the shape of the action potential at nerve terminals.

BK channels are large conductance calcium- and voltage-activated potassium channels whose characteristic is to reduce cell excitability by increasing the open probability following elevation of intracellular calcium. BK channels targeted to active zones in presynaptic nerve terminals would limit calcium entry and transmitter release by reducing the duration of the presynaptic spike. While it has been demonstrated that BK channels regulate secretion and transmitter release at neurosecretory nerve terminals and at the neuromuscular junction, their functional role in central neurones is still uncertain. In the hippocampus BK channels would act as an "emergency brake" that would control transmitter release only under conditions of excessive depolarisation and accumulation of intracellular calcium. Here we show that, under basal experimental conditions, the selective BK channel blockers paxilline and iberiotoxin increase the frequency of spontaneously occurring EPSCs in the CA3 hippocampal region. These drugs did not affect miniature currents suggesting that their action was dependent on action potential firing. Moreover, at hippocampal CA3-CA3 connections paxilline enhanced the probability of transmitter release in target cells by broadening the presynaptic spike. In particular, in the presence of paxilline an increase in successes rate and EPSC amplitude, and a concomitant decrease in paired pulse ratio were observed. BK channel blockers produced also a delayed release, particularly in response to the second action potential following with 50 ms delay the first one in paired pulses. These results are consistent

with the hypothesis that BK channels are powerful modulators of transmitter release and synaptic efficacy in central neurones under normal conditions.

INTRODUCTION

"Molti ignorano e vogliono ignorare il passato e la storia di ieri.
Forse soltanto perché li porrebbe di fronte a un confronto faticoso."
G. P. Motti *Arrampicare a Caprie*, 1983

1. Synaptic transmission

Multicellular organisms depend on the ability of individual cells to communicate with each other. The nervous system and especially the brain consist of ensembles of cells that communicate in a fast, accurate and modifiable way. Electrical signals can travel along the processes of a single neurone over considerable distances (from tens of micrometers up to meters). Therefore, information is transferred from the signal-generating cell (presynaptic) to the receiving one (postsynaptic) at specific contact sites termed synapses. Synaptic contacts between neurones occur primarily between small swellings, known as *boutons*, either at the terminal or along (*en passant*) axonal profiles of the presynaptic neurone and small fingerlike processes (spines) of postsynaptic dentrites (axospinus synpases). However, other types of synapses have been characterised and have been termed depending on the contact elements (for example axosomatic, axoaxonic, dendrodendritic).

Two structurally and functionally distinct forms of synapses exist: electrical and chemical. At electrical synapses, specialised channels (gap junctions) form a direct electrical connection between the presynaptic and the postsynaptic neurones. At chemical synapses, the cells are electrically disconnected from one another: the electrical signal is translated into a chemical one in the presynaptic neurone and only afterwards is re-converted to an electrical signal in the postsynaptic cell. Electrical synapses have the virtue that transmission occurs without delay, but they are far less rich in possibilities for adjustment and control than chemical synapses, that, in fact, represent the predominant way of communication between neurones.

1.1 Chemical synapses

Chemical synapses operate through the release, from the presynaptic neurone, of a neurotransmitter that diffuses in the synaptic cleft and provokes electrical changes in the postsynaptic cell. Neurotransmitter molecules are initially stored in the synaptic vesicles

described, long time ago, by ultrastructural studies (Palade 1954; Palay, 1954). Most vesicles are retained in a large pool behind the plasma membrane, whereas a small part of them approaches the presynaptic membrane, and eventually fuse with it, through a particular cycle that can be divided in several steps and involves a very large number of proteins (reviewed in Südhof, 1995). The vesicles immediately available for release belong to the so-called readily releasable pool of vesicles (Rosenmund & Stevens, 1996; Südhof, 2000). Synaptic vesicles are usually anchored to a network of cytoskeletal filaments by synapsins, a family of protein presenting phosphorylation sites for both cAMP and calcium-calmodulin dependent protein kinases. Phosphorylation is able to free vesicles from the cytoskeleton constrain, allowing them to move into the active zone, where they dock through the interaction between proteins in the vesicular membrane and proteins in the plasma membrane. Candidates for these protein-protein interactions include the vesicular membrane proteins synaptotagmin and Rab3 (Benfenati *et al.*, 1999). After docking, synaptic vesicles go through a maturation process, known as priming, during which a highly stable core complex is formed between the synaptic vesicle protein VAMP/synaptobrevin and the presynaptic membrane proteins syntaxin and SNAP-25. These three proteins are known as SNAP receptors or SNAREs, as they form a high affinity binding site for cytosolic α-SNAP (soluble NSF attachment protein), which itself becomes a receptor site for NSF (N-emthylmaleimide-sensitive ATP-ase). Under steady-state conditions, specific protein interactions, between VAMP and synaptophysin at the vesicle membrane and between syntaxin and munc 18 (or munc 13) at the plasma membrane, inhibit the formation of the core complex that would otherwise assemble outside the active zones (Thomson, 2000). The "unlocking" of these proteins occurs during docking and is modulated by phosphorylation and by calcium, suggesting that it may be an important rate-dependent step in the supply of fusion-competent vesicles during repetitive activity. Once the fusion core complexes are formed, they have to be disassembled for release to occur. The hydrolisis of ATP by NSF provides the energy required and regenerates the SNARE monomers that will be used in the next cycle. Rapid fusion and exocytosis are triggered by high local calcium concentration during the action potential invasion. Ca^{2+} ions act in a co-operative way, as judged from the steep relationship between change in intracellular calcium concentration ($[Ca^{2+}]_i$) and

transmitter release, thus accounting for a considerable margin of safety for synaptic transmission (Dodge & Rahamimoff, 1967). Different lines of evidence suggest that synaptotagmins function as Ca^{2+} sensors in the final fusion step (Südhof, 1995). According to the classical model, during exocytosis, vesicles collapse completely into the plasma membrane ("total fusion") and release neurotransmitter in the synaptic cleft. During this process Rab proteins dissociate from the vesicle membranes and may retard the activation of neighbouring vesicles, resulting in release site refractoriness (Geppert *et al.*, 1997). Therefore, empty vesicles internalise slowly (endocytosis) at sites distant from the active zones and translocate into the interior for endosomal fusion. New vesicles accumulate again neurotransmitter by means of an active transport driven by an electrochemical gradient created by a proton pump. Finally, filled vesicles translocate back to the active zones and the vesicle cycle ends.

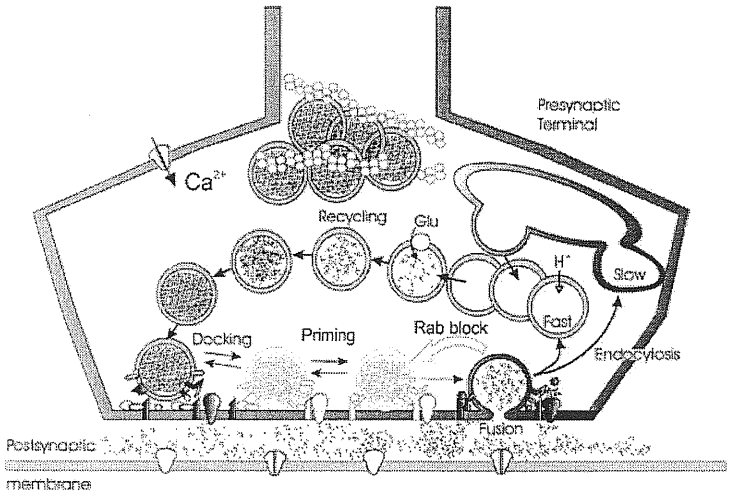

Figure I. Main steps in neurotransmitter release at chemical synapses. A small part of synaptic vesicles approaches the plasma membrane and, through docking and priming processes, gets ready for being rapidly released. The influx of Ca^{2+} ions during action potential invasion triggers vesicle fusion. Thus, the neurotransmitter released diffuses across the narrow synaptic cleft and acts on postsynaptic receptors. Following or during fusion, Rab proteins dissociate from the vesicle and may in some way retard the activation of neighboring vesicles (resulting in release site refractoriness). Synaptic vesicles are therefore recycled, in either slow or fast way, and may accumulate neurotransmitter again. (Modified from Thomson, 2000)

Once neurotransmitter is released in the cleft, it diffuses to act mainly on ionotropic or metabotropic receptors that are clustered in an electron-dense thickening of the postsynaptic membrane, known as postsynaptic density. Then neurotransmitter is rapidly eliminated by diffusion, enzymatic degradation or by uptake into nerve terminals or glial cells. Ionotropic receptors are ion channels that open when they are bound by neurotransmitter molecules, allowing ions to flow within the cell membrane. This process changes the membrane potential of the postsynaptic neurone either in the positive (depolarisation) or in the negative direction (hyperpolarisation). When receptors are permeable to calcium ions, this flow can also activate intracellular processes. In the mammalian brain, glutamate and γ-amonibutyric acid (GABA) represent the principal excitatory and inhibitory neuotransmitters, respectively.

1.2 Synaptic efficacy

The neuromuscular junction (NMJ) represents the standard model for studying synaptic transmission. At the beginning of the 1950s del Castillo and Katz (1954) proposed the quantal hypothesis of transmitter release, according to which neurotransmitter is packaged in discrete quantities of fixed size, called *quanta*, which are generally identified with synaptic vesicles. Thus, when a nerve impulse reaches the terminal, an integer number of *quanta* release their content in the synaptic cleft. In line with this idea, miniature currents, which can be recorded in the absence of action potential, are due to the release of a single *quantum*. Each site operates in an all-or-none fashion, meaning that it can release either zero or one *quantum* and each *quantum* is released probabilistically and independently of the others. If the probability of release is uniform among the different sites, synaptic transmission can be fit with a simple binomial model. Thus, in a synapse with N functional releasing sites and a probability p of release at each individual site, the probability that n *quanta* are released by a single action potential is:

$$P(n) = \frac{N!}{n!(N-n)!} p^n (1-p)^{N-n} \tag{1.1}$$

While the probability of detecting no response (failure rate) is:

$$P(0) = (1-p)^N \tag{1.2}$$

The mean number of *quanta* released, known as quantal content, is given by:

$$m = Np \tag{1.3}$$

If Q represents the magnitude of the postsynaptic response to a single transmitter *quantum* (quantal size), the mean response I and its standard deviation σ are:

$$I = NPQ \tag{1.4}$$

$$\sigma = Q\sqrt{Np(1-p)} \tag{1.5}$$

Interestingly the coefficient of variation (CV), defined as the ratio between the standard deviation and the mean, is independent of Q:

$$CV = \frac{\sigma}{I} = \sqrt{\frac{(1-p)}{Np}} \tag{1.6}$$

In case of a very low probability of release ($p<<1$) and large N, the binomial distribution approximates the Poisson one and eqns.1.1-1.2-1.6 convert respectively into:

$$P(n) = \frac{e^{-m}m^n}{n!} \tag{1.7}$$

$$P(0) = e^{-m} \tag{1.8}$$

$$CV^2 = \frac{1}{m} \tag{1.9}$$

Thus, strength or efficacy of a synaptic connection depends on N, p and Q. Changes in one or more of these parameters account for modifications in synaptic strength. It is clear that, while Q depends on both pre and postsynaptic mechanisms, N and p are controlled only by presynaptic factors. On the basis of the previous equations, quantal analysis has been developed to find estimates of these parameters and/or to define the site of changes in case of modifications in synaptic efficacy, for example through the evaluation of failure rate or CV (Katz, 1969).

Later on, the basic concepts of neurotransmitter release have been extended to central synaptic transmission, despite the obvious differences, both functional and morphological, that distinguish the two systems. In particular, most of central synapses are characterised by a very small quantal size, with a change in conductance that is two orders of magnitude smaller than at the NMJ and is due to the opening of tens rather than thousands of postsynaptic receptors (but see calyx of Held; Borst *et al.*, 1995). Moreover

the distribution of miniature currents is found surprisingly skewed (Bekkers *et al.*, 1990; Bekkers & Stevens; 1995).

Modifications of the original quantal hypothesis have been developed. They take into account the possibility of a non-uniform probability of release as well as the existence of both intra and intersite quantal variability, i.e. the variability in the quantal size at individual releasing site and between different releasing sites, respectively (Redman, 1990; Korn & Faber, 1991; Frerking & Wilson, 1996).

2. The hippocampus

2.1 Anatomy of the hippocampus

The hippocampus is among the best characterised brain structures, mainly because its layered organisation (Andersen *et al.*, 1971) is particularly suitable for anatomical and physiological investigations, but also because, since the early 1950s, it has been recognised to play a fundamental role in some forms of learning and memory (Kandel, 2001).

