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Antidepressant phenotype by inhibiting the phospholipase Cβ$_1$ – Protein kinase Cγ pathway in the forced swim test

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ABSTRACT

Although great advances have recently been made in the study of signal transduction, the pathogenesis of affective disorders is still unknown. There is mounting evidence suggesting that elevated phosphoinositide–protein kinase C (PI–PKC) signal transduction pathway may be a pathophysiological feature of bipolar and major depressive disorders. The aim of the present study was to further investigated the phospholipase C–protein kinase C (PLC–PKC) cascade by evaluating the effect produced by an acute blockade of this intracellular pathway at PLC and PKC level. Adult male mice were administered with pharmacological inhibitors of PLC or PKC and then subjected to the forced swim test (FST), an animal model which emulates the behavioural despair paradigm of depression. In this study we also tested the hypothesis that it might be possible to selectively modulate depressive behaviour by inhibiting the expression of specific PLC and PKC isoforms by means of specific antisense oligonucleotides (aODNs). Administration of the PLC inhibitors neomycin and U73122 as well as of the PKC inhibitors calphostin C and chelerytrine dose-dependently reduced the immobility time in the FST producing an antidepressant-like behaviour. Selective knockdown of the PLCβ$_1$ and PKCγ isoforms also induced an antidepressant phenotype. Conversely, the inhibition of the expression of PLCβ$_3$ was unable to modify the immobility time values. The PLC and PKC modulators used, at the highest effective doses, altered neither locomotor activity nor motor coordination. We demonstrate that selective blockade of PLCβ$_1$–PKCγ signalling pathway produces an antidepressant-like phenotype in mice.

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1. Introduction

Research into the pathophysiology of affective disorders and the mechanism of action of antidepressant drugs has primarily focused on altered monoamine neurotransmission. However, extensive studies have failed to prove conclusively abnormalities in neurotransmitter or post-synaptic receptor function. This has led to an examination of post-receptor signal transduction systems (Marazziti et al., 2009).

There is mounting evidence suggesting that an alteration of the phosphoinositide–protein kinase C (PI–PKC) signal transduction pathway may be associated to affective disorders. In the PI-signalling system, agonists' stimulation of G protein coupled receptors causes hydrolysis of the substrate, phosphatidyl inositol 4,5-bisphosphate (PIP_2), by the enzyme phospholipase C (PLC), resulting in the formation of two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge and Irvine, 1989). IP_3 stimulates the release of intracellular calcium from the endoplasmic reticulum, and DAG stimulates the enzyme protein kinase C (PKC) (Nishizuka, 1992).

Platelets from patients with bipolar disorders (Brown et al., 1993; Soares et al., 2001) or major depressive disorders (Dwivedi et al., 1998; Karege et al., 1996) exhibit several indices consistent with an increased PI activity, implying that depression is associated with over-stimulation of the PI pathway in platelets. More direct evidence of an increased PI-related signal transduction in affective disorders comes from studies in post-mortem human brain tissues (Friedman and Wang, 1996). In particular, these alterations might be a consequence of increased PI levels (Silverstone et al., 2005) or $G\alpha_{q/11}$ and PLC immunoreactivity (Mathews et al., 1997). An elevated PKC signalling activity has also been reported in platelets (Wang et al., 1999; Pandey et al., 1998) and brain samples (Wang and Friedman, 1996) from depressed patients. A further confirmation of this hypothesis is given by the observation that antidepressant drugs from different classes down-regulate the PI–PKC

Abbreviations: aODN, Antisense oligonucleotide; FST, Forced swim test; i.c.v., Intracerebroventricular; PI–PKC, Phosphoinositide–protein kinase C; PLC, Phospholipase C; PKC, Protein kinase C.

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signal transduction pathway by reducing PLC expression (Dwivedi et al., 2002), desensitising $G\alpha_q$-coupled receptor-generated PLC-mediated hydrolysis of PIP_2 (Dyck and Boulton, 1989; Pandey et al., 1991) or decreasing PKC activity (Mann et al., 1995; Morishita and Watanabe, 1997) in animal and human studies.

