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INHIBITION OF ACETYLCHOLINE-INDUCED ACTIVATION OF EXTRACELLULAR REGULATED PROTEIN KINASE PREVENTS THE ENCODING OF AN INHIBITORY AVOIDANCE RESPONSE IN THE RAT

M. G. GIOVANNINI,* M. PAZZAGLI, P. MALMBERG-AIELLO, L. DELLA CORTE, A. D. RAKOVSKA,[1] F. CERBAI, F. CASAMENTI AND G. PEPEU

Dipartimento di Farmacologia, Università di Firenze, Viale Pieraccini 6, 50139 Firenze, Italy

Abstract—It has been demonstrated that the forebrain cholinergic system and the extracellular regulated kinase signal transduction pathway are involved in the mechanisms of learning, encoding, and storage of information. We investigated the involvement of the cholinergic and glutamatergic systems projecting to the medial prefrontal cortex and ventral hippocampus and of the extracellular regulated kinase signal transduction pathway in the acquisition and recall of the step-down inhibitory avoidance response in the rat, a relatively simple behavioral test acquired in a one-trial session. To this aim we studied by microdialysis the release of acetylcholine and glutamate, and by immunohistochemistry the activation of extracellular regulated kinase during acquisition, encoding and recall of the behavior. Cholinergic, but not glutamatergic, neurons projecting to the medial prefrontal cortex and ventral hippocampus were activated during acquisition of the task, as shown by increase in cortical and hippocampal acetylcholine release. Released acetylcholine in turn activated extracellular regulated kinase in neurons located in the target structures, since the muscarinic receptor antagonist scopolamine blocked extracellular regulated kinase activation. Both increased acetylcholine release and extracellular regulated kinase activation were necessary for memory formation, as administration of scopolamine and of extracellular regulated kinase inhibitors was followed by blockade of extracellular regulated kinase activation and amnesia. Our data indicate that a critical function of the learning-associated increase in acetylcholine release is to promote the activation of the extracellular regulated kinase signal transduction pathway and help understanding the role of these systems in the encoding of an inhibitory avoidance memory.

Key words: acetylcholine release, glutamate, medial prefrontal cortex, ventral hippocampus, MAPK, short term memory.

The forebrain cholinergic system is salient for arousal, attention, learning and memory (Everitt and Robbins, 1997; Sarter and Bruno, 2000; Sarter et al., 2003). Activation of basalocortical cholinergic afferents, revealed *in vivo* by increased cortical ACh release (Giovannini et al., 2001b; Sarter and Bruno, 2000; Arnold et al., 2002), is involved in the attentional processing central to memory formation. Cortical acetylcholine (ACh) release increases during acquisition of operant tasks, when demands on attentional processing are high (Muir, 1996), during acquisition, but not during recall, of a rewarded operant behavior (Orsetti et al., 1996), in rats performing a visual attentional task (Dalley et al., 2001), and it has been correlated with the attentional effort (Himmelheber et al., 2000). However, a correlation between attention and ACh release has not always been found (Passetti et al., 2000). Furthermore, in the hippocampus, ACh release increases during performance of a learned spatial memory task (Ragozzino et al., 1999; Stancampiano et al., 1999), and the increase in ACh is positively correlated to the performance improvement during task learning (Fadda et al., 2000), showing that cholinergic neurons are modified functionally during learning, becoming progressively more active. The cholinergic system activates in response to external inputs (Inglis and Fibiger, 1995) when they are novel, but not when the stimuli are repeated, leading to habituation (Acquas et al., 1996). It appears therefore that the forebrain cholinergic system becomes activated by tasks which require the analysis of novel stimuli representing a threat or offering a reward. Moreover, the involvement of the cholinergic system not only in attention but also in the consolidation of new memories (for rev. see Power et al., 2003) is also demonstrated by the enhancing or inhibitory effects of post-training administration of cholinergic agonists (Baratti et al., 1979; Kopf and Baratti, 1996) or antagonists (Kopf et al., 1998; Rudy, 1996; Schroeder and Packard, 2002).