The hippocampus is an elongated structure located on the medial wall of the lateral ventricle, whose longitudinal axis forms a semicircle around the thalamus. Due to its layered organisation through this axis, when the hippocampus is cut across its transverse axis (the septotemporal one), it is possible to identify a particular structure that is preserved in all slices taken with this orientation. The hippocampus *proper* and its neighbouring cortical regions, the dentate gyrus (DG), subiculum and enthorinal cortex, are collectively termed "hippocampal formation". As shown in Figure II, the hippocampus *proper* is divided in *stratum oriens* (1), *stratum pyramidale* (2), *stratum radiatum* (3) and *stratum lacunosum-moleculare* (4). Excitatory neurones (pyramidal cells) are arranged in a layer that forms the *stratum pyramidale*, traditionally divided in four regions CA1-CA4. In general, CA4 is considered the initial part of CA3 and the small CA2, which is indistinct in some species, is included in CA1. All pyramidal neurones bear basal dendrites that arborise and form the *stratum oriens* and apical dendrites that are radially oriented in the *stratum radiatum* and *lacunosum-moleculare*. In

the DG, granule cells represent the principal neurones, while the area between DG and the CA3 region is called the *hilus*.

2.2 The trisynaptic circuit

The main inputs to the hippocampus come from the enthorinal cortex, the septum and the contralateral hippocampus, whereas a unique unidirectional progression of excitatory pathways links each region of the hippocampus, creating a sort of trisynaptic circuit

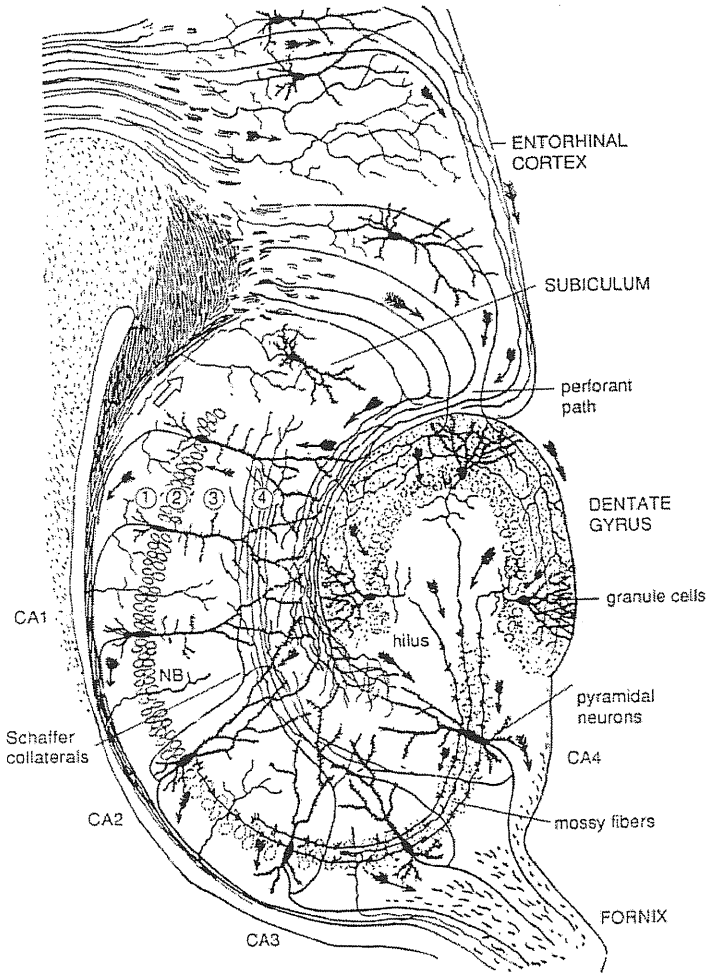

Figure II. Neuronal elements of the hippocampal formation. Labelled areas include the subiculum, part of the enthorinal cortex, the fornix, the dentate gyrus and the region CA1 to CA4. The hippocampus *proper* is divided into *stratum oriens* (1), *stratum pyramidal*e (2; cell bodies drawn as ovals), *stratum radiatum* (3) and *stratum lacunosum-moleculare* (4). (Modified from Ramon y Cajal, 1911)

(Figure II). The perforant path, originated from the enthorinal cortex, passes through the subicular complex and terminates mainly in the dentate gyrus, making synapses on granule cells. Then, the distinctive unmyelinated axons of the granule cells (mossy fibres) project to the *hilus* and to the *stratum lucidum* of the CA3 region. Here they make synapses *en passant* on CA3 pyramidal neurones showing the large, presynaptic varicosities typical of mossy fibres-CA3 contacts. These presynaptic expansions form a unique synaptic complex with equally intricate postsynaptic processes called *thorny excrescences* and may contain tens of releasing sites (Jonas *et al.*, 1993). Information is therefore transferred, through Schaffer collaterals, from CA3 to CA1 pyramidal neurones, which therefore send their axons to the subiculum and the deep layers of enthorinal cortex. Then, signal is sent back to many of the same cortical areas. Thus, information entering the enthorinal cortex from a particular cortical area can traverse the entire hippocampus and return to the cortical area from which it originated. The transformations that take place during this process are presumably essential for information storage (Johnston & Amaral, 1998). Furthermore, commissural associative fibres provide synaptic contacts between CA3 pyramidal neurones and between the two hippocampi, via the fornix. The recurrent connections between pyramidal neurones are particular of the CA3 region and are responsible for making this region quite unstable. The simultaneous activation of a certain percentage of these connections is sufficient for generating epileptiform activity, characterised by spontaneous, synchronised and rhythmic firing in a large number of neurones (Miles & Wong, 1986; Traub & Miles, 1991). This feature accounts for the selective generation of seizures in this region following the application of convulsive agents (Ben-Ari & Cossart, 2000).

The hippocampus is known to be crucial for certain forms of learning and memory, and the recurrent associative network formed by CA3 pyramidal cells have recently been shown to play an important role in associative memory recall (Nakazawa *et al.*, 2002).

A critical role in controlling the communication between pyramidal neurones, especially but not only in this case, is accomplished by local inhibitory interneurones and a balance is set between excitation and inhibition. In contrast to the rather uniform population of excitatory neurones, interneurones, which are distributed in the entire hippocampus, show a great variability and are classified in several families according to their morphology and

axonal and dendritic arborization (for a review see Freund & Buzsaki, 1996). Among all the differences in morphology and in active and passive membrane properties between neurones, a particular feature of pyramidal cells, known as accomodation, is the possibility to limit the maximal firing frequency upon injection of a depolarising current step. This property distinguishes pyramidal neurones from interneurones that, on the contrary, are able to fire up to 400 Hz (Lacaille, 1991).

2.3 Excitatory synaptic transmission in the hippocampus

Excitatory synaptic transmission in the hippocampus is mainly mediated by the action of glutamate on both ionotropic and metabotropic receptors (reviewed by Ozawa *et al.*, 1998). Three kinds of ionotropic glutamate receptors, all selective for cations but distinguishable on the basis of agonist specificity, are present in the central nervous system: NMDA, AMPA and kainate receptors.

2.3.1 NMDA receptors

NMDA receptors show very slow kinetics, with synaptic currents lasting tens or even hundreds of ms (Randall & Collingridge, 1992). They are characterised by a voltage-dependent block by extracellular Mg^{2+} and a high permeability to Ca^{2+}. Thus, NMDA receptors have the particular feature to work as "coincident detectors" as they allow ions flowing into the cell only if glutamate release occurs in coincidence with the depolarisation required for the relief from Mg^{2+} block. NMDA receptors are also blocked by competitive antagonists, such as 2-amino-5-phosphopentanoic acid (AP5) and 3-((R)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), and are sensitive to other endogenous ligands and modulators, such as Zn^{2+} and protons.

2.3.2 AMPA receptors

AMPA receptors show faster kinetics than NMDA receptors, with synaptic currents lasting a few ms (Ozawa *et al.*, 1998) and are voltage independent. AMPA receptors are homomeric or heteromeric tetramers assembled by four different subunits, named GluR1-4 (typical conductance <20 pS). *In situ* hybridisation studies have shown that all GluR1-3 mRNAs are abundantly expressed in the hippocampus, both in the *pyramidal layer* and in

the dentate gyrus. Expression of GluR4 mRNA is much less abundant and is relatively higher in CA1 and DG than in CA3.

AMPA receptors in most central neurones are Ca^{2+} impermeable.

2.3.3 Kainate receptors

AMPA and kainate receptors are often collectively classified as non-NMDA receptors. This is due to the absence of selective agonist or antagonist that, until few years ago, prevented from distinguishing between them. Thus, despite the epileptogenic action of kainate has been well known for decades, the real involvement of specific kainate receptors has only recently started to get clarified (reviewed in Lerma *et al.*, 2001). A particular interest is addressed to the hippocampus, and mainly to the CA3 region, where kainate receptors activation generates a seizure and brain-damage syndrome that provides an animal model for temporal-lobe epilepsy (Ben-Ari, 1985).

Kainate receptors are supposed to be homomeric or heteromeric tetramers formed by the assembling of five different subunits: GluR5-7, KA1 and KA2. GluR5-7 represent the low-affinity binding sites for kainate (K_d~50 nM), while KA1 and KA2 correspond to the high affinity ones (K_d~5 nM).

3. Modulation of synaptic efficacy

Modifications of synaptic efficacy are thought to be very important for the refinement of neural circuitry, information processing and storage. Thus, the possibility to find out the mechanisms and the sites of changes of synaptic strength is really important for understanding brain functions. At central synapses it is quite difficult to find appropriate experimental approaches and models for the application of quantal analysis. Easier approaches, such as the analysis of apparent transmission failures or *CV*, can be used to have an idea on whether pre and/or postsynaptic changes are responsible for particular modifications of synaptic efficacy (Katz, 1969).

Even if changes in Q and N may be involved in the modulation of synaptic strength and have always to be taken into account, probability of release seems to be a major factor that influences the pattern of transmitter release (Thomson, 2000). A strong variability in p has been found when comparing both synapses from different preparations and the same kind of synapses. p can vary from less than 0.01 at specific cortical connections (Thomson *et al.*, 1995) up to 0.9 at the calyx of Held (von Gersdorff *et al.*, 1997) and a similar range of variability has been detected in the CA1 region of the hippocampus (Dobrunz & Stevens, 1997). It is still difficult to define p in an unambiguous and precise way, as a lot of different processes are involved in transmitter release. This particularity can probably account for the high variability in p values as well as for the many opportunities in controlling and modulating it. In general, p represents the probability that Ca^{2+} entering the nerve terminal during action potential triggers the release of fusion-competent vesicles. Thus, we could imagine that different values of p are due to differences in local calcium affinity or binding at the level of Ca^{2+} sensors. Alternatively, the shape of the presynaptic action potential may be of fundamental importance for determining the strength of synapses, since the degree and the duration of depolarisation control the opening of voltage-gated Ca^{2+} channels, as well as the driving force for calcium influx itself. Therefore, also the number, location and properties of calcium channels are important in modulating the shape and the size of the Ca^{2+} transients themselves. On the other hand, a differential expression of proteins involved in release or differences in their phosphorylation could account for further variability in the

probability of release. The interplay between all these mechanisms determines p and a modification at any of these levels might affect synaptic strength (Thomson, 2000).

3.1 Short-term plasticity

Short-term plasticity refers to use-dependent synaptic changes that are restricted to brief periods of time (reviewed in Zucker & Regehr, 2002). These processes are crucial for regulating temporal coding and information processing between neurones in the brain (Tsodyks & Markram, 1997), where, in fact, information is conveyed by spike train rather than by isolated action potentials. These modifications vary from synapse to synapse and in the same synapse according to its previous history (Debanne *et al.*, 1996; Markram & Tsodyks, 1996). Thus, most synapses in the CNS, including neocortex and hippocampus, undergo dynamic bidirectional regulations of their efficacy following activity-dependent processes.

3.1.1 Facilitation, augmentation and potentiation

Increases in transmitter release by repeated stimulation fall into two categories: those that act over short interval (facilitation) and those that accumulate significantly during prolonged stimulation, augmentation and potentiation. These phenomena have been shown to be presynaptic in origin, with a strong correlation between elevation in $[Ca^{2+}]_i$ and enhancement of synaptic strength (Zucker & Regehr, 2002; Thomson, 2000). In particular, the "residual calcium hypothesis" has been suggested for explaining facilitation. According to this hypothesis, the small fraction of calcium remaining in the terminal after a conditioning pulse increases the probability of transmitter release to a second stimulus (Zucker, 1989). More complex mechanisms, involving Na^+ accumulation and intracellular buffering of Ca^{2+}, modulate the dynamics of Ca^{2+} removal upon brief and long trains of stimulation, thus accounting for augmentation and potentiation, respectively (Regehr, 1997). A particular form of short-term potentiation has been recently described at the mossy fibres boutons upon high frequency stimulation (Geiger & Jonas, 2000). In this case the cumulative inactivation of fast-inactivating potassium channels accounts for activity-dependent spike broadening, resulting in an increased influx of calcium and enhanced synaptic strength.

