

ORIGINAL RESEARCH ARTICLE

Decreased phorbol ester binding in the parahippocampal gyrus from subjects with schizophrenia is not associated with changes in protein kinase C

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Combining *in situ* radioligand binding with autoradiography, we previously identified a reduction of [³H]phorbol 12,13-dibutyrate binding in the parahippocampal gyrus from schizophrenic subjects. To determine whether these changes were due to decreases in the level of protein kinase C, we measured [³H]phorbol 12,13-dibutyrate binding, levels of the protein kinase C isoforms α , β , δ , ϵ , γ , η and θ , as well as protein kinase C activity in crude particulate membranes from parahippocampal gyri of 15 schizophrenic and 15 control subjects. There was a significant decrease in the density (mean \pm SEM: 6.56 ± 0.73 pmol mg⁻¹ vs 9.68 ± 1.22 pmol mg⁻¹; $P < 0.05$) and affinity (mean $K_D \pm$ SEM: 4.64 ± 0.34 nM vs 2.95 ± 0.35 nM; $P < 0.005$) of [³H]phorbol 12,13-dibutyrate binding in homogenates from schizophrenic subjects. There were no significant changes in levels of the protein kinase C isoforms which are known to bind phorbol esters or in the activity of protein kinase C in membranes from schizophrenic subjects. These results suggest that there are changes in molecules capable of binding [³H]phorbol 12,13-dibutyrate, other than protein kinase C, in the parahippocampal gyrus from subjects with schizophrenia.

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Introduction

We previously demonstrated a decrease in the density of [³H]phorbol 12,13-dibutyrate (PDBu) binding in the parahippocampal gyrus (PHG) from schizophrenic subjects.¹ This change in [³H]PDBu binding was not present in other areas of the temporal lobe, the frontal cortex or the striatum.² Protein kinase C (PKC), an important component of second messenger cascades, was proposed to be the major binding site for phorbol esters.^{3–5} However, it is now known that PKC exists as a number of isoforms varying in both distribution and cofactor requirements,⁶ although only eight (α , β I, β II, δ , ϵ , γ , η and θ) of the eleven PKC isoforms thus far identified have the structural requirements that allow the binding of phorbol esters.⁷ Thus, our [³H]PDBu binding data could suggest that there is a decrease in PKC in the PHG from subjects with schizophrenia. Therefore, identifying which PKC isoform is altered in the PHG from subjects with schizophrenia would be an important step in our understanding of the mechanism

by which PKC could be involved in the neurobiology of the illness. To determine whether levels of the PKC isoforms were the cause of the reported change in [³H]PDBu binding, we measured parameters of [³H]PDBu binding and levels of the relevant isoforms in the PHG from subjects with schizophrenia. In addition, PKC activity was measured in membrane preparations of the PHG obtained from the same schizophrenic and control subjects.

Materials and methods

[³H]Phorbol 12,13-dibutyrate was obtained from New England Nuclear, Complete™ protease inhibitor cocktail from Boehringer Mannheim, whilst the Sephadex DEAE column was obtained from Pharmacia. Antibodies to PKC α , PKC β , PKC δ , PKC ϵ , PKC γ and PKC θ were obtained from Transduction Laboratories, the goat anti-mouse IgG:HRP conjugate from Bio-Rad. Anti-PKC η was obtained from Santa Cruz Biotechnology and goat anti-rabbit IgG:HRP conjugate from Vector. The Signa TECT™ protein kinase C assay kit was obtained from Promega. ECL™ came from Amersham, as did the Hyperfilm™-ECL. Gö6976 was obtained from Research Biochemicals International.