On this matter, there is not a general consensus. In contrast to the hypothesis of an over-stimulated PI cascade, it has been suggested that a hypofunctionality, rather than a hyperactivity, of the PI–PKC pathway might be related to affective disorders. Studies performed in platelets and human brain samples reported no difference (Coull et al., 2000) or decreases in indices of PI or PKC signalling (Jope et al., 1996; Pandey et al., 2002) in patients suffering from bipolar or major depressive disorders. Specifically, a decreased activity and expression of PLC and PKC have been observed in teenage suicide victims (Pandey et al., 1999, 2004).

As a step towards better understanding of the involvement of PI–PKC signalling pathway in mood disorders, we further investigated the PLC–PKC cascade by evaluating the effect produced by a pharmacological blockade of this intracellular pathway in mice exposed to the forced swim test, an animal model which emulates the behavioural despair paradigm of depression. In this study we also tested the hypothesis that it might be possible to selectively modulate depressive behaviour in antidepressant-responsive behavioural paradigms by inhibiting the expression of specific PLC and PKC isoforms by using an antisense strategy.

2. Materials and methods

2.1. Animals

Male Swiss albino mice (20–22 g) from the Morini (San Polo d'Enza, Italy) breeding farm were used. Ten mice were housed per cage (26 × 41 cm). The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were fed a standard laboratory diet and tap water *ad libitum* and kept at 23 ± 1 °C with a 12 h light/dark cycle, light on at 7 a.m. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimise animal suffering, and to reduce the number of animals used.

2.2. Intracerebroventricular injection technique

Intracerebroventricular (i.c.v.) administration was performed under ether anaesthesia, as previously described (Galeotti et al., 2003). Briefly, during anaesthesia, mice were grasped firmly by the loose skin behind the head. A 0.4 mm external diameter, hypodermic needle attached to a 10 μl syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse, where 5 μl were then administered. The injection site was 1 mm to the right or left from the mid-point on a line drawn through to the anterior base of the ears. Injections were performed into the right or left ventricle randomly. To ascertain that the drugs were administered exactly into the cerebral ventricle, some mice (20%) were injected with 5 μl of diluted 1:10 India ink and their brains examined macroscopically after sectioning. The accuracy of the injection technique was evaluated and the percentage of correct injections was determined to be 95%. Drug concentrations were prepared so that the necessary dose could be administered in a volume of 5 μl per mouse.

2.3. Forced swim test

The forced swimming test used was the same as described by Porsolt et al. (1977). Briefly, mice were placed individually into glass cylinders (height: 25 cm, diameter: 10 cm) containing 6 cm of water maintained at 22–23 °C and left there for 6 min. A mouse was judged to be immobile when it floated in the water, in an upright position, and made only small movements to keep its head above water. The duration of immobility was recorded during the last 4 min of the 6-min test. A decrease in the duration of immobility is indicative of an antidepressant-like effect. 12–15 mice per group were tested.

2.4. Motor coordination

The motor coordination was assessed by using the rota rod test. The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a non-slippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into 5 equal sections by 6 disks. Thus, up to 5 mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 r.p.m. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s. Those mice scoring less than 3 and more than 6 falls in the pretest were rejected (20%). The number of falls was measured before (pretest) and 15, 30 and 45 min after the beginning of the test. 10 mice per group were used.

2.5. Locomotor activity

The locomotor activity was evaluated by using the hole–board test. The apparatus consisted of a 40 cm square plane with 16 flush mounted cylindrical holes (3 cm diameter) distributed 4 by 4 in an equidistant, grid-like manner. Mice were placed on the centre of the board one by one and allowed to move about freely for a period of 10 min each. Two photobeams, crossing the plane from mid-point to mid-point of opposite sides, thus dividing the plane into 4 equal quadrants, automatically signalled the movement of the animal (counts in 5 min) on the surface of the plane (locomotor activity). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice. 12–15 mice per group were tested.