3.1.2 Short-term depression

Another common form of short-term plasticity lasting from seconds to minutes is depression upon repeated use (Thomson & Deuchars, 1994; Nelson & Turrigiano, 1998). This may provide a dynamic gain control over a variety of presynaptic afferent firing action potentials at different rates (Markram & Tsodyks, 1996). This form of plasticity is supposed to reflect mainly presynaptic depletion of the RRP of vesicles (Rosenmund & Stevens, 1996). However, many other mechanisms may be involved. Presynaptic mechanisms of depression may include a reduction in the Ca^{2+} influx as well as the adaptation of the Ca^{2+} sensor for release (Hsu *et al.*, 1996) or activity-dependent inactivation of the release machinery (Betz, 1970). Therefore changes in the action potential shape, calcium channels inactivation (Gingrich & Byrne, 1985) and calcium depletion from the synaptic cleft (Borst & Sakmann, 1999) may be involved. Synaptic depression may be due to inhibition via metabotropic receptors, activation of presynaptic ionotropic receptors (MacDermott *et al.*, 1999), release of retrograde messengers from the postsynaptic neurone (Zilberter *et al.*, 1999) or release-independent mechanisms such as propagation failures (Debanne *et al.*, 1997). Postsynaptic mechanisms of depression include mainly receptor desensitisation (Otis *et al.*, 1996), but also receptor saturation.

3.1.2 The interplay between facilitation and depression

Repeated use can either enhance or decrease synaptic efficacy, but in some cases multiple processes are present and the result is a combination of facilitation and depression. A particular example is obtained through the application of a paired-pulse protocol within a short time interval. In this case, the ratio between the mean amplitudes of the second response over the first one, known as paired-pulse ratio (PPR), is inversely related to the initial release probability. This means that either paired-pulse facilitation or paired-pulse depression can be observed in case of low or high release probability, respectively (Debanne *et al.*, 1996; Dobrunz & Stevens, 1997).

In case of high release probability, most of the vesicles will be released upon arrival of the first spike, thus leaving few ready to be released with the second. Hence PPD. On the

contrary, if the release probability is low, the dominant effect will be that of the residual calcium and will lead to facilitation.

3.2 Role of potassium conductances

Potassium channels constitute a large group of proteins that carry out a variety of different functions, including stabilisation of the resting membrane potential, repolarisation of the membrane after action potentials and regulation of the firing rate, both at the level of soma and nerve endings (Rudy, 1988; Storm, 1990; Roeper & Pongs, 1996). Therefore, presynaptic K^+ channels localised on nerve terminals, as a result of these different actions, play a fundamental role in modulating temporal coding and synaptic efficacy.

3.2.1 Potassium channels

The multiple roles that K^+ channels play in the nervous system suggest that a wide variety of these proteins might exist and might be differentially expressed according to their specific function. Indeed, over 80 related mammalian genes for subunits of K^+-selective channels have been revealed (Hille, 2001). Although they come in several architectural forms, they are characterised by some common features, such as the presence of a pore-lining P-loop with a consensus amino acid sequence, called the K^+-channel "signature sequence". These residues, repeated in all the principal subunits, line the selectivity filter. However, two transmembrane segments flanking the P-region are at least necessary for completing the pore-forming core. In addition to this two-transmembrane core, most of the subunits of potassium channels have four additional transmembrane regions that, in voltage-activated channels, account for the voltage sensitivity, mainly through the fourth transmembrane segment (S4). Fully assembled potassium channels are formed by homo or heteromeric tetramers of principal subunits (named α) and are often supplemented by accessory subunits, known as β-subunits, that mainly seem to affect the voltage sensitivity and inactivation properties of the channels (Jan & Jan, 1997).

The cloning and sequencing of many mammalian α subunits have shown that most of them belong to two main families, encoding for voltage-activated (Kv) or inwardly rectifying (Kir) K^+ channels. Kv channel α subunits fall into different subfamilies Kv1-

Kv9 according to their amino acid sequence similarity (Hille, 2001). Each of these subfamilies comprises several members, denoted Kvm.n thus indicating the subfamily (m) and the order of discovery (n). In particular, the four major subfamilies Kv1, Kv2, Kv3 and Kv4 are the diversified vertebrate homologous of the channels encoded by the four *Drosophila* genes previously introduced. However, according to this classification, channels that are fast inactivating, delayed rectifier and channels in between are found in the same subfamily. It is known that various α subunits belonging to the same subfamily can co-assemble, whereas those from different subunits cannot. The possibility of forming heteromultimeric channels and the existence of auxiliary β subunits account for the high variability and complexity of kinetics and pharmacology of K^+ currents (Rudy, 1988; Storm, 1990).

3.2.2 Potassium currents in hippocampal pyramidal cells

Hippocampal pyramidal cells represent a particular example of how multiple potassium currents can co-exist and function in central mammalian neurones (Storm, 1990). Six principal voltage or calcium activated outward potassium current have been described mainly in CA1 pyramidal neurones. Four of them (I_A, I_D, I_K, I_M) are activated by depolarisation, whereas, the two others (I_C, I_{AHP}) are activated by voltage-dependent influx of Ca^{2+}. I_A is a fast activating (10 ms) and fast inactivating (15-50 ms) current that may be activated at membrane potential positive to -60 mV. It contributes to action potential repolarisation and can be blocked by millimolar concentration of 4-AP (Rudy, 1988; Storm 1990). The "delay" current (I_D) activates rapidly (within 20 ms) but inactivates slowly (seconds), causing a long delay in the onset of firing, and is already active at rest (Storm, 1988). Due to its slow recovery from inactivation (seconds), it may "integrate" separate depolarising inputs. As I_A, also I_D participates in spike repolarisation, but, in contrast to I_A, it is selectively blocked by micromolar concentration of 4-AP (Storm, 1988). Differently, I_K activates slowly (20-60 ms) in response to depolarisation positive to -40 mV and inactivates slowly too (seconds). It participates in spike repolarisation and may be blocked by millimolar concentration of tetraetylammonium. I_M has the particular feature of being suppressed by acethylcoline and other muscarinic agonist (from which the name M-current; Brown & Adams, 1980). It activates slowly (50

ms), but it does not inactivate at all. Although it does not seem to contribute to the stabilisation of the resting potential as much as in ganglia, in the hippocampus I_M still tends to reset the membrane potential towards the resting level in response to long-lasting depolarisations. In particular, it is involved in the modulation of firing properties, accounting for the early phase of the characteristic accomodation of pyramidal neurones and the medium afterhyperpolarisation (AHP) following sustained firing. A similar role is accomplished by I_{AHP} that is slowly activated by Ca^{2+} influx during action potentials, causing spike-frequency adaptation and the slow AHP. Finally, I_C has the particular characteristic of being both Ca^{2+} and voltage-activated and is thought to account for the final part of the spike repolarisation.

Several neurotransmitters have been found to modify neuronal excitability by modulating the function of K^+ channels (Rudy, 1988). Beside the already mentioned suppression of I_M by muscarinic agonists, for example it has also been shown that I_{AHP} is reduced by norepinephrine, serotonin or histamine (Madison & Nicoll, 1982). As already stated in paragraph 2.3.4, a novel modulation of I_D by mGluRs of group I and II has been recently reported in cultured hippocampal neurones. A critical role in the modulation of potassium currents is also accomplished by intracellular Ca^{2+} itself, that is well known to activate I_{AHP} and I_C, but has also been shown to suppress, and eventually block, I_A current in hippocampal neurones (Chen & Wong, 1991).

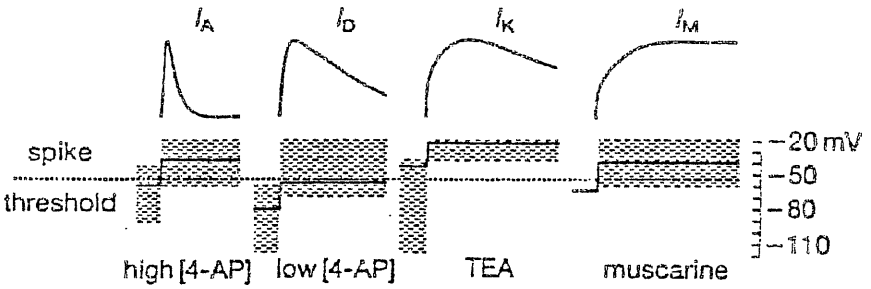

Figure III. Schematic representation of the four voltage-gated, Ca2+-independent K+ currents I_A, I_D, I_K, I_M. For each of these currents, the time course is roughly shown as drawing of idealised currents (top). The approximate voltage ranges of inactivation and activation (shaded areas) and typical voltage steps used to activate them (solid lines) are also indicated (middle). Effective blocking agents and their concentrations are listed on the bottom. (Modified from Storm, 1988)

3.2.3 BK channels

BK channels are very large conductance channels (of ~250 pS) activated by both calcium and voltage that regulate cell excitability (Vergara et al., 1998). They are widely expressed throughout the vertebrate nervous system (Hille, 2001; Knaus et al., 1996) where they are often co-localised with voltage-dependent calcium channels (VDCCs, Robitaille et al., 1993; Lancaster and Nicoll, 1987; Storm, 1987a; Marrion and Tavalin, 1998; Stanley, 1997). The interplay between these two channels is very tight and has very important functional consequences (see for instance in the cochlea, Roberts et al., 1990). Activated by membrane depolarisation during action potentials, BK channels tend to hyperpolarise the cell driving the membrane potential towards the equilibrium potential for potassium. By doing so they regulate cell excitability and contribute to action potential repolarization and spike-frequency adaptation (Adams et al., 1982; Lancaster and Nicoll, 1987; Storm, 1987b; Schwindt et al., 1988; Shao et al., 1999). Immunohistochemical and radioligand binding studies have revealed the presence of BK channels on neuronal soma, processes (but see Poolos and Johnston, 1999) and axon terminals of several brain structures including the hippocampus, where they are particularly abundant (Knaus et al., 1996; Wanner et al., 1999). Here, they have been localised in presynaptic nerve endings, on the membranes facing the synaptic cleft, at the calyx synapse isolated from the chick ciliary ganglion (Sun et al., 1999), and at Schaffer collateral-CA1 synapses (Hu et al., 2001). This presynaptic localisation suggests a role for BK channels in controlling transmitter release. Thus, by shaping presynaptic action potentials they would regulate calcium signals necessary to trigger fusion of synaptic vesicles, exocytosis and transmitter release (Sabatini and Regher, 1997; Sun et al., 1999). Indeed, BK channels have been shown to regulate secretion at neurosecretory nerve terminals (Petersen and Maruyama, 1984; Obaid et al., 1989; Dopico et al., 1999) and transmitter release at the frog neuromuscular junction (Robitaille and Charlton, 1992). In this preparation, block of BK channels with charybdotoxin produced a two-fold increase of transmitter release. This suggests that under physiological conditions BK channels diminish transmitter release by narrowing presynaptic action potentials and by reducing calcium entry into the cytosol (Robitaille and Charlton, 1992; Robitaille et al., 1993). In central neurones however, the role of BK channels in regulating transmitter release is still

uncertain and has been indirectly inferred from their action at the somatic level (Shao et al., 1999). It has been even hypothesised that, under basal conditions, BK channels targeted to active zones of presynaptic glutamatergic terminals do not exert any effect on transmitter release (Hu et al., 2001). They would provide an "emergence brake" only under conditions of excessive depolarisation and accumulation of intracellular calcium, such as brain ischemia and epilepsy (Hu et al., 2001; Runden-Pran et al., 2002).

