

## Effect of halothane on conventional protein kinase C translocation and down-regulation in rat cerebrocortical synaptosomes

H. C. HEMMINGS JR AND A. I. B. ADAMO

### Summary

Protein kinase C (PKC) is a key regulatory enzyme that has been implicated as a molecular target for the action of general anaesthetics. We have determined the effects of halothane on the translocation and down-regulation of conventional PKC (cPKC) by analysing the subcellular distribution of PKC activity, [<sup>3</sup>H]phorbol-12,13-dibutyrate ([<sup>3</sup>H]PDBu) binding and PKC immunoreactivity in intact rat cerebrocortical synaptosomes, a subcellular fraction that contains functional nerve terminals. Halothane alone (2.4 vol%) reduced membrane-associated ( $P < 0.05$ ) and increased cytosol ( $P < 0.01$ ) PKC activity, while phorbol-12-myristate, 13-acetate (PMA) 0.1  $\mu\text{mol litre}^{-1}$ , a metabolically stable activator of PKC, reduced membrane ( $P < 0.01$ ) without altering cytosol PKC activity. Halothane and PMA in combination reduced membrane PKC activity to undetectable levels and reduced cytosol PKC activity ( $P < 0.01$ ). Halothane alone had no significant effects on the distribution of [<sup>3</sup>H]PDBu binding, while PMA alone significantly reduced both membrane and cytosol [<sup>3</sup>H]PDBu binding ( $P < 0.01$ ). Halothane and PMA in combination reduced membrane and cytosol [<sup>3</sup>H]PDBu binding further, but this effect was not significantly different from the effect of PMA alone. Experiments using isoform-selective antibodies to PKC $\alpha$ , PKC $\beta$  or PKC $\gamma$  demonstrated synergistic interactions between halothane and PMA in promoting translocation of the three conventional PKC isoforms from the cytosol to the membrane fraction of synaptosomes and down-regulation of their immunoreactivity. Halothane and PMA together reduced cytosol PKC $\alpha/\beta/\gamma$  immunoreactivity significantly more ( $P < 0.05$ ) than PMA alone. Halothane thus has two distinct actions on PKC in synaptosomes: activation of endogenous PKC activity and potentiation of activation-induced cPKC translocation and down-regulation. These potentially competing effects may underlie some of the conflicting results obtained with halothane on PKC-mediated processes in intact cells. (*Br. J. Anaesth.* 1997; 78: 189–196)

### Key words

Anaesthetics volatile, halothane. Enzymes, protein kinase C. Nerve, synaptosomes. Rat.

Protein kinase C (PKC) is an important family of protein kinases that regulates neuronal function.<sup>1,2</sup> Many studies have implicated PKC as a potential target for general anaesthetic effects in various tissues.<sup>3–17</sup> PKC exists as a number of structurally related isoforms distinguished by their regulatory domains and cofactor dependence<sup>18–20</sup>: the conventional PKC isoforms (cPKC:  $\alpha$ ,  $\beta_1$ ,  $\beta_2$  and  $\gamma$ ) are activated by  $\text{Ca}^{2+}$ , diacylglycerol and phorbol esters; the new PKC isoforms (nPKC:  $\delta$ ,  $\epsilon$ ,  $\eta$  and  $\theta$ ) do not require  $\text{Ca}^{2+}$  but are activated by diacylglycerol and phorbol esters; and the atypical PKC isoforms (aPKC:  $\zeta$ ,  $\lambda$ ,  $\mu$  and PKC $\zeta$ ) do not require  $\text{Ca}^{2+}$  or diacylglycerol for activity and are not affected by phorbol esters.

