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DEPENDENCE RECEPTOR INVOLVEMENT IN SUBTILISIN-INDUCED LONG-TERM DEPRESSION AND IN LONG-TERM POTENTIATION

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Abstract—The serine protease subtilisin induces a form of long-term depression (LTD) which is accompanied by a reduced expression of the axo-dendritic guidance molecule Unco-ordinated-5C (Unc-5C). One objective of the present work was to determine whether a loss of Unc-5C function contributed to subtilisin-induced LTD by using Unc-5C antibodies in combination with the pore-forming agents Triton X-100 (0.005%) or streptolysin O in rat hippocampal slices. In addition we have assessed the effect of subtilisin on the related dependence receptor Deleted in Colorectal Cancer (DCC) and used antibodies to this protein for functional studies. Field excitatory postsynaptic potentials (fEPSPs) were analyzed in rat hippocampal slices and protein extracts were used for Western blotting. Subtilisin produced a greater loss of DCC than of Unc-5C, but the antibodies had no effect on resting excitability or fEPSPs and did not modify subtilisin-induced LTD. However, antibodies to DCC but not Unc-5C did reduce the amplitude of theta-burst long-term potentiation (LTP). In addition, two inhibitors of endocytosis – dynasore and tat-gluR2(3Y) – were tested and, although the former compound had no effect on neurophysiological responses, tat-gluR2(3Y) did reduce the amplitude of subtilisin-induced LTD without affecting the expression of DCC or Unc-5C but with some loss of PostSynaptic Density Protein-95. The results support the view that the dependence receptor DCC may be involved in LTP and suggest that the endocytotic removal of a membrane protein or proteins may contribute to subtilisin-induced LTD, although it appears that neither Unc-5C nor DCC are involved in this process.

Key words: Deleted in Colorectal Cancer, DCC, plasticity, hippocampus, subtilisin, serine proteases.

INTRODUCTION

Long-term depression (LTD) has been recognized as one of the major forms of neural plasticity in the central nervous system (CNS). Often induced by low-frequency electrical stimulation, the phenomenon results in a significant reduction in the amplitude of synaptic potentials recorded *in vitro* (Christie et al., 1994) or *in vivo* (Goh and Manahan-Vaughan, 2013) with the depressed synaptic transmission correlating with alterations in specific aspects of learning, memory and other cognitive behaviors (Kemp and Manahan-Vaughan, 2004; Lemon and Manahan-Vaughan, 2012; Dong et al., 2013; Goh and Manahan-Vaughan, 2013). LTD can also be induced by chemical stimuli, notably the activation of *N*-methyl-D-aspartate (NMDA) (Mulkey and Malenka, 1992) or metabotropic glutamate receptors (Anwyl, 1999, 2006) but can also be generated by some serine proteases, including tissue plasminogen activator (tPA) (Tsirka et al., 1995; Calabresi et al., 2000; Pawlak et al., 2002; Pang and Lu, 2004), neuropsin (Komai et al., 2000) and neurotrypsin (Frischknecht et al., 2008). The enzymes do not necessarily act by the same or similar mechanisms, since tPA is dependent on Brain-Derived Neurotrophic Factor (Pang et al., 2004; Mou et al., 2009; Rodier et al., 2014) but neuropsin modifies the expression or function of adhesion molecules and fibronectin (Tani et al., 2001; Matsumoto-Miyai et al., 2003).

We have demonstrated a similar LTD in hippocampal slices in response to the bacterial serine protease subtilisin (MacGregor et al., 2007) and have reported its association with the loss of specific proteins including Unco-ordinated-5C (Unc-5C, *formerly known as Unc-5H3 in rodents*) (Forrest et al., 2011). Levels of several other proteins remain largely unchanged, including the closely related Unc-5A, the synaptic protein synaptotagmin, the cytoskeletal organisers RhoA and RhoB and the morphogenetic protein sonic hedgehog (Shh) (Forrest et al., 2011). In addition, subtilisin-induced LTD is not a reflection of generalized cellular toxicity as it is not associated with a classical activation of caspase-3 or caspase-9 and it is not modified by inhibitors of caspase activation (Forrest et al., 2013). These observations indicate that the loss of Unc-5C is not simply the result of a general proteolytic effect of subtilisin and raise the possibility of a specific relationship between Unc-5C and subtilisin induced-LTD.

The family of Unc-5 proteins contains four members with markedly different regional distributions and functional properties. In many cell types they function as

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Abbreviations: aCSF, artificial cerebrospinal fluid; CED, Cambridge Electronic Design; CNS, central nervous system; DCC, Deleted in Colorectal Cancer; fEPSPs, field excitatory postsynaptic potentials; HRP, horse radish peroxidase; LTD, long-term depression; LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate; Shh, sonic hedgehog; tPA, tissue plasminogen activator; Unc-5C, Unco-ordinated-5C.

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receptors for the secreted protein ligand netrin and have become known as 'dependence receptors' since cell survival is dependent on the interaction between netrin and the receptor: a loss of netrin results in the initiation of apoptotic processes leading to cell death (Mehlen and Guenebeaud, 2010; Castets et al., 2012).