3.2.4 Modulation of neurotransmitter release by potassium currents

As already mentioned at the beginning of this chapter, the shape of presynaptic action potential is of fundamental importance in determining transmitter release and synaptic efficacy. The duration of the depolarisation determines the calcium signal available to trigger fusion of synaptic vesicles with the plasma membrane, by controlling both the opening of voltage-activated Ca^{2+} channels and the driving force for Ca^{2+} itself (Augustine, 1990; Sabatini & Regher, 1997). Although modifications of presynaptic action potential have been shown to modulate neurotransmitter release in many systems (Klein & Kandel, 1980; Llinas *et al.* 1981; Augustine, 1990; Sabatini & Regehr, 1997; Geiger & Jonas, 2000), most of our knowledge comes from the giant synapse of the squid (Katz & Miledi, 1967; Augustine 1990) and much remains to be understood about the coupling between the presynaptic waveform and release. In fact, the enhancement produced by spike broadening depends on the properties of presynaptic calcium channels and the calcium sensitivity of the release machinery. Spike broadening causes the same percentage increase in calcium influx and in the amplitude of postsynaptic responses supporting the idea that calcium entering through one single channel triggers vesicle fusion at each individual site (Augustine, 1990). Thus, action potential broadening increases neurotransmitter release primarily by opening more calcium channels. On the contrary, a different arrangement for central synapse has been suggested by different studies. They propose that the distance that Ca^{2+} ions must cover to reach the calcium sensor is relatively long and therefore Ca^{2+} entry through multiple channels is needed to release a single vesicle (Wu & Saggau, 1994; Borst & Sakmann, 1996).

As stated in the previous paragraph, several K^+ channels are involved in shaping the action potential and are therefore suggested to participate in the modulation of synaptic

efficacy. As the spike shape may change between soma and terminals (Geiger & Jonas, 2000), a proper estimate of electrical events that precede transmitter release should be based on direct recordings from presynaptic elements. As a direct examination of mammalian central synapses is hampered by the small size of presynaptic nerve endings, much of our present knowledge on the functional correlation between changes in spike shaping and transmitter release comes from studies on invertebrate models.

In the last decades, some groups have succeeded in performing direct recordings from central nerve endings, few of them focusing on the specific role of presynaptic K^+ currents in modulating action potential shape and, consequently, Ca^{2+} influx and neurotransmitter release. Indeed, the first report of $[Ca^{2+}]_i$ signals from individual vertebrate nerve endings was performed on pituitary nerve terminals during high frequency stimulation (Jackson *et al.*, 1991). K^+ channels inactivation was found responsible for the observed increase in spike duration and the consecutive enhancement of AP-induced changes in $[Ca^{2+}]_i$. Similarly, patch-clamp recordings from mossy fibre boutons in rat hippocampal slices have allowed the characterisation of voltage-gated rapidly inactivating potassium channels that, during repetitive activity, accumulate in the inactivating state, thus leading to spike broadening, increase in Ca^{2+} influx and enhancement of synaptic efficacy (Geiger & Jonas, 2000). Also at the calyx of Held synapse in auditory brainstem, that offers unique advantages because of the extraordinary size of its presynaptic terminals, a rapidly activating delayed rectifier K^+ current has been found involved in the modulation of presynaptic waveform (Forsythe, 1994). Namely, it allows the occurrence of fast action potentials with high firing rate, a feature that is crucial for the rapid and high-fidelity transmission typical of auditory system. Broadening the presynaptic waveform, by blocking K^+ channels, was found to prolong the calcium current, without affecting significantly its peak. This increase in Ca^{2+} influx in turn enhanced EPSCs amplitude in a supralinear way.

AIM OF THE STUDY

It has been demonstrated that BK channels regulate secretion and transmitter release at neurosecretory nerve terminals and at the neuromuscular junction. However the functional role of these channels in central neurones is still uncertain. It has been proposed that in the hippocampus BK channels act as an "emergency brake" that would control transmitter release only under conditions of excessive depolarisation and accumulation of intracellular calcium, such as during ischemia. Aim of the present work was to understand if, under basal experimental conditions, BK channels localized on nerve terminals can modulate glutamate release in the hippocampus. To study this we have used the selective BK channel blockers paxilline and iberiotoxin and test their effect on spontaneously occurring glutamatergic excitatory postsynaptic currents (EPSC) in the CA3 region of hippocampal slice cultures. Moreover, using double patch recordings from pairs of interconnected CA3-CA3 neurons, the role of BK channels on evoked glutamate release was also tested. In particular, the effect of paxilline on successes rate, EPSC amplitude and paired pulse ratio was assessed.

These experiments clearly demonstrate that in normal conditions BK channels can modulate transmitter release and synaptic efficacy in central neurones by changing the shape of the spike.

METHODS

"Scorrete lacrime, disse il poliziotto"
P.K. Dick

1. Organotypic hippocampal slice cultures

Experiments were performed on organotypic hippocampal slice cultures as this preparation offers a lot of advantages for studying synaptic transmission and plasticity. In fact, more convincing results on these topics can be obtained when recording from monosynaptically interconnected neurones. It is known that in acute hippocampal slices the probability of connectivity between excitatory neurones is rather low (less than 5% in adult rat; Miles, 1990) and therefore it is very hard to find pairs of interconnected cells. On the contrary, in organotypic hippocampal slices, where the cytoarchitecture of the tissue of origin is properly maintained, many connections are regenerated with time. Thus, 56% of CA3 pyramidal neurones and 76% of CA3-CA1 pyramidal cells are found connected after 2-4 weeks in vitro (Debanne et al., 1995). In view of this high degree of connectivity, hippocampal slice cultures offer a unique opportunity to study mechanisms involved in the modulation of synaptic efficacy.

1.1 Preparation of organotypic hippocampal slice cultures

For the present work, organotypic hippocampal slice cultures were prepared using the roller-tube technique (Gähwiler, 1981). The main steps of the preparation, carried out under a laminar flow hood in sterile conditions, are described below (Figure IV), while the properties of hippocampal slice cultures are discussed in paragraph 2.2.

1.1.1 Dissection

Young rats, between P4 and P7, were killed by decapitation. Brain was quickly removed from the skull and placed in a Petri dish, containing few drops of Gey's balanced salt solution (GBSS, Gibco) enriched with D-glucose (5 mg/ml) and kynurenic acid (1 mM). Hippocampi were carefully isolated and transverse 400 µm thick slices were cut with a tissue chopper (McIlwain). The sections were therefore placed in a second Petri dish containing the same dissection medium and were properly separated under the

microscope (Olympus SZ40). The best slices were therefore selected and stored in a new Petri dish at +4°C.

D-glucose was dissolved at 50% in sterile distilled water (Gibco). Kynurenic acid was dissolved 100 mM in alkaline solution.

1.1.2 Embedding

After recovering for one hour at +4°C, single slices were placed in a drop (20 µl) of reconstituted chicken plasma (Cocalico) on a glass coverslip. To fix the slice, the plasma was coagulated by addition of 30 µl of thrombin (Merck). Coagulation took 30-45 min to occur. Then the coverslips were placed into tubes (Nunc).

Prior to be used, glass coverslips (12x24 mm^2, thickness 1 mm, Vitromed) had to be specially treated. They were inserted in a teflon holder, submerged in HCl 0.5 N for 24 h, repetitively washed with distilled water, and soaked for 30 min in absolute alcohol. After further repetitive washes in distilled water, coverslips were then dried and sterilised al +150°C in an oven overnight.

Lyophilised chicken plasma was diluted in distilled water and then centrifuged for 20 min at +4°C at 3500 rpm. Lyophilised thrombin was diluted in distilled water (200 u/ml).

1.1.3 Feeding of cultures and incubation

The coverslips bearing the slices were transferred to plastic tubes containing 0.75 ml of nutrient medium. The medium consists of 50% Basal medium (BME, Eagle, with Hanks's Salts, without L-Glutamine; Gibco), 25% Hanks' Balanced Salt Solution (HBSS; Gibco), 25% Horse Serum (Gibco). L-glutamine (Gibco, 1 mM) and D-glucose (5 mg/ml) were also added (osmolarity 310 mOsm, pH 7.4). The tubes were placed in a roller drum (6 revolutions h^{-1}, 5° tilted) located inside an incubator at 36°C, without any control in CO_2 and O_2 concentration. Through rotation, cultures were submerged in medium for half a turn and covered by a film of medium for the other half. This was essential for feeding, aeration and flattening of the cultures. The medium was replaced with fresh one after the first two days *in vitro* and, thereafter, once a week.

After at least 10-14 days *in vitro* (DIV), an individual coverslip was transferred to a particular recording chamber for electrophysiological experiments.

1.2 Properties of organotypic hippocampal slices

Organotypic hippocampal slice cultures have been widely studied to determine whether the properties of the tissue of origin were maintained and to compare them with those observed in living tissue *in situ* (reviewed in Gähwiler *et al.*, 1997). During the preparation of hippocampal slice cultures, afferent fibres are cut and therefore degenerate. This process might be supposed to account for synaptic rearrangement, but only few changes of this kind, similar to those obtained in lesion studies, have been observed only at the level of the DG.

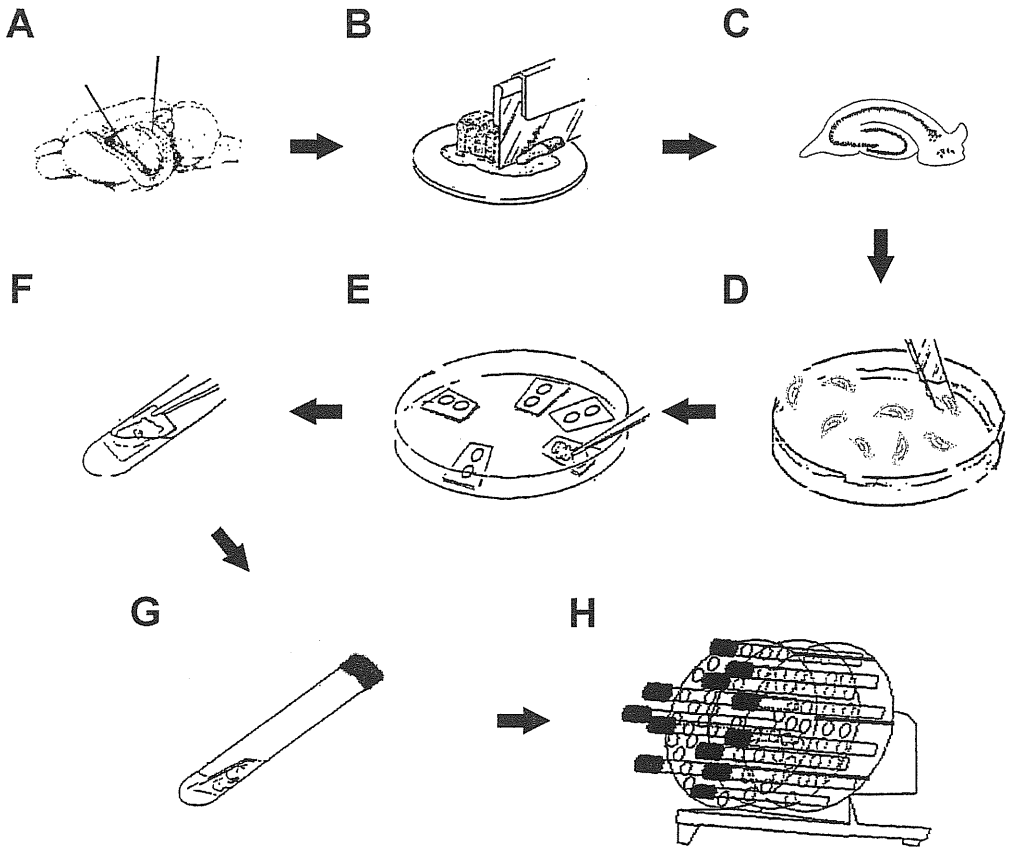

Figure IV. Preparation of organotypic hippocampal slice cultures. A) Following brain removal from the skull, hippocampi were carefully isolated. B) Transverse slices were obtained by means of a tissue chopper. C-D) After proper separation and selection, hippocampal slices were stored at +4°C in Petri dishes containing dissection medium, for recovery. E) Slices were attached to coverslips in a film of reconstituted chicken plasma clotted with thrombin. F-G) Coverslips with slices were transferred in plastic tubes containing nutrient medium. H) Tubes were inserted in the roller drum inside the incubator.

All nerve and glial cell types survive and the phenotypic morphology of neuronal types and the "gross" tissue organisation are similar to those *in situ.* Immediately after dissection, the density of synapses decreases, but then the development is comparable to synaptogenesis *in situ.*

Pyramidal cells show normal synaptic transmission and both short-term and long-term changes in synaptic strength occur, despite the absence of extra-hippocampal afferent. No loss of neurotransmitter receptors or major alterations of the voltage or ligand-gated membrane currents were found. Finally, the degree of connectivity between cells increases with time in cultures, up to at least ten times that reported for acute slices.