Physiological activation of cPKC isoforms, which are abundant in mammalian brain,<sup>19</sup> occurs when diacylglycerol is generated in response to activation of cell surface receptors coupled to activation of phospholipase C or in response to  $\text{Ca}^{2+}$ -induced activation of phospholipase D.<sup>1,21</sup> Binding of diacylglycerol increases the affinity of cPKC for  $\text{Ca}^{2+}$  and phosphatidylserine, facilitates cPKC translocation and binding to cell membranes, and increases cPKC catalytic activity.<sup>22</sup> Phosphorylation of specific proteins by activated PKC is the effector mechanism for regulation of several neuronal processes,<sup>2</sup> many of which are also sensitive to general anaesthetics, such as neurotransmitter release,<sup>23</sup> ion channel function<sup>24</sup> and neurotransmitter receptor desensitization.<sup>25</sup> On agonist-induced stimulation of a number of cell types, cPKC and nPKC, but not aPKC, isoforms<sup>26</sup> rapidly translocate from the cytosol to the membrane where they undergo proteolysis-mediated down-regulation in an isoform-specific manner.<sup>27,28</sup> The role of these important regulatory mechanisms in determining the ultimate effects of anaesthetics and other drugs on the PKC signalling pathway in specific tissues and cells is unknown.

Previous studies of general anaesthetic effects on PKC activity have yielded contradictory results. For example, halothane has been found to inhibit<sup>13</sup> or stimulate<sup>14</sup> purified brain PKC *in vitro*, and to

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stimulate PKC activity in brain cytosol,<sup>6</sup> in synaptosomes<sup>15</sup> and in intact neurosecretory cells.<sup>9</sup> These differences may be explained in part by variations in the conditions used to analyse PKC activity *in vitro*,<sup>14</sup> or by differences in the particular PKC isoforms present. PKC has been implicated in the effects of general anaesthetics on neurotransmitter release from PC12 cells,<sup>9</sup> prostacyclin production in endothelial cells,<sup>16</sup> protein phosphorylation in neuronal growth cones,<sup>12</sup> smooth muscle contraction,<sup>10</sup> hepatic blood flow<sup>17</sup> and loss of righting reflex in tadpoles.<sup>11</sup> Several of these studies inferred anaesthetic inhibition, rather than activation, of PKC activity based on indirect evidence. We now report evidence that halothane can potentiate cPKC translocation and down-regulation induced by phorbol ester treatment in synaptosomes. The results provide a potential mechanism for biphasic effects of halothane on PKC activity in intact cells: initial stimulation of PKC activity as a result of a direct effect on enzymatic activity,<sup>15</sup> followed by alterations in PKC-dependent processes caused by PKC translocation and down-regulation.

## Materials and methods

### SYNAPTOSOME PREPARATION AND INCUBATION

Synaptosomes were prepared from rat cerebral cortex by the method of Dunkley, Jarvie and Heath,<sup>29</sup> as described previously,<sup>30</sup> and were stored as pellets on ice for up to 4 h until use. Synaptosome pellets were resuspended to a protein concentration of 2 mg ml<sup>-1</sup> (determined by the method of Bradford<sup>31</sup> with bovine serum albumin as standard) in incubation medium containing the following (mmol litre<sup>-1</sup>): NaCl 140, KCl 5, NaHCO<sub>3</sub> 5, MgCl<sub>2</sub> 1, D-glucose 10 and HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid) 10, at pH 7.4 with NaOH containing 1 mg ml<sup>-1</sup> of fatty acid-free bovine serum albumin equilibrated with 95% oxygen-5% carbon dioxide (v/v). After a 5-min incubation period in a shaking water bath at 37°C to allow ion gradient re-equilibration, CaCl<sub>2</sub> 1 mmol litre<sup>-1</sup> was added to a 1-ml aliquot of synaptosomes. After another incubation period of 1 min, halothane, phorbol-12-myristate, 13-acetate (PMA) or drug vehicle (dimethyl sulphoxide; DMSO) was added where appropriate. Translocation reactions were carried out in 8.8 ml glass vials sealed with Teflon/silicone septa, through which liquid halothane (1–2 µl, either pure or diluted with DMSO, injected onto the side of the vial to vaporize) was injected directly with a Hamilton microsyringe. Final vapour phase halothane concentrations, which equilibrate by 2 min,<sup>32</sup> were measured in similar samples by gas chromatography.<sup>33</sup> The incubation was continued for another 10 min, after which the reaction mixtures were transferred rapidly to polycarbonate centrifuge tubes on ice and centrifuged at 9000 × g for 5 min at 4°C.