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Tissue preparation

The tissue for this study came from subjects who were part of the cohort in which levels of [³H]PDBu binding had previously been measured using autoradiography.¹ Tissue was obtained at autopsy, with ethical approval, from 15 subjects with a preliminary diagnosis of schizophrenia and from 15 subjects, matched for age and sex, with no history of mental illness (controls). The diagnosis of schizophrenia was confirmed, using the criteria for DSM-IV,⁸ by a senior psychologist and a psychiatrist using the Diagnostic Instrument for Brain Studies.⁹ From case histories, the duration of illness (DOI—the interval between the first hospital admission to time of death) and the last recorded antipsychotic doses, expressed as chlorpromazine equivalents,¹⁰ were calculated (Table 1). Where death was witnessed, the postmortem interval (PMI) was the time between death and autopsy. In cases where the death was not witnessed, the PMI was taken as being the interval halfway between the individual last being seen alive and being found dead. For all samples, the length of time the tissue was frozen at -70°C (freezer time, FT) was the time from autopsy to the date the tissue was removed for this study. In all cases the agonal status of the brain tissue was assessed by measuring the pH¹¹ of cerebellar tissue.

Crude particulate membranes were prepared as a total homogenate of the parahippocampal gyrus (5% w/v) in 20 mM Tris (pH 7.4), containing 0.1 mM EDTA, 0.2 mM EGTA, leupeptin, pepstatin A and chymotrypsin at 10 µg ml⁻¹, benzamidine at 1 mM and bacitracin at 1 mg ml⁻¹, as well as Complete™ protease inhibitor cocktail at three times the recommended strength. The membranes were prepared on ice, using a handheld glass Teflon homogeniser. The protein concentrations were determined using the Bio-Rad modified Lowry protein assay adapted for the microplate. The membrane was then stored at -70°C in 200-µl aliquots until required for the measurement of [³H]PDBu binding and Western blots.

Tissue extracts for the PKC assay were prepared by homogenizing parahippocampal gyrus in 20% w/v extraction buffer (25 mM Tris pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM mercaptoethanol, leupeptin and aprotinin at 1 µg ml⁻¹ and 0.5 mM phenylmethylsulphonyl fluoride) using a polytron. The homogenate was centrifuged at 14 000 × *g* for 5 min at 4°C. The supernatant was partially purified by chromatography through a Sephadex DEAE column. The samples were batch eluted with extraction buffer containing 200 mM NaCl. The concentration of protein in each fraction was measured using the Bio-Rad detergent compatible protein assay adapted for the microplate. The measurement of PKC activity was performed on the day that the extract was prepared.

[³H]Phorbol-12,13-dibutyrate binding assay

[³H]PDBu binding was measured essentially as described previously,¹² crude particulate membrane was used at a final protein concentration of 0.1 mg ml⁻¹ in 50 mM Tris-HCl (pH 7.7), 100 mM NaCl and 1 mM

CaCl₂. [³H]PDBu was used at a range of concentrations, from 7.5 nM to 30 nM. Incubation was carried out at room temperature for 1 h. [³H]PDBu binding was measured in the presence (non-specific) or absence (total) of non-radioactive phorbol-12, 13-dibutyrate at a concentration of 1 µM. Bound and free radioligand were separated by filtration over Whatman GF/B using a Brandel Cell Harvester. Scatchard analyses and Hill plots were carried out using the EBDA program.

Western blots

Crude particulate membrane preparations were diluted in reducing buffer (0.125 M Tris-HCl pH 6.8, 5% glycerol, 4% sodium dodecyl sulphate, 1.42 M 2-mercaptoethanol, 0.0025% bromophenol blue) to give final protein levels of 0.33 mg ml⁻¹. The proteins in each sample (10 µg per lane) were separated using polyacrylamide gel electrophoresis (4% stacking gel and 10% running gel) at a constant voltage of 150 V. They were then transferred to nitrocellulose membranes overnight in Towbin transfer buffer¹³ at a constant current of 40 mA.

After blocking in 5% non-fat milk powder, made with 0.1% Tween-20 in Tris-buffered saline, the nitrocellulose membranes were probed with antibodies for the PKC isoforms at empirically determined concentrations (mouse anti-PKCα 1/1000, anti-PKCβ 1/250, anti-PKCδ 1/500, anti-PKCε 1/1000, anti-PKCγ 1/5000, anti-PKCθ 1/250 and rabbit anti-PKCη 1/1000 in 5% non-fat milk powder) for 1 h at room temperature. After rinsing with 0.1% Tween-20 in Tris-buffered saline, the membranes were incubated with either goat anti-mouse IgG:HRP conjugate (1/2000 in 5% non-fat milk powder) or goat anti-rabbit IgG:HRP conjugate (1/2000 in 5% non-fat milk powder) for 1 h at room temperature.