2. Identification of neurones

2.1 Staining with Cresyl violet

Some organotypic slices were stained with Cresyl violet in order to look at developmental changes in their structure. Cresyl violet binds to nucleic acid thus revealing nuclei, nucleoli, and cytoplasmic Nissl substance (cytoplasmic ribonucleoprotein) of neurones. It allowed *pyramidal layer* and DG at different stages of development to be distinguished (Figure V). Slices were fixed in 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) and kept at room temperature for 1 h. After few rinses in PBS, they could be stored in the same buffer at +4°C for few days. Before staining, they were washed twice for 10 min in distilled water. Then they were incubated in 0.5% of Cresyl violet (Sigma) for 10 min and rinsed twice briefly in distilled water. Slices were dehydrated by immersion in solutions containing an increasing concentration of ethanol up to 100%, then further soaked in xylene-ethanol 50% and xylene 100% and finally mounted on coverslips with Eukitt mounting medium (O. Kindler Gmbh & CO).

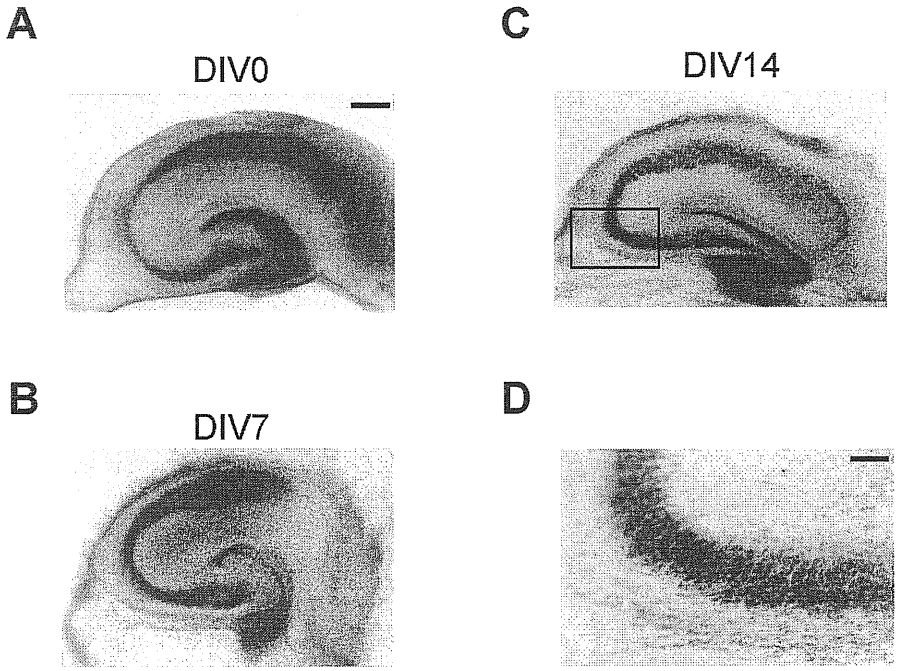

Figure V. Organotypic hippocampal slice cultures at different days in culture (cresyl violet staining). A) An hippocampal slice was stained the day of culture preparation (DIV0). **B)** After one week *in vitro* the flattening of the culture could be already observed. **C-D)** Experiments were performed after about 2 weeks *in vitro* from CA3 pyramidal neurones (calibration bar is 400 or 100 μm in A-C or D, respectively). The part of the CA3 region included in the black rectangle in C is shown at higher magnification in D.

2.2 Biocytin injection

Biocytin injection was often used to confirm the identity of cells as pyramidal neurones (Figure VI). Biocytin (0.2-0.3 %; Sigma) was added to the intracellular solution just before use and injected into the cell during electrophysiological recordings. Then, the slices were fixed and stored as described in the previous paragraph. To label biocytin-injected cells, either the avidin-biotinilated alkaline phosphatase or the avidin-biotinilated horseradish peroxidase complex (ABC, Vector Laboratories) was used.

In the first case, after washing slices in PBS for 5 min, membranes were permeabilised by incubation in PBS 0.1% Triton (Sigma) for 30 min. After three washes in PBS (5 min each), slices were incubated in ABC kit (1:100 in PBS) for 40 min and washed again in PBS for three times (5 min each). Slices were then incubated in DIG developing buffer (100 mM TRIS-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 1 mM

levamisolhydrochloride) for 10 min. The staining reaction was developed by incubation of slices in new DIG developing buffer where p-nitro blue tetrazolium chloride (NBT; 0.5 mg/ml, Sigma) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP; 0.25 mg/ml, Sigma) were added. The reaction was stopped with P4DIG buffer containing (in mM): Tris-HCl 10 (pH 8), EDTA 1 (pH 8).

When the avidin-biotinilated horseradish peroxidase complex was used, slices were first washed in PBS for 5 min, then immersed in 1% H_2O_2 in 10% methanol for 10 min. After five washes in PBS (3 min each), membranes were permeabilised by incubation in PBS + 2% Triton (Sigma) for 1 h. Slices were incubated in ABC kit (1:100 in PBS) for 40 min and washed again in PBS for three times (5 min each). Slices were then incubated in 3,3'-diaminobenzidine (DAB) solution containing 0.03% $CoCl_2$, 0.02% nickel ammonium sulphate and 0.001 % H_2O_2. Once the cells were identified, the reaction was stopped by washing the slices in PBS three times (10 min each).

In both cases, after some washes in distilled water, slices were mounted on slides as previously described.

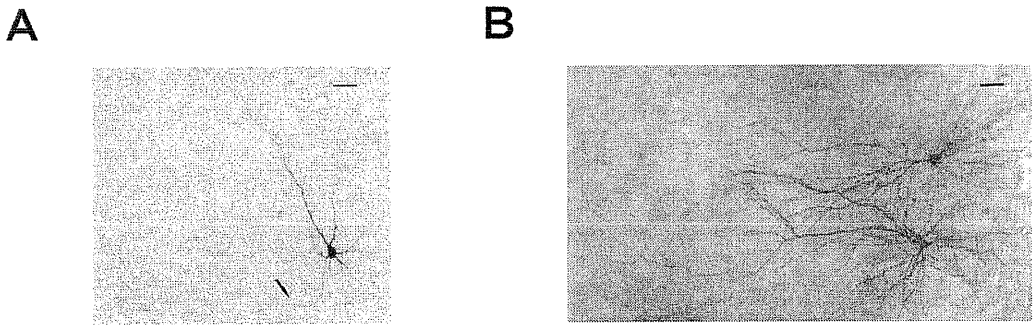

Figure VI. Biocytin injected neurones. A) A CA3 pyramidal neurone was injected with biocytin during electrophysiological recording (alkaline phosphatase complex). Note the presence of a prominent apical dendrite and shorter basal dendrites. The thin process departing from the left side of the cell body might be the axon (see arrow). **B)** Pair of interconnected CA3 neurones labeled with biocytin (horseradish peroxidase complex). In this case, at the end of the experiment the slice was placed again in the incubator to allow a further diffusion of biocytin in the cells. In this way, a very high number of processes could be stained. (Calibration bar is 50 μm in A-B).

3 Electrophysiological recordings

After 10-14 days *in vitro* the cultures, which had flattened near monolayer thickness, were transferred to a recording chamber fixed to the stage of an upright microscope.

Cultured slices in the recording chamber were superfused at room temperature (22-24°C) with a bath solution containing (in mM): NaCl 150, KCl 3, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose 10 (pH 7.3, adjusted with NaOH). A low concentration of tetrodotoxin (TTX, 10 nM; Affinity Research Products, Nottingham, U.K.) was added in order to reduce polysynaptic activity. Electrophysiological experiments were performed on CA3 pyramidal cells using the whole-cell configuration of the patch-clamp technique in current or voltage-clamp mode. CA3 neurones were identified both visually (using infrared differential interference contrast video microscopy) and on the basis of their firing properties, i.e. their ability to accommodate in response to long (800 ms) depolarising current pulses. The identity of cells as pyramidal neurones was confirmed in some experiments in which cells were labelled with biocytin (0.2-0.3 %; purchased from Sigma, Milan, Italy; see Figure 3). Patch electrodes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany). They had a resistance of 3-6 MΩ when filled with an intracellular solution containing (in mM): $KMeSO_4$ 135 (125 when adding 10 mM BAPTA), KCl 10, HEPES 10, $MgCl_2$ 1, Na_2ATP 2, Na_2GTP 0.4; pH was adjusted to 7.3 with KOH. Single or pairs of action potentials (50 ms interval) were evoked in current-clamp mode by short (5 ms) depolarising current pulses at 0.05 Hz. Spontaneous or evoked EPSCs were recorded from the postsynaptic neurones, loaded with the calcium chelator BAPTA (10 mM) in order to block activation of BK channels. EPSCs were detected in voltage-clamp mode at the holding potential of -60 mV. Under our experimental conditions the reversal potential for Cl^- was–66 mV. Membrane potential values were not corrected for the liquid junction potential of 9 mV.

Stock solution of the BK channel blockers iberiotoxin (from Latoxan, Valence, France) and paxilline (from Sigma-Aldrich, Milan, Italy) were obtained by dissolving the drugs in water or dimethylsulphoxide (DMSO) and applied at the final concentration of 100 nM and 10 μM, respectively. The final concentration of DMSO in the working solution was 0.1% (v/v). At this concentration, DMSO alone did not modify the shape of action potentials or the kinetics properties of EPSCs.

4 Data acquisition and analysis

Data were stored on a magnetic tape and transferred to a computer after digitisation with an A/D converter (Digidata 1322, Axon Instruments, Foster City, CA). Data acquisition was done using pClamp 8.2 (Axon Instruments, Foster City, CA). Data were sampled at 20-100 kHz and filtered with a cut off frequency of 1 kHz. Series resistance compensation was used for current-clamp recordings. Membrane input resistance was calculated by measuring the amplitude of voltage responses to steady hyperpolarising current steps of 100-200 pA.

Spontaneous EPSCs were analysed with the AxoGraph 4.6 program (Axon Instruments, Foster City, CA), which uses a detection algorithm based on the minimisation of the sum of squared errors between data and a template function approximating the width and the time-course of a typical synaptic event as described by Clements and Bekkers (1997). Miniature EPSCs were recorded in the presence of TTX (1 μM) at the holding potential of –60 mV, which is close to the reversal for Cl^-.

Evoked EPSCs were analysed with Clampfit software and transmission failures were identified visually. The onset of the EPSC was given by the intersection of a line through the 10 and 90% of EPSC rise time with the baseline. Onset, rise and decay times were calculated after averaging only the successes. EPSC latency was calculated as the time gap between the onset of the mean EPSC and the peak of the presynaptic spike. Mean EPSC amplitude was obtained by averaging successes and failures. Paired-pulse ratio (PPR) was calculated as the ratio between the mean amplitude of EPSC2 over EPSC1.

Action potentials were analysed with Clampfit software. They were characterised by their firing threshold, their amplitude (from threshold to peak) and their width at the threshold level.

Values are given as mean ± SEM. Significance of differences was assessed by Student's *t*-test or Wilcoxon test. The differences were considered significant when P was <0.05.

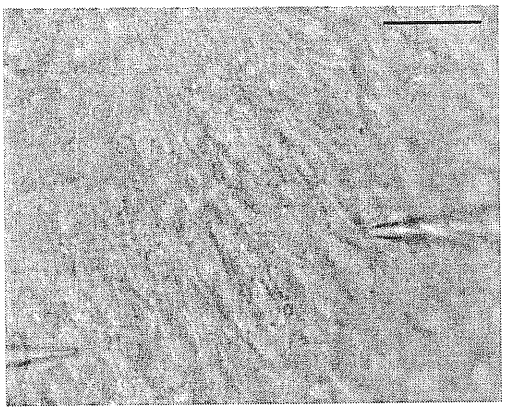

Figure VII. Visual identification of neurones. Pyramidal neurones were visually identified using an infrared differential interference contrast video microscope. The picture, acquired by frame grabber (ATI Technologies), shows an example of paired recording from CA3 neurones in organotypic slice. (Calibration bar is 50 μm)

RESULTS

"Cancelliere, metta a verbale: 1'14'' e 4"
Febbre da cavallo

1. BK channels contribute to action potential repolarisation in CA3 neurones

Patch-clamp recordings, in whole-cell configuration and current-clamp mode, were performed from CA3 pyramidal neurones in organotypic hippocampal slice cultures. These neurones were identified as principal cells both visually and on the basis of their firing properties, i.e. their ability to accommodate in response to long depolarising current pulses. In some experiments (n=16), cells were morphologically identified as pyramidal neurones by biocytin injection (see methods).