### SYNAPTOSOME FRACTIONATION

After incubation synaptosomes were fractionated by

a modification of the method of Diaz-Guerra and colleagues.<sup>34</sup> The synaptosome pellet was lysed immediately in 2 ml of ice-cold hypotonic lysis buffer (HEPES 10 mmol litre<sup>-1</sup>, pH 7.4, with NaOH, EGTA 1 mmol litre<sup>-1</sup>, EDTA 1 mmol litre<sup>-1</sup>, dithiothreitol 1 mmol litre<sup>-1</sup>, leupeptin 1 µg ml<sup>-1</sup>) by homogenization with a glass/Teflon homogenizer for five up-and-down strokes. The homogenate was centrifuged at 200 000 × g for 15 min at 4°C to yield supernatant ("cytosol") and pellet ("membrane") fractions, which were separated. The pellet fraction was resuspended in 1 ml of lysis buffer containing 0.5% (v/v) Nonidet P-40 by homogenization and incubated on ice for 30 min to extract PKC activity. An additional 1 ml of lysis buffer was added to dilute the detergent concentration, the pellet was centrifuged at 200 000 × g for 15 min at 4°C, and the supernatant fraction ("membrane extract") was removed. The cytosol and membrane extract fractions were then filtered through 1-ml (bed volume) columns of DEAE-cellulose (DE-52, Whatman) equilibrated in lysis buffer to remove endogenous PKC inhibitors<sup>35</sup> and detergent. The columns were washed with 4 ml of lysis buffer, followed by 2 ml of lysis buffer containing NaCl 0.1 mol litre<sup>-1</sup> to elute PKC activity. The samples containing PKC were then concentrated and their buffer changed to HEPES 10 mmol litre<sup>-1</sup>, pH 7.5, with a Centricon-30 concentrator (Amicon, Inc., Beverly, MA, USA). The concentrated samples were adjusted to equal volumes and protein concentrations were measured by the method of Bradford.<sup>31</sup> Protein recoveries were comparable between the different treatments. Aliquots were stored frozen at -80°C until analysis.

### PKC ACTIVITY

The activity of PKC in the synaptosome extracts was measured at 30°C in a reaction volume of 0.1 ml containing HEPES 50 mmol litre<sup>-1</sup>, pH 7.4, with NaOH, MgCl<sub>2</sub> 10 mmol litre<sup>-1</sup>, bovine serum albumin 20 µg ml<sup>-1</sup> (fraction V; Baker, Phillipsburg, NJ, USA), dithiothreitol 0.1 mmol litre<sup>-1</sup>, [ $\gamma$ -<sup>32</sup>P]ATP 100 µmol litre<sup>-1</sup> (100–250 cpm pmol<sup>-1</sup>; DuPont-NEN, Boston, MA), lysine-rich histone H1 0.2 mg ml<sup>-1</sup> (9.3 µmol litre<sup>-1</sup>) (histone HL; Worthington Biochemical, Freehold, NJ, USA), an aliquot of synaptosome extract (protein 0.25–10 µg), CaCl<sub>2</sub> 1.5 mmol litre<sup>-1</sup>, EGTA 1 mmol litre<sup>-1</sup> (free [Ca<sup>2+</sup>] = 500 µmol litre<sup>-1</sup>), and small unilamellar lipid vesicles composed of bovine brain L- $\alpha$ -phosphatidylserine 20 µmol litre<sup>-1</sup> (PS; Avanti Polar Lipids, Alabaster, AL, USA), chicken egg L- $\alpha$ -phosphatidylcholine (PC; Avanti Polar Lipids) 80 µmol litre<sup>-1</sup> and 1,2-dioleoyl-sn-glycerol (Avanti Polar Lipids) 2 µmol litre<sup>-1</sup> prepared as described previously.<sup>14</sup> The reaction mixture, containing all assay components except ATP, was equilibrated for 5 min, after which reactions were initiated by addition of ATP and terminated after 5 min by addition of glacial acetic acid 10 µl. Histone phosphorylation was determined by the phosphocellulose paper method as described previously.<sup>14</sup> At least two concentrations of each synaptosome

extract were analysed to ensure that the reaction was in the linear range. Each assay was performed in triplicate in the absence and presence of the selective PKC inhibitor peptide PKC<sub>19-36</sub><sup>36</sup> at 10  $\mu\text{mol litre}^{-1}$ . PKC activity was defined as the difference between histone kinase activity in the absence or presence of the PKC inhibitor (1 unit of activity = 1 pmol min<sup>-1</sup> of <sup>32</sup>P incorporation into histone H1). Background phosphorylation in the absence of histone H1 as a result of endogenous substrates was negligible.