[1] Present address: Institute of Physiology, Bulgarian Academy of Sciences, Laboratory of Neuropeptides, Acad. G. Bonchev Str., bl. 23, 1113 Sofia, Bulgaria.

*Corresponding author. Tel: +39-055-4271-238; fax: +39-055-4271-280.
E-mail address: mariagrazia.giovannini@unifi.it (M. Grazia Giovannini).

Abbreviations: ACd, anterior dorsal cingulate cortex; ACh, acetylcholine; aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; AUC, area under the curve; BB, (blocking buffer) 5% non fat dry milk in phosphate-buffered saline containing 0.05% Tween 20; BLA, basolateral amygdala; CA1, cornu ammonis area 1; CA3, cornu ammonis area 3; DAB, 3,3′-diaminobenzidine; DG, dentate gyrus of the hippocampus; DMSO, dimethylsulphoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol bis(βaminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; ERK, extracellular regulated protein kinase; FITC, fluorescein; HPLC, high performance liquid chromatography; IL, infralimbic cortex; MEK, mitogen activated kinase kinase; mPFC, medial prefrontal cortex; NeuN, neuronal specific enolase; OPA, *o*-phthalaldehyde; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline containing 0.05% Tween 20; PBS-TX, phosphate-buffered saline containing 0.3% Triton X-100; PBS-TX-NGS, 10% normal goat serum in phosphate-buffered saline containing 0.3% Triton X-100; PD98059, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one; PrL, prelimbic cortex; S.E.M., standard error of the mean; U0126, 1,4-diamino2,3dicyano-1,4-bis[2-aminophenyltio]butadiene; VH, ventral hippocampus.

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The inhibitory avoidance response is a learning task depending upon the activation of the cholinergic system, as shown by the impairment by pre- (Giovannini et al., 1999; Izquierdo et al., 1998b) or post-training administration of muscarinic receptor antagonists (Giovannini et al., 1999; McGaugh and Izquierdo, 2000; Izquierdo et al., 1998b), and enhanced by muscarinic receptor agonists (Barros et al., 2002; Baratti et al., 1979). However, so far no investigation exists directly correlating increased ACh release to performance of an inhibitory avoidance task in the rat.

If the forebrain cholinergic system is activated during learning, the questions arises as to which neurotransmitter systems trigger and/or accompany this activation. The interaction of forebrain cholinergic neurons with the glutamatergic system, whose involvement in the retrieval of aversive memory has been postulated (Szapiro et al., 2001), in the encoding of the inhibitory avoidance has not yet been unraveled. The *in vivo* microdialysis allows to detect more than one neurotransmitter from the same animal and, by studying their changes in the behaving animal, to identify the behaviors and cognitive functions in which they may play a role.

The further question concerns which downstream transduction pathway is switched on during acquisition thereby activating intracellular mechanisms leading to learning and memory formation. The extracellular regulated protein kinase (ERK) cascade, a transduction pathway that conveys signals from cell membranes to the nucleus (Orban et al., 1999), is involved in synaptic plasticity and is necessary for the induction of long term potentiation in the hippocampus (English and Sweatt, 1997; Giovannini et al., 2001a). The ERK cascade is also required for associative learning (Atkins et al., 1998), is activated in response to visual stimulation (Kaminska et al., 1999), is essential for long term spatial memory (Blum et al., 1999), and is necessary for step-down inhibitory avoidance (Walz et al., 2000; Cammarota et al., 2000). Furthermore, activation of ERK *in vitro* (Rosenblum et al., 2000) by muscarinic receptor stimulation indicates strong biochemical connections between two systems involved in learning and memory, namely ACh and ERK.