2.6. Antisense oligonucleotides

Phosphodiester oligonucleotides (ODNs) protected by terminal phosphorothioate double substitution (capped ODNs) against possible exonuclease-mediated degradation were obtained from Tib Molbiol (Genoa, Italy). The following aODN was produced against **PLCβ$_3$**: 5′-T*G G*TGGTCATCTGGGATG*T*A-3′ where* indicates the phosphorothioate residue. Anti-PLCβ$_3$ ODN was designed from the sequence 2649–2669 of the gi: 31982121 NCBI Sequence Wiewer; this sequence was searched through GenBank and found to be unique to its respective PLCβ$_3$ isoenzyme and was based on mouse sequence. The aODN against **PKC**γ was the following: 5′-A*C*GAAGTCCGGGTTCACA *T*A-3′. Anti-PKCγ ODN was designed from the sequence 2359–2378 of the gi: 31982442 NCBI Sequence Wiewer; this sequence was searched through GenBank and found to be unique to its respective PKCγ isoenzyme and was based on mouse sequence. The aODN against **PLCβ$_1$** was the following: 5′- G*C*T GTC GGA CAC G*C*A -3′, corresponding to nucleotides 49–63 of the PLCβ$_1$ gene sequence. Anti-PLCβ$_3$ was previously characterised by in vitro and in vivo experiments in our laboratory (Galeotti et al., 2006). Anti-PLCβ$_1$ was previously characterised by Sanchez-Blazquez and Garzon (1998) and by us. We confirmed the aODN effect on PLCβ$_1$ protein levels by performing immunoblotting experiments. We observed a statistically significant reduction of the expression of PLCβ$_1$ (45.1 ± 6.9) after aODN treatment in comparison with mice treated with dODN. The 15 and 20mer fully degenerated ODNs (dODNs), where each base was randomly G, or C, or A, or T, were used as control ODN treatment. Antisense ODNs and dODNs were pre-incubated at 37 °C for 30 min with an artificial cationic lipid (13 μM DOTAP, Sigma, Milan, Italy) and i.c.v. injected to mice in a 5 μl final volume. Mice received a single i.c.v. injection on day 1, 2 and 3. Behavioural and western blotting experiments were performed on day 4, 24 h after the last i.c.v. injection.

2.7. Preparation of membranes

Brain areas to conduct western blotting experiments were collected 24 h after the end of the anti-PKCγ i.c.v treatment. Mouse brains were dissected to separate specific areas. Mouse hippocampus and frontal cortex were homogenised in a homogenisation buffer containing 25 mM Tris–HCl pH = 7.5, 25 mM NaCl, 5 mM EGTA, 2.5 mM EDTA, 2 mM NaPP, 4 mM PNFF, 1 mM Na_3VO_4, 1 mM PMSF, 20 μg/ml leupeptin, 50 μg/ml aprotinin, 0.1% SDS. The homogenate was centrifuged at 9000 × *g* for 15 min at 4 °C, the low speed pellet was discarded and the supernatant was stored at −80 °C. Protein concentration was quantified using Bradford's method (protein assay kit, Bio-Rad Laboratories, Milan, Italy).

2.8. Western blot analysis

Membrane homogenates (10 μg) made from hippocampus and frontal cortex regions of dODN and aODN treated mice were separated on 10% SDS–PAGE and transferred onto nitrocellulose membranes (110 min at 120 V) using standard procedures. Membrane were blocked in PBST (PBS containing 0.1% Tween) containing 5% nonfat dry milk for 120 min. Following washings, blots were incubated overnight at 4 °C with specific antibodies against PKCγ (1:1000; Santa Cruz Biotechnology Inc, CA, USA) or β-actin (1:1000 dilution). After being washed with PBS containing 0.1% Tween, the nitrocellulose membrane was incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antisera (1:10,000) and left for 1 h at room temperature. Blots were then extensively washed according to the manufacturer's instruction and developed using enhanced chemiluminescence detection system (Pierce, Milan, Italy). Exposition and developing time used was standardised for all the blots. Densitometric analysis of scanned images was performed on a Macintosh iMac computer using the public domain NIH Image program. Measurements in control samples were assigned a relative value of 100%. Measurements were normalised relative to β-actin, used as loading control.

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