Visualization of the antigenic reaction was carried out using the enhanced chemiluminescence (ECL) technique. The membrane was then exposed to ECL film for empirically determined periods and the optical density × area of the chemiluminescent reaction was determined using computer assisted densitometry (MCID M1 analysis system, Imaging Research Inc, Catherine, Ont, Canada).

Protein kinase C assay

The PKC assay was carried out using the Signa TECT™ protein kinase C assay kit from Promega. The basal reactions were carried out in 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.25 mM EGTA, 0.4 mM CaCl₂, and 0.1 mg ml⁻¹ bovine serum albumin with 0.1 mM ATP [^γ-³²P]ATP at a final concentration of 0.1 µCi and the biotinylated substrate (neurogranin peptide) present at a final concentration of 100 µM. Activated reactions were carried out as for basal reactions, but in the presence of 0.32 mg ml⁻¹ phosphatidylserine and 0.032 mg ml⁻¹ 1,2-diacylglycerol. The reactions were incubated at 30°C for 5 min before being terminated with 7.5 M guanidine hydrochloride. The reaction mixtures were dotted onto the SAM²™ biotin capture membrane, which was thoroughly washed before being counted in

Table 1 Demographic data relating to the donor and tissue status of the schizophrenic and control subjects in whom [³H]phorbol-12, 13-dibutyrate binding and parameters of protein kinase C were measured (***P* < 0.005)

Diagnosis	Identification number	Age (yrs)	Sex	Cause of death	PMI (h)	Brain pH	Freezer time (days)	Duration of illness (yrs)	Chlorpromazine equivalent (mg)
Schizophrenia	1	40	F	Poisoning	61.0	5.71	1723	24	550
	2	35	M	Perforated gastric ulcer	47.0	6.27	1611	17	400
	3	81	F	Aspiration	25.0	6.31	1442	40	100
	4	46	M	Ischaemic heart disease	42.0	5.53	1380	12	160
	5	22	M	Asphyxia	48.5	6.29	1715	2	2920
	6	38	M	Hanging	36.0	6.44	1703	11	200
	7	38	M	Meningococcal meningitis	50.0	6.02	1294	4	50
	8	71	M	Aspiration	48.0	6.45	1397	53	150
	9	21	F	CO poisoning	56.0	6.24	1925	2	NK
	10	27	F	asphyxia	41.0	5.85	1395	10	NK
	11	53	M	Intestinal ischaemia	37.0	5.98	1905	30	1700
	12	69	M	Ischaemic heart disease	44.5	6.38	1379	47	100
	13	58	M	Ischaemic heart disease	36.0	6.28	1926	23	
	14	72	F	Aspiration	58.5	6.48	1935	37	25
	15	32	M	CO poisoning	17.0	6.05	1393	15	670
Mean ± SEM		46.9 ± 5.1			43.2 ± 3.1	6.15 ± 0.1	1608 ± 61.6	23 ± 4.0	585.4 ± 251.2
Control	1	62	M	Thoracic aortic dissection	62.5	6.54	1725		
	2	35	M	Acute myocardial infarct	27.0	6.4	1521		
	3	88	F	Ischaemic heart disease	55.0	6.28	1421		
	4	46	M	Acute myocardial infarct	46.5	6.08	1340		
	5	22	M	Exsanguination	51.0	6.58	1437		
	6	36	M	Crush accident	42.0	6.46	2069		
	7	37	M	Cardiomyopathy	47.0	6.4	1549		
	8	71	M	Ischaemic heart disease	50.0	6.33	1738		
	9	22	F	Multiple injuries	42.0	6.31	1451		
	10	29	F	Combined drug toxicity	34.0	6.38	1688		
	11	50	M	Ischaemic heart disease	69.0	6.43	1537		
	12	65	M	Acute myocardial infarct	20.5	6.47	1527		
	13	50	M	Ischaemic heart disease	65.0	6.4	1521		
	14	60	F	Hanging	22.0	6.47	1975		
	15	33	M	Ruptured thoracic aorta	39.0	6.51	1576		
Mean ± SEM		46.5 ± 4.7			44.8 ± 3.8	6.4 ± 0.03**	1605 ± 52.7		