Action potentials were induced by the injection of short depolarising current steps from the resting membrane potential. The impact of BK channels on action potentials was examined by applying paxilline, a tremorgenic indole alkaloid that selectively blocks these channels (Knaus et al., 1994). As shown in Figure VIIIA and B, application of paxilline (10 μM) significantly broadened the action potential and induced the

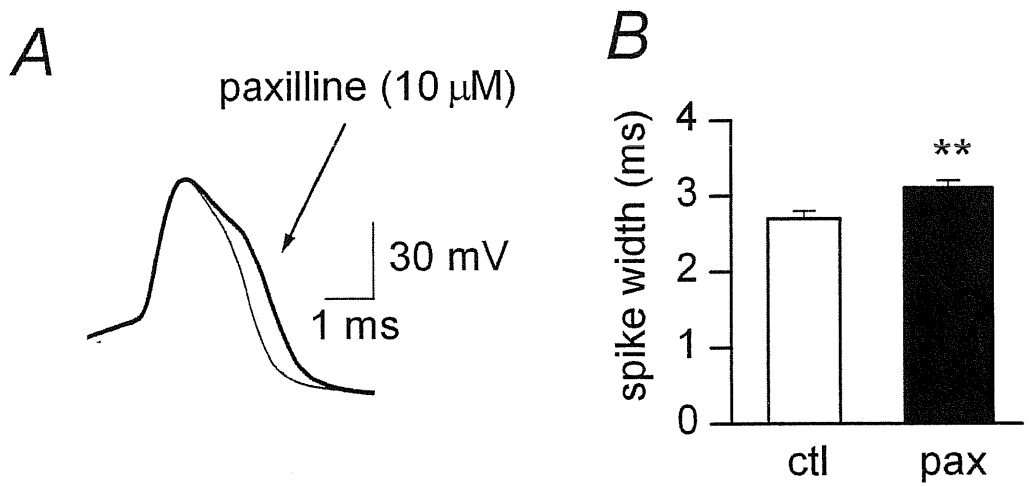

Figure VIII. BK channels are involved in action potential repolarisation
A. Action potentials generated under control conditions (thin line) and in the presence of paxilline (thick line) are aligned at threshold and superimposed. B. Mean changes in spike width before (white column) and during paxilline application (black column; n=10). ± 0.1 ms, n=12, P<0.001). These data indicate that BK channels exert a strong control on spike repolarisation. Paxilline also slightly, but significantly decreased spike amplitude (from 70 ± 3 to 66 ± 3 mV, n=11, P<0.05).

development of a shoulder (on average spike duration increased from 2.7 ± 0.1 ms to 3.1 In CA1 pyramidal neurones, fast inactivation of a transient BK channel-mediated current substantially contributes to frequency-dependent spike broadening (Shao et al., 1999). In order to see whether this occurred also in our preparation, bursts of five consecutive action potentials were generated at the frequency of 50 Hz. Spike duration increased by 4 ± 2 % from the first to the second spike, but less for the last three consecutive spikes (n=3). The involvement of BK channels in spike broadening was tested by blocking their activity with paxilline. Interestingly, paxilline (10 μM) broadened the first two spikes (by10 ± 5 % and 7 ± 4%, respectively; Figure IX,C-D), but had only a small effect on the

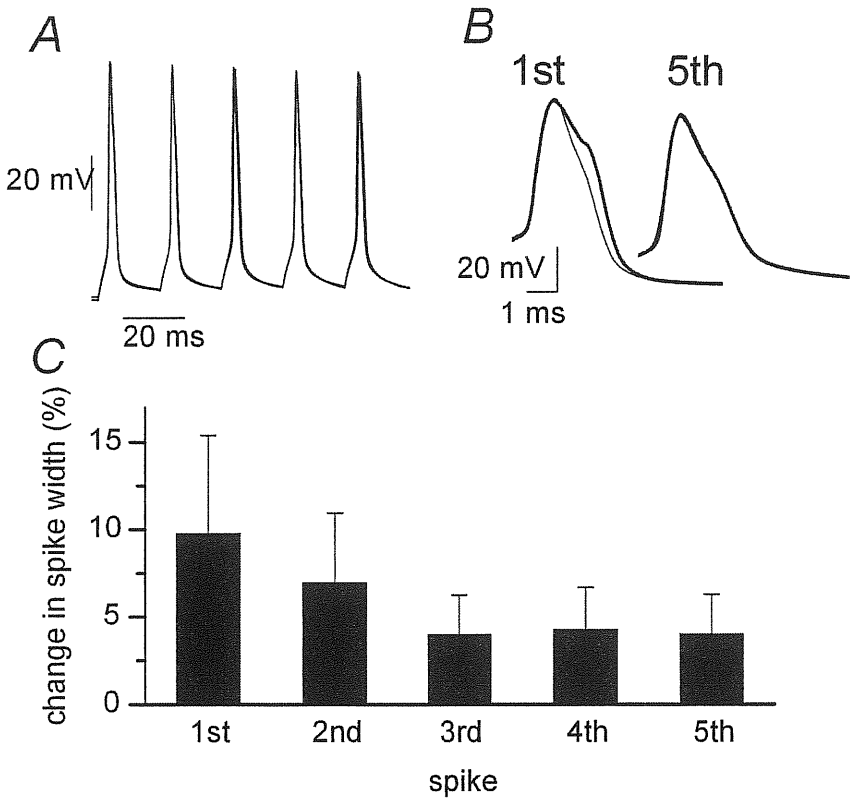

Figure IX. Trains of action potentials at 50 Hz.
A. Two bursts of five consecutive action potentials generated by brief depolarising current pulses (5ms duration each delivered at 50 Hz) under control conditions and in the presence of paxilline (10 μM) are superimposed. B. The first and the fifth spike in the train before (thin line) and during paxilline (thick line) are superimposed. C. Mean changes in spike duration (as percentage of controls) obtained in the presence of paxilline (10 μM) during repetitive firing (n=3). Note that paxilline clearly broadened only the first two action potentials. ** P<0.001.

last three (data from 3 experiments are shown in Figure IX,E). Paxilline-induced modifications of the first spikes were similar to those obtained under control conditions with development of a shoulder during the repolarising phase of the action potential.
In agreement with a previous study on CA1 pyramidal cells (Shao et al., 1999), the present results clearly show that BK channels participate in action potential repolarisation, but in CA3 neurones their role is less pronounced during repetitive firing.

2. BK channels modulate spontaneous release of glutamate

To evaluate the possibility that BK channels localised on presynaptic nerve endings may control transmitter release, spontaneous action potential-dependent EPSCs were recorded from CA3 pyramidal neurones before and after application of selective BK channel blockers. Application of the non-NMDA receptor antagonist CNQX (10 μM) completely blocked spontaneously occurring synaptic events indicating that they were mediated by non-NMDA receptors (not shown). In a first set of experiments BK channels were selectively blocked with iberiotoxin, a toxin from the scorpion Buthulus tamulus (Galvez et al. 1990; Candia et al. 1992) known to block BK channels and increase transmitter release at the neuromuscular junction (Robitaille et al., 1993). As shown in the representative traces of Figure XA, iberiotoxin (100 nM) increased the occurrence of spontaneous events but did not modify their amplitude. This is shown by the cumulative distribution plots of Figure XB and XC, where a clear shift to the left of the inter-event intervals but not of the amplitude curves is seen. Overall in seven neurones iberiotoxin significantly reduced the inter-event interval from 1.2 ± 0.2 s to 0.8 ± 0.1 s ($P<0.05$) without modifying current amplitude (23 ± 5 pA and 23 ± 7 pA in control and iberiotoxin, respectively) or EPSC kinetics (the rise time was 2.1 ± 0.3 ms under both conditions; Figure X,D). In another set of experiments, BK channels were selectively blocked with paxilline (10 μM). As shown in the summary data of Figure X,D, paxilline significantly reduced the inter-event interval from 1.1 ± 0.2 s to 0.9 ± 0.2 s ($n=7$; $P<0.05$). Again, in the presence of paxilline no significant changes in EPSC amplitude (24 ± 3 pA and 21 ± 2 pA, in control and in the presence of paxilline, respectively) or rise time (2.2 ± 0.3 ms and 2.3 ± 0.3 ms, in control and paxilline, respectively) were detected (Figure X,D). The significant reduction in inter-event- interval duration after treatment with

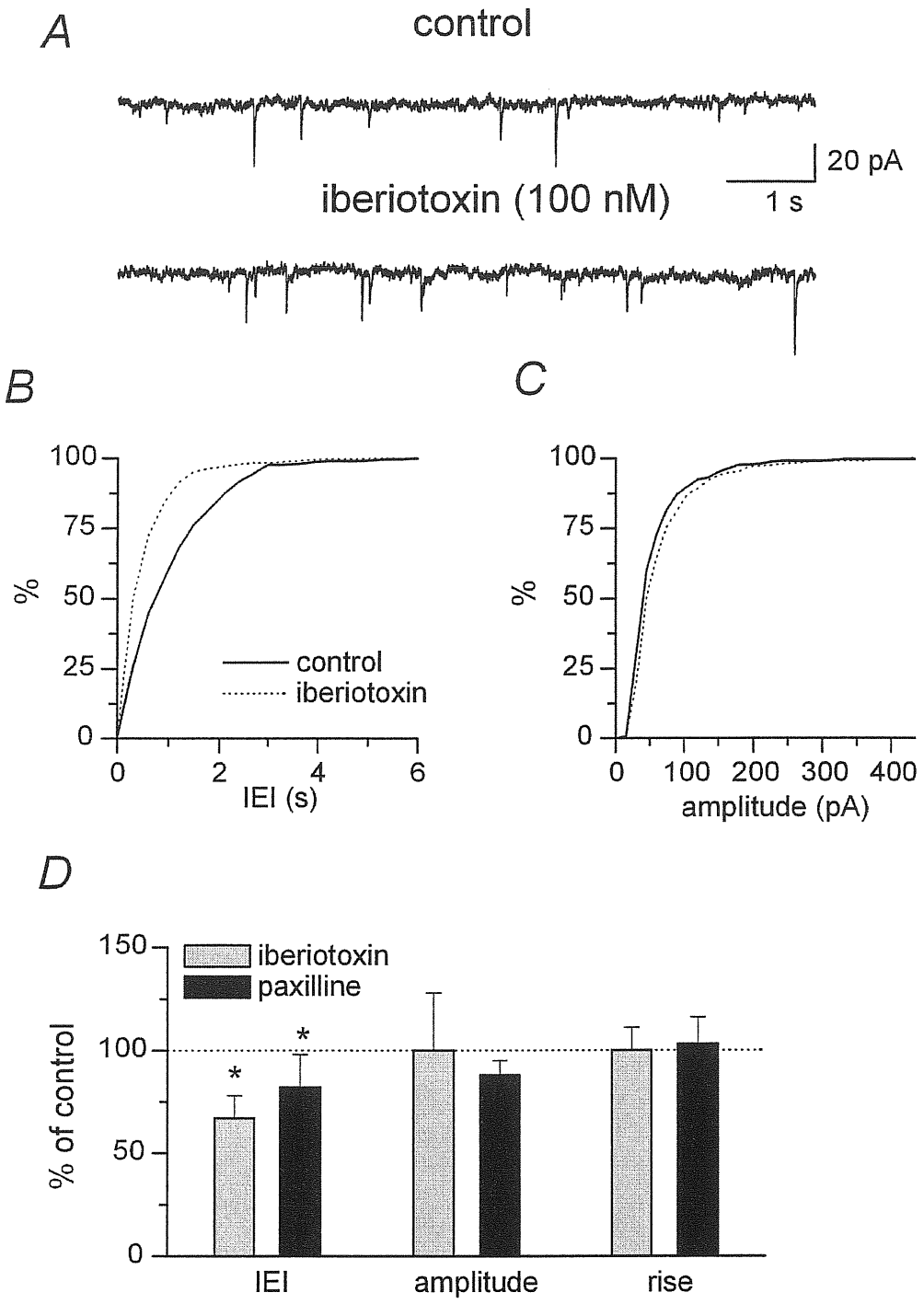

Figure X. BK channels increase the frequency but not the amplitude of spontaneous action-potential dependent EPSCs

A. Traces showing spontaneous EPSCs recorded from a CA3 pyramidal neurone at the holding potential of –60 mV under control conditions and in the presence of iberiotoxin (100 nM). B-C. Cumulative inter-event-interval (B) and amplitude distribution (C) of spontaneous EPSCs (same neurone shown in A) under control conditions (continuous line) and during iberiotoxin application (dotted line). Bin size was 0.3 s in B and 15 pA in C. D. Mean changes of inter-event interval (IEI), amplitude and rise time, as compared to control (dotted line) during application of iberiotoxin (n=7) or paxilline (n=7). *P<0.05.

iberiotoxin and paxilline suggests that BK channels are involved in the modulation of transmitter release. In order to elucidate whether the observed effects depended on action potential broadening following BK channels block with iberiotoxin and paxilline or to changes in the release machinery downstream to calcium entry, additional experiments (n=3) were performed in the presence of TTX (1μM) in order to record miniature EPSCs. In line with the occurrence of BK activation during action potentials, no significant change in the mean inter-event interval of mini EPSCs was noticed (1.49 ± 0.04 s and 1.5 ± 0.2 s, in control and paxilline, respectively; data not shown).