Here we describe the involvement of the forebrain cholinergic system and ERK pathway in acquisition and recall of a step-down inhibitory avoidance in the rat. The step-down inhibitory avoidance paradigm was chosen because it is a relatively simple behavioral test which is acquired in a one-trial session. Recall can be performed 60 min after acquisition, a time sufficient for short term memory to be formed (Izquierdo et al., 1998a,b). Microdialysis samples can be collected during the entire behavioral session and neurotransmitter's release can be correlated to the behavior performance. We present data to show that activation of the ERK pathway is downstream of cholinergic activation and is necessary for acquisition of this form of short term memory.

EXPERIMENTAL PROCEDURES

Male adult Wistar rats, weighing 260–280 g, were used (Harlan Nossan, Milano, Italy). The rats were individually housed in macrolon cages until experiment with *ad libitum* food and water and maintained on a 16 h light/8 h dark cycle with light at 7:00 a.m. The room temperature was 23±1 °C. All rats were kept for at least 1 week in the animal house facility of the University of Florence before experiment and were frequently handled. All animal manipulations were carried out according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC). Formal approval to conduct the experiments described has been obtained from the animal subjects review board of the University of Florence. All efforts were made to minimize animal sufferings and to use only the number of animals necessary to produce reliable scientific data. No alternatives to *in vivo* techniques are available for this type of experiment.

Step-down inhibitory avoidance task

In the step-down inhibitory avoidance task rodents, put on an elevated platform placed by one wall of an arena, learn to associate exploration of the adjacent compartment with a foot shock delivered through the floor grid. On a subsequent exposure to the same environment, the animal will avoid stepping down, or will increase the latency before "stepping down" onto the floor grid. We used a standard step-down apparatus placed in a soundproof room. Rats were handled and habituated to the experimenter and to the handling procedure the day before the test. Rats were positioned on an elevated platform placed in a dark compartment facing an open arena equipped with an electrified floor grid. We recorded the "Acquisition latency," i.e. the time spent before stepping down onto the grid where an aversive stimulus (10 electric shocks, 20 ms/0.5 mA/5 Hz) was delivered to the animal. Rats were immediately removed from the arena and placed in their home cage for consolidation ("Encoding"). Recall tests, given 60 min after the training test, were identical to training sessions, except that the footshock was omitted. At the recall test the time spent in the dark compartment before stepping down onto the arena was also recorded ("Recall latency"). All trained rats acquired the behavior. A 300 s ceiling was imposed on recall test latencies.

Surgery

Rats were deeply anesthetized with ketamine/xylazine (80/120 mg/kg, i.p.) and placed in a stereotaxic frame (Stellar, Stoelting Co., Wood Dale, IL, USA) for surgery. At the end of surgery, rats were treated with diaminocilline (1,000,000 U/rat, s.c.) and put back in their home cages (one rat per cage) to recover. All coordinates were taken from (Paxinos and Watson, 1982) and are relative to bregma and dural surface.

Implantation of microdialysis probes. Two vertical microdialysis guide cannulae (CMA 12, CMA Microdialysis AB, Solna, Sweden) were inserted stereotaxically, one into the medial prefrontal cortex (mPFC; coordinates: AP: +3.2; L: ±0.8; H: 2.3 mm, angle 12°) and the other one into the ventral hippocampus (VH; coordinates: AP: −5.5; L: ±4.8; H: 7.8 mm) (Fig. 1a–b). The guide cannulae were secured to the parietal bone with acrylic dental cement and the skin was sutured. Microdialysis experiments were performed seven days after surgery.

Implantation of cannulae for i.c.v. injection of drugs. A polyethylene cannula was implanted in the right lateral ventricle (coordinates: AP: −1.5; L: −1.5; H: 4.0 mm) for the i.c.v. injection of 1,4-diamino2,3dicyano-1,4-bis[2-aminophenyltio]butadiene (U0126) or 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD98059) (both from Tocris, Cockson, UK) two inhibitors of the enzyme mitogen activated kinase kinase (MEK), which activates ERK. The cannula was secured to the parietal bone with acrylic dental cement and the skin sutured. i.c.v. injection of drugs was performed 7 days after surgery.