However, the same ligand (netrin) and receptors are also involved in axonal guidance, spine development and synapse stabilization (Masuda et al., 2008; Muramatsu et al., 2010), raising the possibility that the loss of Unc-5C produced by subtilisin could affect these processes and also, therefore, aspects of neural plasticity. The present study was designed to obtain further information on the role of Unc-5C by applying antibodies to Unc-5C receptors to hippocampal slices and assessing their influence on subtilisin-LTD. In addition, the previous finding that subtilisin-LTD was associated with a loss of Unc-5C raised the possibility of some relationship between this protein and other forms of synaptic plasticity.

In their regulation of axon and dendrite formation and guidance Unc-5 receptors operate alone or in conjunction with a second member of the netrin receptor family, Deleted in Colorectal Cancer (DCC). The expression of DCC alone promotes chemoattraction between growing axons and postsynaptic sites (Xu et al., 2010) whereas the expression of Unc-5C, alone or in conjunction with DCC, generates repulsion (Muramatsu et al., 2010). The balance in the expression of these two receptors is, therefore, a critical feature of brain development and anatomical plasticity. On the hypothesis that one or both of these dependence receptors may also be involved in the LTD response to subtilisin, we have examined the expression of both proteins in response to subtilisin and have tested antibodies to both proteins against electrically-induced LTP and LTD.

Finally, since the removal of proteins from neuronal membranes seems to be largely responsible for several forms of LTD (Kim et al., 2001; Hu et al., 2007) we have used two inhibitors of membrane protein endocytosis: the cell permeant dynamin inhibitor dynasore, and the viral protein derivative tat-GluR2(3Y), to assess whether the internalization of a membrane protein, possibly including DCC or unc-5C, is relevant to subtilisin-induced LTD.

EXPERIMENTAL PROCEDURES

Electrophysiology

All experiments were performed in accordance with Home Office regulations and the Animals (Scientific Procedures) Act, 1986, and approved by the Glasgow University Ethics Committee. Male Wistar rats weighing 100–150 g (approximately postnatal days 28–35) were anaesthetized with urethane (1.5 g/kg) and immediately killed by cervical dislocation. The brain was removed into ice-cold artificial cerebrospinal fluid (aCSF) of composition: (in mM) NaCl 115; KH_2PO_4 2.2; KCl 2; $MgSO_4$ 1.2; $NaHCO_3$ 25; $CaCl_2$ 2.5; glucose 10, gassed with 5%CO_2 in oxygen. The hippocampi were rapidly removed and chopped into 450-μm transverse slices using a McIlwain tissue chopper. The slices were pre-incubated at room temperature for at least 1 h in a water-saturated atmosphere of 5%CO_2 in O_2 before individual slices were transferred to a 1 ml capacity superfusion chamber for recording. Slices were superfused at 28–30 °C using aCSF at a flow rate of 3–4 ml/min. A concentric bipolar electrode was used for stimulation of the Schaffer collateral and commissural fibers in stratum radiatum, using stimuli delivered at 0.1 Hz or 0.05 Hz with a pulse width of 50–300 μs, adjusted to evoke a response amplitude of approximately 70% of maximum to allow increases or decreases in size to be detected. Extracellular recordings were made via glass microelectrodes containing 1 M NaCl (tip diameter approximately 2 μs 12 m, 2–5 MΩ) with the tip positioned under microscopic visualization in the stratum radiatum of the CA1 region to evoke field excitatory postsynaptic potentials (fEPSPs). Potentials were amplified, digitized and stored on computer via a CED (Cambridge Electronic Design) micro1401 interface. The fEPSPs were routinely quantified by measurement of the early negative-going slope using cursor positions in Signal software (CED, Cambridge, UK). The axonal volley was monitored wherever it was possible to distinguish it clearly from the fEPSP to ensure that no change in synaptic input occurred during experiments.

The fEPSP was allowed to stabilize for a minimum period of 10 min before the application of compounds. The degree of LTD and its alteration by the compounds tested was quantified by measuring the size of the evoked potential 40 min after terminating the subtilisin application. This allowed standardization and comparison between slices since the plateau of depression was normally attained approximately 30 min after the end of subtilisin application. One data point per minute (one every six stimuli) is used in the graphical records of fEPSP size for clarity. The fEPSP slope is expressed as a percentage of the potential size obtained immediately prior to the subtilisin perfusion, taken as the mean of the last 10 EPSPs before starting the superfusion of subtilisin.

Long-term potentiation (LTP) was induced by a theta-burst pattern of stimulation as described by Larson et al. (1986), using groups of four pulses at 100 Hz, delivered 5 times per second for 2 s. Electrical LTD was induced using a paradigm modified from that described by Kemp and Bashir (1999) and Kemp et al. (2000). Stimulation was effected by a triplet of pulses, 200 ms apart, delivered at a frequency of 1 Hz for 5 min (stim1). Repetition of this sequence (stim2) increased the magnitude of the induced LTD and in this study two sequences were therefore used routinely, the second being applied 20 min after the end of the first sequence.

Use of antibodies

For the examination of Unc-5C antibodies, exposure of the slices was maximized by pre-incubation with antibodies in the holding chamber for at least 1 h before transferring them to the recording bath. After the initial preparation of slices they were placed alternately into aCSF in two petri dishes in the holding chamber. The