NK for the chlorpromazine equivalent denotes subjects with schizophrenia who were known to have been receiving anti-psychotic medication but had no recorded dose. The subject with no entry in the chlorpromazine equivalent column had not been receiving antipsychotic medication for 5 years prior to death.

a Packard Tricarb LSA2100. From these results basal PKC activity, activated PKC activity, PKC activity (activated minus basal) and percentage activation were calculated. In order to assess the specificity of the assay, the experiments were repeated in the presence of the selective PKC inhibitor, Gö6976, at a final concentration of 5 nM.

Statistical analysis

Having ascertained that the results met the criteria for normal distribution using the Kolmogorov–Smirnov

test, comparisons between the diagnostic groups were made with the Student's *t*-test. Relationships between experimental parameters and various potential confounding factors were analyzed using Pearson single product moment correlations calculated using an assumed straight line fit. Finally, the relatively high incidence of suicide in schizophrenia makes it a possible confounding factor as protein kinase C has been reported to be altered in suicide.¹² Therefore, where significant differences are identified between schizophrenics and controls, further analysis using *t*-tests

will be carried out to determine the effect of suicide in the population as a whole and within diagnostic cohorts. All statistical analyses were carried out using GraphPad Prism Version 3.0 for Windows (GraphPad Software Inc, San Diego, CA, USA).

Results

Demographics

There were no significant differences between the age, PMI or FT of the schizophrenic and control subjects. Brain pH was significantly lower (6.15 ± 0.1 vs 6.40 ± 0.03) for the schizophrenic group compared to the control group (Table 1).

[³H]Phorbol-12,13-dibutyrate binding assay

There was a significant decrease in the density of [³H]PDBu binding to crude particulate membrane from the PHG of schizophrenic subjects compared to those from the control subjects (mean \pm SEM: 6.56 ± 0.73 pmol mg⁻¹ vs 9.68 ± 1.22 pmol mg⁻¹; $P < 0.05$, see Figure 1). There was also a small, but significant, reduction in the affinity of [³H]PDBu binding in the tissue from schizophrenic subjects compared to that from control subjects (mean $K_D \pm$ SEM: 4.64 ± 0.34 nM vs 2.95 ± 0.35 nM; $P < 0.005$, see Figure 1). All Scatchard plots were linear and the Hill coefficients were close to unity (mean \pm SEM: 1.005 ± 0.011 for the schizophrenic group vs 1.027 ± 0.009 for the control group).

Western blots

There were no significant differences between the levels of the PKC α , β , γ , ϵ or η isoforms in the PHG of schizophrenic and control subjects (Figure 2). Levels of both δ and θ isoforms in the PHG were below the detection limits of this Western blot technique.

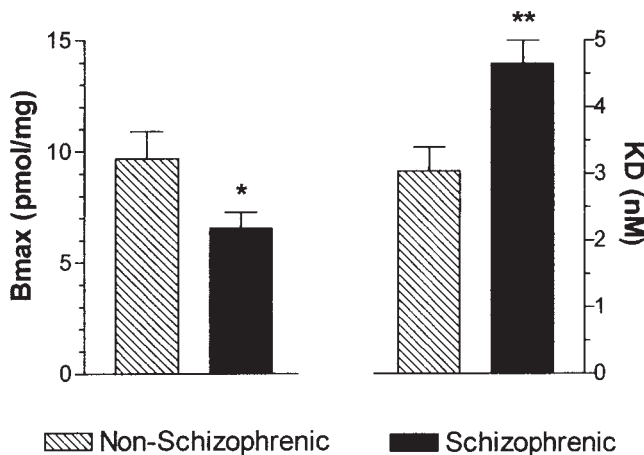


Figure 1 The density (B_{max}) and affinity (K_D) (mean \pm SEM) of [³H]PDBu binding to crude particulate membrane from the PHG, obtained at autopsy, from 15 schizophrenic and 15 control subjects (* $P < 0.05$; ** $P < 0.005$).