#### 4 $\beta$ -[<sup>3</sup>H]PHORBOL-12,13-DIBUTYRATE BINDING

Binding of the phorbol ester 4 $\beta$ -[<sup>3</sup>H]phorbol-12,13-dibutyrate (PDBu) to PKC was determined at 37 °C in a reaction volume of 0.1 ml containing HEPES 50 mmol litre<sup>-1</sup>, pH 7.4, with NaOH, MgCl<sub>2</sub> 10 mmol litre<sup>-1</sup>, EGTA 1 mmol litre<sup>-1</sup>, CaCl<sub>2</sub> 1.5 mmol litre<sup>-1</sup>, PS 20  $\mu\text{mol litre}^{-1}$ , PC 80  $\mu\text{mol litre}^{-1}$  and [<sup>3</sup>H]PDBu 0.1  $\mu\text{mol litre}^{-1}$  (0.2  $\mu\text{Ci}$ ; DuPont-NEN). The reaction was initiated by addition of [<sup>3</sup>H]PDBu, incubated for 15 min (sufficient for equilibration of binding; data not shown), and terminated by addition of 4 ml of ice-cold Tris 20 mmol litre<sup>-1</sup>, pH 7.5, with HCl, CaCl<sub>2</sub> 200  $\mu\text{mol litre}^{-1}$  and 20% (v/v) methanol, followed by rapid filtration through polyethyleneimine-treated<sup>37</sup> GF/C filters (Whatman, Hillsboro, OR, USA), and then by three washes of 2 ml each with the same buffer. Protein-bound radioactivity retained on the filters was quantified by liquid scintillation spectrometry in 4 ml of Bio-Safe NA scintillant (Research Products International Corp., Mount Prospect, IL, USA). Non-specific binding, determined by inclusion of non-radioactive PDBu 10  $\mu\text{mol litre}^{-1}$ , was negligible. These assays were saturated with respect to [<sup>3</sup>H]PDBu concentration and provided a quantitative assay of the phorbol ester receptor as an index of PKC.<sup>38</sup> Residual PMA from the preincubation does not affect subsequent binding of [<sup>3</sup>H]PDBu to membranes as these subcellular fractions are subjected to chromatography and washing by centrifugation.<sup>37</sup>

In a separate series of experiments, the effect of halothane on equilibrium [<sup>3</sup>H]PDBu binding to synaptosomes was analysed using a range of [<sup>3</sup>H]PDBu concentrations (10–200 nmol litre<sup>-1</sup>). The dissociation constant ( $K_d$ ) and maximum number of binding sites (Bmax) were calculated from Scatchard plots using the Enzfit kinetic program (Elsevier-Biosoft, Cambridge, UK).

#### PKC IMMUNOBLOT ANALYSIS

Immunoreactive PKC was measured by immunoblotting of synaptosome extracts separated by Sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Cytosolic and membrane extracts were prepared as described above, but were not subjected to detergent extraction or DE-52 column chromatography. Aliquots (protein 20–60  $\mu\text{g}$ ) of the crude extracts were separated by SDS-PAGE using a 10% acrylamide running gel.<sup>39</sup> Proteins were