3. BK channels modulate the evoked release of glutamate

To further investigate whether BK channels modulate neurotransmitter release, double patch-clamp recordings were performed from monosynaptically interconnected CA3 pyramidal neurones. Evidence for monosynaptic connections between neurones was given by the short latency of evoked EPSCs (3.3 ± 0.7ms, n=6; see also Debanne et al., 1995). Seven different pairs of neurones were studied under control conditions and in the presence of paxilline. Usually, pairs of presynaptic action potentials (50 ms apart), delivered at the frequency of 0.05 Hz, evoked two sequential EPSCs that fluctuated in amplitude from trial to trial, with occasional transmission failures. The example of Figure XI shows a low probability synapse in which, under control conditions, the first spike evoked few small amplitude EPSCs (characterised by a first peak marked by an arrow, followed by a second one occurring with a longer delay), associated with response failures. This kind of response is not rare in recordings from CA3-CA3 connections and may reflect concomitant activation of a monosynaptic and disynaptic pathway (Debanne et al., 1995). Due to paired-pulse facilitation (PPF), which largely depends on presynaptic increase in release probability (Zucker, 1989), responses to the second spike were larger and associated with less transmitter failures. Addition of paxilline (10 μM) produced a clear potentiation of the synaptic responses. It increased the success rate to both first and second spike (from 17 to 58 % and from 44 to 85 %, respectively) and the amplitude of individual EPSCs (from 1.9 to 6.57 pA and from 4.4 to 9.1 pA for the first and the second EPSC, respectively; Figure XI). As expected from an enhanced release probability due to

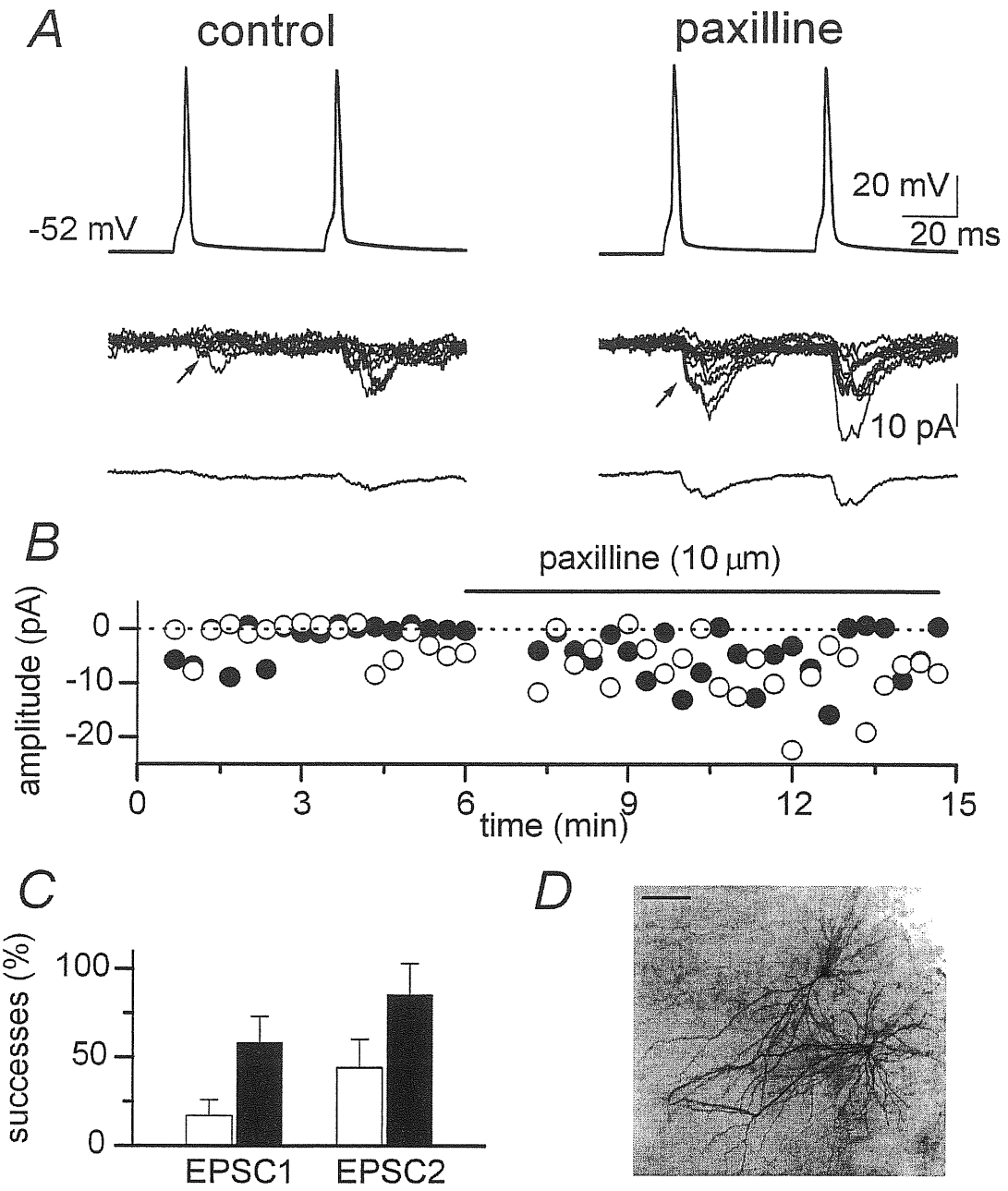

Figure XI. Blocking BK channels with paxilline increases synaptic efficacy at low probability CA3-CA3 connections

Pairs of action potentials are generated (50 ms intervals, 0.05 Hz) in the presynaptic cell (upper traces) while EPSCs are recorded from the postsynaptic cell in control (left) and in the presence of paxilline (10 µM, right). Eight traces are superimposed and shown in the middle, while the average of all responses (successes plus failures) is shown at the bottom. Note reduced failure rate and increased amplitude of successes after paxilline B. Time course of the peak amplitude of the first (closed circles) and second (open circles) EPSCs recorded from the cell shown in A. C. Mean percentage of successes in response to the first and second spike for the cell shown in A and B in control (white column) and during paxilline application (black columns). Bars represent the SEM. D. A pair of interconnected cells labelled with biocytin.

an increased calcium entry in presynaptic terminals, in the presence of paxilline a reduction of PPR was observed (from 2.3 to 1.4, see the average responses of Figure XIIIA).

Figure XII illustrates a high probability CA3-CA3 synapse that under control conditions showed only successes both to the first and second spike. This type of synapse was found in two out of seven cases. In line with a high probability of release, these synapses did not exhibit paired pulse facilitation (see average traces of Figure XIIA). Application of paxilline (10 μM) increased the peak amplitude of the responses to the first action potential (from 118 to 142 pA) and produced paired pulse depression (PPR changed from 0.96 to 0.77), suggesting that also in the case of highly reliable synapses, block of BK channels is able to increase transmitter release. Overall, as shown in the summary data of Figure XIII A-C, paxilline significantly increased the percentage of successes to the first and second responses (from 75 ± 10 % and 81 ± 8 % to 87 ± 6 % and 95 ± 7 %, for the first and second EPSC, respectively; $P<0.05$), significantly reduced the paired pulse ratio (from 1.26 ± 0.2 to 1.03 ± 0.1; $P<0.05$) and increased the mean peak amplitude of the first and second EPSCs (by 50 % and 16 %, respectively). Although the first and second EPSC amplitudes in the absence or presence of paxilline were not significantly different, a clear trend towards potentiation was observed. It should be stressed that the reported paxilline-induced increase in success rate is underestimated. In fact, summary data include also those connections (n=2) with no failures, in which the number of successes could not have been increased further with paxilline. Indeed, if these neurons are excluded, the percentage of successes increased from 66 ± 11 % to 82 ± 6 % and from 70 ± 9 % to 92 ± 3 % for the first and second spike, respectively.

As shown in Figure XIIA (arrow), in some cases in the presence of paxilline a second EPSC peak appeared with a delay. This may be due to a delayed release caused by a rise of calcium into the nerve terminal after blockade of BK channels with paxilline or alternatively to the activation of a previously silent connection.

Mean EPSCs latency was not affected by paxilline. However, in the presence of the BK channel blocker, the jitter of the EPSC evoked by the second (but not the first) spike was significantly increased (from 0.41 ± 0.14 ms to 0.61 ± 0.22 ms, n=5, $P<0.05$; Figure 5D). The jitter was defined as the standard deviation of the latencies measured in each pair.

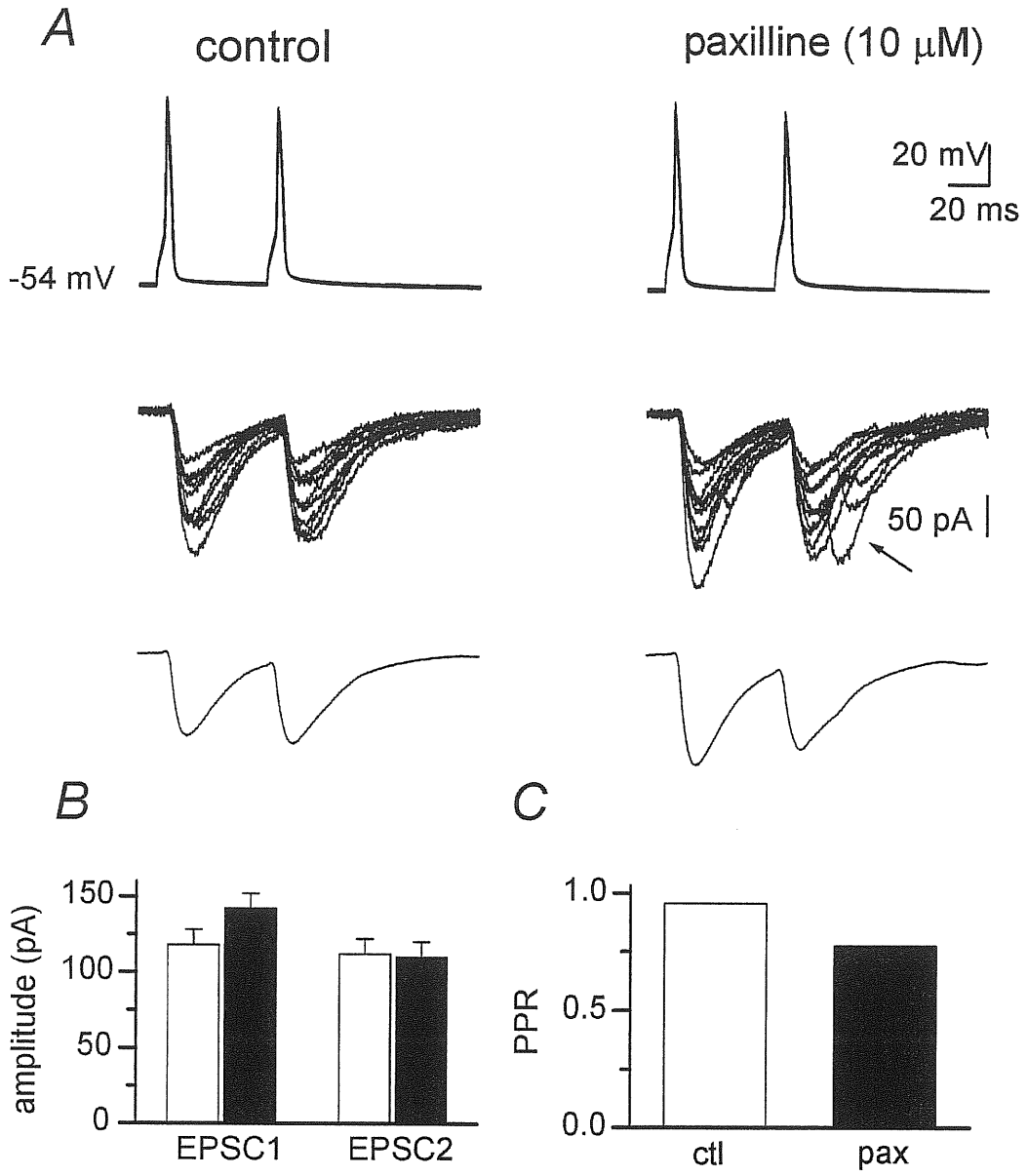

Figure XII. In high probability CA3-CA3 connections paxilline increases the amplitude of evoked EPSCs and decreases the PPR

A. The upper traces represent pairs of ten superimposed action potentials generated at 0.05 Hz in the presynaptic cell. Pairs of ten EPSCs recorded from the postsynaptic cell are superimposed in the middle. Average EPSCs (in this particular neurone no failures were detected) are recorded at the bottom. Note that paxilline (right, 10 μM) increased EPSCs amplitude and induced the appearance of delayed responses. B. Amplitude of EPSC1 and EPSC2 before (white column) and during paxilline (black columns) for the cell shown in A. C. mean paired-pulse ratio, calculated as the ratio between the mean amplitude of the second and the first response in control conditions (white column) and during paxilline (black column).