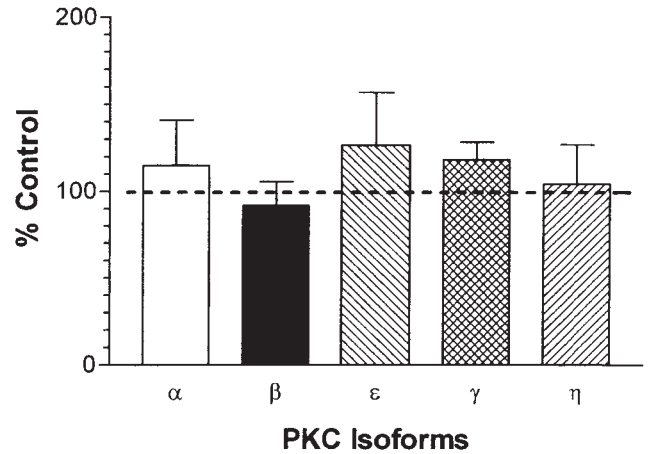


Figure 2 Levels of PKC α ($n = 15$), β ($n = 15$), ϵ ($n = 13$), γ ($n = 15$) and η ($n = 12$) (mean \pm SEM), expressed as a percent of the value obtained from the relevant control subject, in crude particulate membrane preparations from the PHG, obtained at autopsy, from schizophrenic subjects. The data were obtained as optical density \times area of film after the chemiluminescent detection of Western blots.

Protein kinase C assay

There were no significant differences between the basal, activated or percentage activation levels of PKC in PHG from schizophrenic and control subjects (Figure 3). Gö6976 at a final concentration of 5 nM reduced the PKC activity by 48.8% in extracts from subjects with schizophrenia and 49.5% in extracts from control subjects (data not shown).

Confounding factors

There were no significant correlations between any of the measures of [³H]PDBu binding, levels of any PKC isoform or PKC activity. There were no significant correlations between any of the biochemical parameters measured in this study and the variables related to donor/tissue status (age, PMI, pH, FT or DOI and

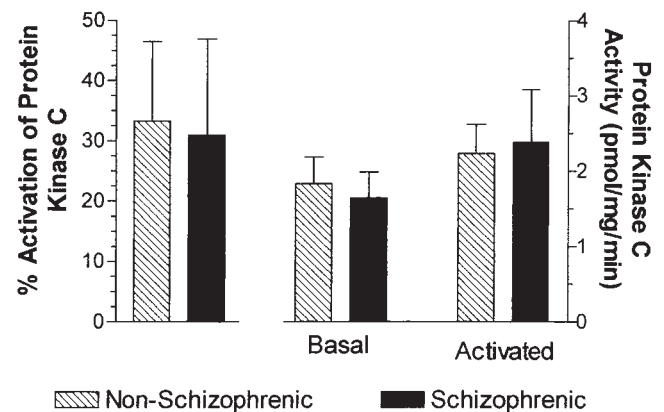


Figure 3 Levels of protein kinase C activity (mean \pm SEM), expressed as activity, basal and activated, and percent activation in partially purified membrane preparations of the PHG, obtained at autopsy, from 12 schizophrenic and 12 control subjects.

chlorpromazine equivalents for subjects with schizophrenia). Further analysis showed that in neither the population as a whole nor the schizophrenic cohort was suicide a significant factor in relation to either the density or affinity of [³H]PDBu binding. An analysis of the control as a separate cohort was not completed because only two of the subjects died as a result of suicide.

Discussion

Using particulate membranes, this study confirmed the results from our previous study,¹ with a decrease in the density of [³H]PDBu binding in the PHG from subjects with schizophrenia. In addition, the current study also showed a small, but significant, decrease in the affinity of [³H]PDBu binding to particulate membranes from the PHG from schizophrenic subjects. At this stage it is not possible to predict whether a change in affinity, of the magnitude we observed in the tissue from schizophrenic subjects who died by suicide, would have physiological consequences.