transferred from gels to 0.2  $\mu\text{m}$  nitrocellulose sheets (Schleicher and Schuell, Keene, NH, USA) at 0.2 amp for 20 h by the method of Towbin, Staehelin and Gordon.<sup>40</sup> The nitrocellulose sheets were blocked with 2.5% (w/v) non-fat dry milk (Carnation) and 0.05% (v/v) Tween 20 in PBS buffer (potassium phosphate 10 mmol litre<sup>-1</sup>, pH 7.4, NaCl 150 mmol litre<sup>-1</sup>, 0.02% (v/v) sodium azide) overnight. After a brief rinse in immunoblot buffer (PBS containing 0.2% (v/v) Tween 20), the nitrocellulose transfers were immunoblotted for 2 h with one of three specific mouse monoclonal antibodies to PKC in immunoblot buffer: (1) an antibody to rat brain PKC $\alpha$  used at a dilution of 1:5000 (Transduction Laboratories, Lexington, KY, USA; #P16520); (2) an antibody to human PKC $\beta$  used at a dilution of 1:2500 (Transduction Laboratories, #P17720); and (3) an antibody to rat PKC $\gamma$  used at a dilution of 1:5000 (Transduction Laboratories, #P20420). The nitrocellulose transfers were then incubated successively with immunoblot buffer three times for 10 min, with rabbit anti-mouse secondary antibody in immunoblot buffer for 1 h (1:500 dilution; Pierce; Rockford, IL, USA), with immunoblot buffer five times for 5 min, with <sup>125</sup>I-labelled protein A (1  $\mu\text{Ci ml}^{-1}$ ; Amersham) in 2.5% non-fat dry milk and 0.05% Tween 20 in immunoblot buffer for 1.5 h, and with immunoblot buffer overnight followed by twice for 5 min. The PKC immunoreactive bands were examined by autoradiography and quantified by scanning and analysis with a PhosphorImager autoradiography system (Molecular Dynamics, Sunnyvale, CA, USA).

Thymol-free halothane was a gift from Halocarbon Products (River Edge, NJ, USA). The PKC inhibitor peptide (PKC<sub>19-36</sub>),<sup>36</sup> synthesized by the Rockefeller University Protein Sequencing Facility, was a gift from A. J. Czernik (Rockefeller University). Nonidet P-40, Tween 20 and sodium dodecyl sulphate were from Pierce Chemical Co. (Rockford, IL, USA). PMA and PDBu were from LC Laboratories (Woburn, MA, USA). Bovine serum albumin (fatty acid-free) was from Sigma (St Louis, MO, USA).

#### STATISTICAL ANALYSIS

Statistical significance was assessed by analysis of variance with the Newman-Keuls multiple range test using the PHARM/PCS Pharmacological Calculation System, v. 4.2 (Springer-Verlag, New York, NY).

The studies were approved by the Cornell University Medical College Institutional Animal Care and Use Committee.

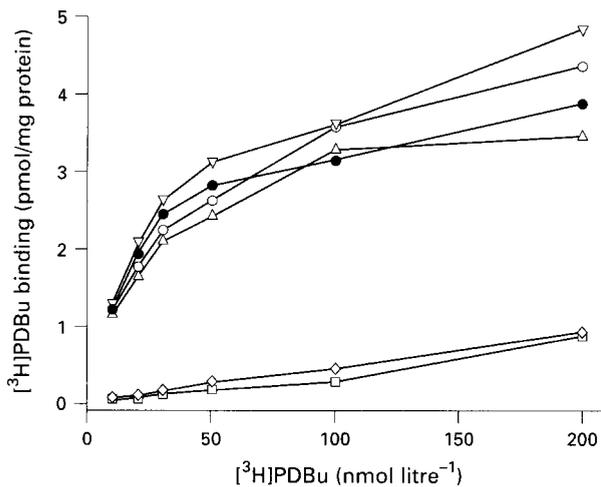
## Results

#### PKC ACTIVITY

The relative content of PKC enzymatic activity in fractionated rat cortical synaptosomes is shown in table 1. The synaptosomal membrane fraction contained a higher specific activity of PKC (defined as PKC<sub>19-36</sub>-sensitive histone H1 activity) than the

**Table 1** Subcellular distribution of PKC in synaptosome exposed to phorbol ester and halothane. Intact synaptosome were incubated at 37°C for 10 min in the presence of 0.2% DMSO (control) or PMA 0.1  $\mu\text{mol litre}^{-1}$  and/or 2.4 vol% halothane with 0.2% DMSO, followed by synaptosome subfractionation as described in materials and methods. Data for PKC activity and [ $^3\text{H}$ ]PDBu binding are mean (SD) of three independent experiments with individual assays performed in triplicate. U = unit (= pmol/min); ND = not detectable; PDBu = phorbol-12,13-dibutyrate; PMA = phorbol-12-myristate, 13-acetate; PKC = protein kinase C. \* $P < 0.05$  vs control; \*\* $P < 0.01$  vs control; † $P < 0.05$  vs halothane; †† $P < 0.01$  vs halothane; ‡ $P < 0.05$  vs PMA; ‡‡ $P < 0.01$  vs PMA