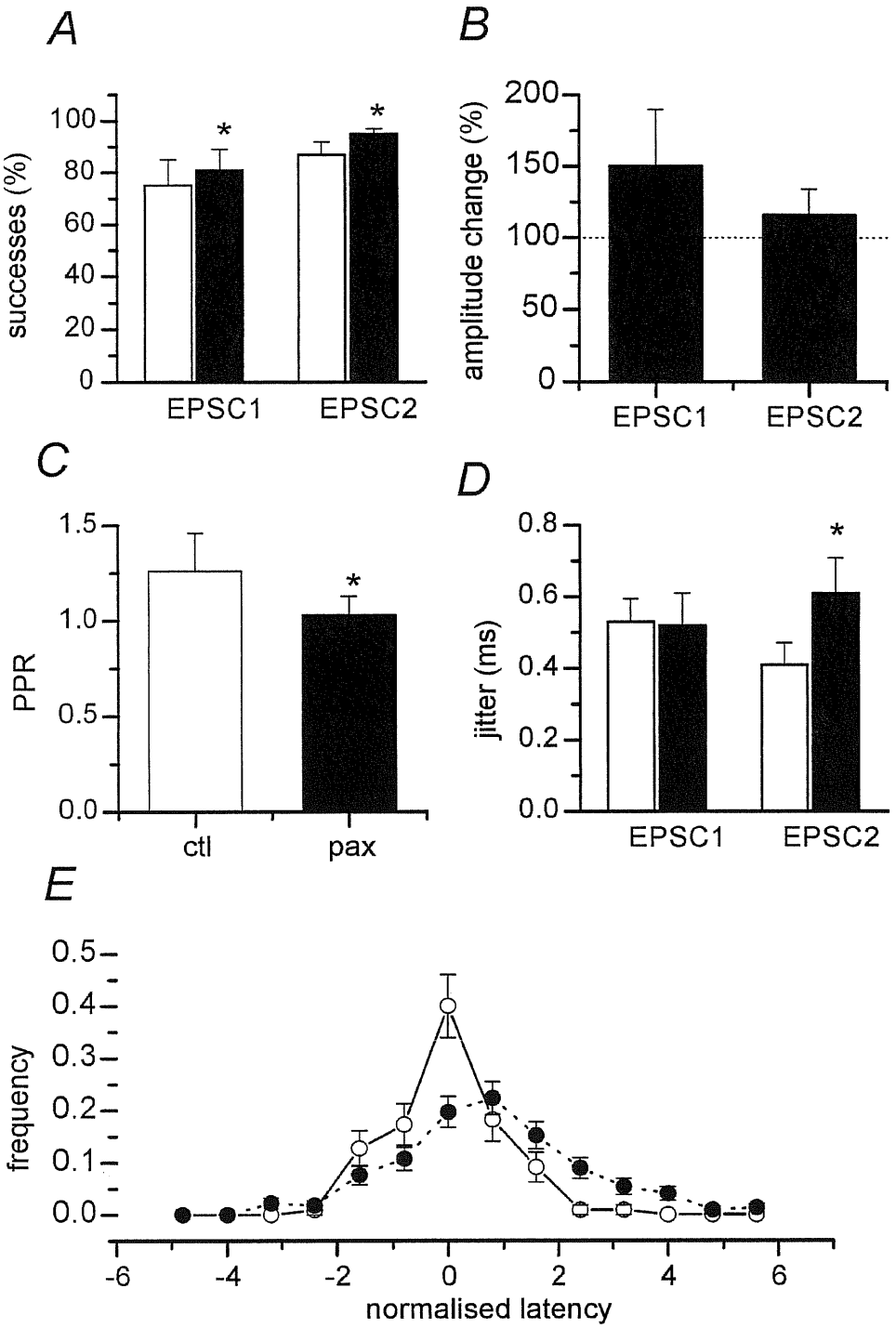

Figure XIII. Effects of paxilline on success rate, EPSC amplitude, PPR and jitter
A. Mean success rate of EPSC1 and EPSC2 under control conditions (white columns) and during paxilline application (black columns, n=7). B. Mean amplitude of EPSC1 and EPSC2 in paxilline, normalised to control values (n=5). C. Mean PPR under control conditions (white column) and during application of paxilline (black column). D. Jitters of responses obtained in control (white columns) and in paxilline (black columns, n=5,) calculated as the standard deviations of the latencies. E. Histogram of the latency of each response in control (open circles) and in paxilline (closed circles, n=5). Each latency value x_i in cell i was normalized to the mean and standard deviation m_i and s_i (measured in control conditions within the same cell) according to $y_i=(x_i-m_i)/s_i$. *P<0.05.

Figure XIIIE illustrates the distribution of latencies (for EPSC2) before and after paxilline. It is clear from the graph that in paxilline the distribution is broader. Altogether these observations suggest that BK channels are involved in the modulation of both synaptic efficacy and temporal coding.

DISCUSSION

"You tried your best and failed miserably. The lesson is, never try"
H. Simpson

The results presented in this study provide evidence that BK channels control transmitter release under basal conditions at CA3-CA3 connections in rat hippocampal slice cultures. We found that both iberiotoxin and paxilline were able to increase the frequency but not the amplitude of spontaneous action potential-mediated EPSCs in CA3 pyramidal neurones. Moreover, broadening the action potential in presynaptic neurones with paxilline enhanced the probability of transmitter release and synaptic strength in target cells. Although evidence for distinct BK channel subtypes at soma and terminals has been provided for neurosecretory neurones (Dopico et al., 1999), an indirect estimate on how spike repolarisation and presynaptic firing affect transmitter release and synaptic efficacy can be inferred with simultaneous recordings from two synaptically connected neurones, particularly in those cases in which the small size of presynaptic nerve endings preclude direct measurements with patch pipettes.

BK channels are widely expressed in the CNS and in the hippocampus. In this region receptor autoradiography and immunocytochemistry have revealed the highest level of protein expression in the middle and outer molecular layer of the dentate gyrus and in the mossy fibre pathway (Knaus et al., 1996; Wanner et al., 1999). Lower but still significant levels have been found in stratum oriens and stratum radiatum (Wanner et al., 1999) within the terminal areas, suggesting a functional role of these channels in regulating transmitter release. Further evidence in favour of a presynaptic localisation of BK channels is given by the experiments of Hu et al., (2001). Using double labeling immunogold analysis with BK channel and glutamate receptor antibodies, these authors have demonstrated that the pore forming BK channel subunits are primarily targeted to presynaptic membranes of CA1 glutamatergic synapses where they face the synaptic cleft. Interestingly, CA3 pyramidal cells give rise to Schaffer collateral which form the majority of glutamatergic axons projecting to CA1 stratum radiatum, and to collaterals which synapse on neighbouring CA3 pyramidal cells (CA3-CA3 connections). Therefore the synapses under examination in this study were formed by collateral of the same axons where presynaptic BK channels have been identified (Hu et al., 2001).

1. BK channels contribute to action potential repolarisation

In the CA1 region of the hippocampus, blocking calcium entry or rapidly chelating intracellular calcium significantly slows down the repolarisation of the action potential suggesting a prominent role for calcium-activated potassium currents in action potential repolarisation (Storm, 1987a, b; Poolos and Johnston, 1999; Shao et al., 1999). Further experiments using selective BK channel blockers provided evidence that BK channels are indeed involved in spike repolarisation (Adams et al., 1982; Lancaster and Nicoll, 1987; Storm, 1987b; Schwindt et al., 1988; Shao et al., 1999). Our experiments with paxilline confirmed and extended to CA3 pyramidal cells previous data on spike broadening obtained on the CA1 hippocampal region. Moreover, the results obtained with bursts of spikes elicited at 50 Hz suggest, in agreement with a previous report on CA1 pyramidal cells (Shao et al., 1999), that fast inactivation of a transient BK-channel current account for frequency-dependent spike broadening of the first few spikes. Such inactivation might be linked to the presence of BK channel beta subunits conferring an inactivating behaviour to the channels, such as for example β2 (Wallner et al., 1999), although its expression in CA3 neurones has not been specifically assessed. As a consequence, during high frequency bursts BK channels would affect transmitter release only during the first two-three spikes even if calcium accumulation can be enhanced by high frequency stimulation.

2. BK channels modulate the spontaneous release of glutamate

As shown in the cerebellum at granule cell to Purkinje cell synapses, a slight broadening of the presynaptic action potential caused by low concentrations of tetraethylammonium modestly increased presynaptic calcium fluxes that in turn led to a greatly enhanced transmitter release (Sabatini and Regehr, 1997). Therefore presynaptic spike broadening may be crucial for the enhancement in frequency of synaptic currents. Indeed, with the present experiments, we showed that both iberiotoxin and paxilline were able to enhance transmitter release as suggested by the increase in frequency of spontaneous events. These data are similar to those obtained at the neuromuscular junction using iberiotoxin and charybdotoxin, another BK channel blocker (Robitaille and Charlton, 1992;

Robitaille et al., 1993). The increase in frequency but not in amplitude of spontaneous EPSCs suggests a presynaptic site of action of the drugs. The observed paxilline- and iberiotoxin-induced potentiation of spontaneously occurring EPSCs, but not minis, is consistent with this hypothesis, and also indicates that BK channels regulate action potential waveform but do not interfere directly with the release machinery.

3 BK channels modulate the evoked release of glutamate

Our data on paired recordings from interconnected cells clearly show that blocking BK channels with paxilline increases synaptic efficacy both at low and high probability synapses. The effect of paxilline on EPSCs was presynaptic in origin as shown by the decrease in transmitter failures and paired-pulse ratio, which are considered traditional indexes of presynaptic modifications (Katz, 1969; Zucker, 1989). In particular, in a paired-pulse protocol, the PPR is inversely related to the initial release probability (Dobrunz and Stevens, 1997). Thus, it is likely that the observed reduction in PPR reflects an increased number of quanta delivered simultaneously by a single nerve pulse. In line with an increased probability of release following blockade of BK channels with paxilline is the appearance in some patches of delayed responses with multi-peaks that could be due to the activation of previously presynaptically "silent" connections (see Gasparini et al., 2000). In this respect our data confirm previous work on neuromuscular junction, where a clear increase of transmitter release was observed after BK channels block under normal experimental conditions (Robitaille and Charlton, 1992; Robitaille et al., 1993; Blundon et al., 1995).

Interestingly, in the presence of paxilline an increased variability in EPSC latency and a delayed release of glutamate was observed particularly after the second action potential. This seems paradoxical since the biggest effect of BK channel is on the first spike (Figure 1; see also Shao et al., 1999). The precise mechanism of this effect is still unclear. However, compatible with a release mechanism mediated by two calcium sensors with different affinities (Goda and Stevens, 1994) we can not exclude the possibility that in paxilline, during the second spike calcium concentration might reach a level that would facilitate the slow component of release. Whatever the mechanism, it is plausible from

the present data that BK channels contribute to precise spike timing facilitating network synchronisation, particularly during repetitive activity

Although in previous work it has been demonstrated that Ca^{2+}- and voltage-activated potassium channels are localised in presynaptic active zones, their role in regulation of synaptic transmission under physiological conditions has been challenged (Hu et al., 2001). According to Hu et al. (2001), presynaptic BK channels would be recruited only in extreme or rare conditions of enhanced calcium accumulation in presynaptic terminals such as those occurring during application of 4-aminopyridine. Although we do not deny the possibility that BK channels may serve as an "emergency brake", protecting against hyperactivity particularly during brain ischemia and epilepsy (Runden-Pran et al., 2002), it is clear from the present experiments that, by modulating presynaptic action potential repolarisation, BK channels regulate calcium entry not only at the somatic level but also at nerve terminals, where they act as powerful regulators of transmitter release.

CONCLUSIONS

The present results show that BK channels modulate glutamate release at CA3-CA3 synapses under basal conditions. They do so by changing the action potential shape, thus modulating the time course and concentration of calcium influx in the nerve terminal upon spike arrival. These findings are in line with previous work showing the presence of BK channels at presynaptic terminals in the rat hippocampus, but contrast the idea that they could act only in extreme conditions, with repetitive firing leading to calcium accumulation in presynaptic terminals.

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Girolamo Frescobaldi, Toccata Nona, 1627

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