In the main, phorbol esters bind with high affinity to PKC, a key enzyme which mediates a second messenger system.⁶ Therefore, the decrease in the density of [³H]PDBu binding we measured in the PHG would be predicted to be due to a change in the levels of PKC isoforms, capable of binding phorbol esters, in the PHG. However, in this study, Western blot analysis suggests that there is no change, of a high enough magnitude to account for the change in [³H]PDBu binding, in any of the PKC isoforms which bind phorbol esters in the PHG from subjects with schizophrenia. It should be taken into account that the levels of δ and θ isoforms were too low to be measured by Western blot analysis. However, such low levels of PKC isoforms would be unlikely to make a significant contribution to phorbol ester binding in the PHG. Furthermore, the antibody raised against PKC β does not distinguish between the splice variants β I and β II, so the values recorded for the β isoform are the sum of the two variants. Therefore, if there are changes in either the β I or β II isoform in the PHG from subjects with schizophrenia, it must be masked by a compensatory change in the other isoform since the overall levels of the β isoforms were unchanged.

The absence of change in any of the PKC isoforms is in agreement with our finding that the overall activity of PKC is not changed in the PHG from schizophrenic subjects. Together, the Western blot and the enzyme activity data suggest that PKC is not altered in the PHG from schizophrenic subjects.

With postmortem studies, there is always the question of whether the drugs ingested by the subjects during their lifetime have affected the outcome of the experiments. Although it has previously been reported that the antipsychotics haloperidol, chlorpromazine and clozapine can reduce the levels of PKC protein, mRNA and activity in rats,¹⁴ these drugs did not affect [³H]PDBu binding following either acute or chronic administration.^{14,15} Whilst the possibility of a drug

effect cannot be completely dismissed, this lack of effect of antipsychotic drugs on [³H]PDBu binding, combined with the fact that neither PKC protein levels nor PKC activity were altered in tissue from schizophrenic subjects, makes it unlikely that the results obtained in this study are an artifact of the pharmacotherapy received by the patients during their illness.

The apparent discrepancy in our data could be accounted for by there being other additional binding sites for [³H]PDBu in the PHG which are altered in schizophrenia. A review of the literature revealed two non-PKC candidate molecules which bind [³H]PDBu with an affinity similar to that of PKC.^{16,17}

One of these sites, n-chimaerin, is a protein with a phorbol ester binding domain similar to that of PKC.¹⁸ N-chimaerin is widely expressed in rat brain and has been shown to be present in the rat hippocampus. N-chimaerin is a rac21 GTPase activating protein, which moves from the cytosol to the membrane upon activation.¹⁹ Rac21 is thought to be involved in cell growth and differentiation.²⁰ Although N-chimaerin in rodents was identified using probes based on human DNA sequences, its presence has yet to be demonstrated in the human central nervous system.

Another protein, which is present in the mammalian brain and binds [³H]PDBu, is Munc13.¹⁷ Munc13 is the mammalian homologue of unc-13p found in *Caenorhabditis elegans*; there are three isoforms, Munc13-1 to Munc13-3. Interestingly, in the context of changes in brain tissue obtained from schizophrenic subjects, all Munc13 isoforms are found presynaptically and are thought to be involved in the regulation of neurotransmitter release¹⁷ and so could play an important role in the pathology of schizophrenia. More specifically, Munc13-1 has been shown to be necessary for the maturation of glutamatergic vesicles in presynaptic neurons,²¹ a neurotransmitter system thought to be altered in schizophrenia.²² To date, all the work relating to Munc13 isoforms has been carried out in rodents, although a human homologue has been reported.²³

In conclusion, our data suggest that the decrease in [³H]PDBu binding in the PHG from subjects with schizophrenia is not due to changes in the levels of those PKC isoforms known to be capable of binding phorbol esters. This raises the possibility that changes in the levels of other molecules capable of binding phorbol esters are occurring in the PHG of schizophrenic subjects. The exact cause of these changes in [³H]PDBu binding and its possible implications in the pathogenesis of schizophrenia remain to be elucidated but point to other candidate molecules which may have a role in the pathology of schizophrenia.

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