Treatment	PKC activity		[ $^3\text{H}$ ]PDBu binding	
	U/mg	% Control	fmol/mg	% Control
Control				
Cytosol	1252 (110)	—	2027 (86)	—
Membrane	2425 (639)	—	459 (29)	—
PMA				
Cytosol	1251 (256)	100	140 (18)**	7
Membrane	400 (221)**	16	82.9 (53)**	18
Halothane				
Cytosol	3676 (1209)**‡‡	294	1983 (206)‡‡	98
Membrane	1538 (146)*‡‡	63	297 (78)‡	65
Halothane+PMA				
Cytosol	311 (100)**††‡‡	25	102 (31)**††	5
Membrane	ND	—	66.3 (31)**†	14



**Figure 1** Effect of halothane on equilibrium [ $^3\text{H}$ ]PDBu binding to rat cerebrocortical synaptosomes as a function of [ $^3\text{H}$ ]PDBu concentration in the absence ( $\circ$  = total binding;  $\square$  = non-specific binding in the presence of PDBu 10  $\mu\text{mol litre}^{-1}$ ;  $\triangle$  = specific binding) or presence ( $\nabla$  = total binding;  $\diamond$  = non-specific binding in the presence of PDBu 10  $\mu\text{mol litre}^{-1}$ ;  $\bullet$  = specific binding) of 2.4 vol% halothane. Scatchard analysis yielded  $K_d$  values of 18 (1.7)  $\text{nmol litre}^{-1}$  and 16 (2.1)  $\text{nmol litre}^{-1}$ , and  $B_{\text{max}}$  values of 3.9 (0.11)  $\text{pmol/mg protein}$  and 4.1 (0.16)  $\text{pmol/mg protein}$  in the absence or presence of halothane, respectively. A representative experiment, which was repeated four times, is shown.

cytosol fraction under basal conditions, which is consistent with the results of previous studies.<sup>34 41</sup> Treatment with phorbol esters has been shown to induce translocation of cytosolic PKC activity and down-regulation of membrane PKC activity in crude<sup>27</sup> and purified<sup>34</sup> synaptosomes. Exposure of intact purified synaptosomes to PMA 0.1  $\mu\text{mol litre}^{-1}$  for 10 min resulted in a significant reduction in membrane PKC activity with no change in cytosol PKC activity. Exposure of synaptosomes to 2.4 vol% halothane for 10 min also reduced membrane PKC activity, although not as much as PMA alone, but increased cytosol PKC activity. Exposure of synaptosomes to both PMA and halothane further reduced membrane PKC activity to undetectable

levels and significantly reduced cytosol PKC activity compared with control, PMA alone or halothane alone.

#### PHORBOL ESTER BINDING

Phorbol esters bind to the regulatory domains of and activate cPKC and nPKC, but not aPKC.<sup>27 42</sup> The effects of various treatments on the distribution of [ $^3\text{H}$ ]PDBu binding sites in intact synaptosomes is shown in table 1. The cytosol fraction of rat cortical synaptosomes contained a four-fold excess of [ $^3\text{H}$ ]PDBu binding sites compared with the membrane fraction. Exposure to PMA markedly reduced both membrane and cytosol [ $^3\text{H}$ ]PDBu binding as a result of translocation and down-regulation.<sup>27 34</sup> The reduction in binding could be caused by residual PMA from the synaptosome preincubation competing with [ $^3\text{H}$ ]PDBu binding, however, this effect has been reported to be reduced by anion exchange chromatography and washing before assay.<sup>34</sup> Exposure of synaptosomes to halothane had no significant effect on the subcellular distribution or amount of [ $^3\text{H}$ ]PDBu binding. Treatment with a combination of both halothane and PMA reduced [ $^3\text{H}$ ]PDBu binding to both the membrane and cytosol fractions, but this effect was not statistically different from treatment with PMA alone.

The effect of halothane on equilibrium binding of [ $^3\text{H}$ ]PDBu was also assessed. A representative experiment is shown in figure 1. There was no significant difference between [ $^3\text{H}$ ]PDBu binding to cerebrocortical synaptosomes in the absence ( $K_d = 15$  (SD 2)  $\text{nmol litre}^{-1}$ ;  $B_{\text{max}} = 4.5$  (1.1)  $\text{pmol/mg protein}$ ) or presence ( $K_d = 16$  (7)  $\text{nmol litre}^{-1}$ ;  $B_{\text{max}} = 4.7$  (1.3)  $\text{pmol/mg protein}$ ) of 2.4 vol% halothane ( $n = 4$ ).

#### PKC IMMUNOREACTIVITY

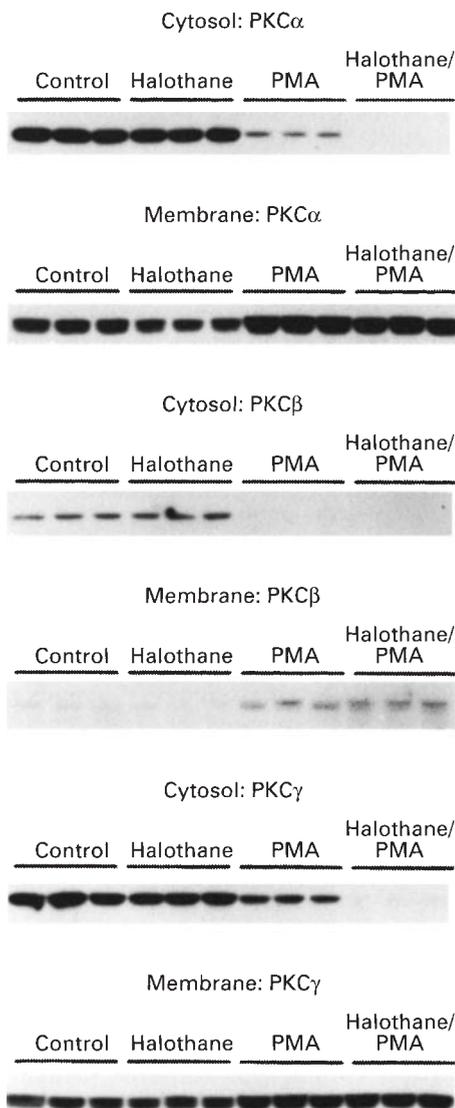
Isoform-selective antibodies to PKC were used to determine the distribution of cPKC $\alpha$ ,  $\beta$  and  $\gamma$  immunoreactive protein in control and treated synaptosomes by immunoblotting (fig. 2). All three

**Table 2** Subcellular distribution of conventional PKC isoforms in synaptosomes exposed to phorbol ester or halothane, or both. Intact synaptosomes were incubated at 37°C for 10 min in the presence of 0.2% DMSO (control) or PMA 0.1  $\mu\text{mol litre}^{-1}$  and/or 2.4 vol% halothane with 0.2% DMSO, followed by synaptosome subfractionation as described in Materials and methods. Data are mean (SD) of one independent experiments with individual assays performed in triplicate. This experiment was repeated twice with similar results. AU = arbitrary units; U = unit (= pmol/min); PDBu = phorbol-12,13-dibutyrate; PMA = phorbol-12-myristate, 13-acetate; PKC = protein kinase C. \* $P < 0.05$  vs control; \*\* $P < 0.01$  vs control; † $P < 0.05$  vs halothane; †† $P < 0.01$  vs halothane; ‡ $P < 0.05$  vs PMA; ‡‡ $P < 0.01$  vs PMA

Treatment	PKC $\alpha$ immunoreactivity		PKC $\beta$ immunoreactivity		PKC $\gamma$ immunoreactivity	
	AU/ng	% Control	AU/ng	% Control	AU/ng	% Control
Control						
Cytosol	38.4 (4.4)	—	2.06 (0.32)	—	29.5 (7.6)	—
Membrane	6.58 (0.58)	—	0.15 (0.02)	—	4.77 (0.33)	—
PMA						
Cytosol	4.43 (0.36)**††	12	0.25 (0.03)**††	12	9.76 (0.93)**††	33
Membrane	14.3 (1.0)**††	217	0.47 (0.08)**††	313	7.67 (0.09)**††	161
Halothane						
Cytosol	33.2 (1.4)*	86	2.95 (0.64)**	143	25.6 (2.0)	86
Membrane	5.30 (0.12)‡	80	0.10 (0.02)‡	67	4.62 (0.47)	97
Halothane+PMA						
Cytosol	0.22 (0.18)**††‡	0.5	0.014 (0.011)**††‡	1	0.56 (0.09)**††‡	2
Membrane	13.6 (0.5)**††	206	0.55 (0.04)**††	367	8.00 (0.63)**††	168

cPKC isoforms (PKC $\alpha$ ,  $M_r = 82\ 000$ ; PKC $\beta$ ,  $M_r = 80\ 000$ ; PKC $\gamma$ ,  $M_r = 80\ 000$ ) were enriched in the cytosol fraction compared with the membrane fraction under control conditions (table 2). The

relative amounts of the three cPKC isoforms in purified rat cerebrocortical synaptosomes detected with these antibodies (PKC $\alpha > \text{PKC}\gamma > \text{PKC}\beta$ ) do not reflect the actual relative amounts caused by differences in antibody cross-reactivity. Treatment with PMA induced both translocation of all three isoforms of PKC from the cytosolic to the membrane fraction (evident in the significant increase in membrane-associated PKC) and down-regulation of PKC (evident in the reduced cytosolic and total PKC). The effect of PMA on PKC $\gamma$  was less marked. This is consistent with a previous study that showed PKC $\gamma$  to be less sensitive to PMA-induced translocation and down-regulation than PKC $\alpha$  or PKC $\beta$ .<sup>27</sup> Halothane caused a small reduction in cytosolic PKC $\alpha$  and an increase in cytosolic PKC $\beta$ . Combined treatment with halothane and PMA resulted in synergistic reductions in the cytosolic levels of all three cPKC isoforms. There were no significant changes in membrane levels, although total immunoreactivity was reduced; this indicates an increase in both translocation and degradation of PKC, as proteolysis occurs primarily with the membrane bound, and not the cytosolic, form of the enzyme.



## Discussion

Most neurones in the CNS contain the  $\alpha$ ,  $\beta$  and/or  $\gamma$  subspecies of the cPKC isoforms.<sup>19 47</sup> PKC $\alpha$  and

**Figure 2** Effects of halothane and phorbol ester on the subcellular localization of specific conventional PKC isoforms in rat cerebrocortical synaptosomes. Synaptosomes were incubated at 37°C for 10 min in the absence or presence of 2.4 vol% halothane, phorbol-12-myristate 13-acetate (PMA) 0.1  $\mu\text{mol litre}^{-1}$ , or both, and separated into cytosolic and membrane fractions as described in the Materials and methods. Aliquots of each fraction (cytosol protein 20  $\mu\text{g}$ ; membrane protein 60  $\mu\text{g}$ ) in triplicate were separated by SDS-PAGE, transferred to nitrocellulose sheets and immunoblotted with antibodies to PKC $\alpha$ , PKC $\beta$ 1 and PKC $\beta$ 2, or PKC $\gamma$ . PKC immunoreactivity was detected by the binding of <sup>125</sup>I-labelled protein A and autoradiography (shown), and quantified by PhosphorImager analysis. The portion of the immunoblot containing PKC immunoreactive bands ( $M_r = 80\text{--}82\ 000$ ) is shown for clarity; no other immunoreactive bands were identified.

PKC $\beta$  are expressed in both CNS and peripheral tissues, while PKC $\gamma$  is restricted to the brain and adrenal glands.<sup>19</sup> Within the CNS, PKC $\gamma$  and PKC $\beta$ 1 are found predominantly in nerve terminals and growth cones, while PKC $\gamma$  and PKC $\beta$ 2 are predominantly postsynaptic<sup>19 43 44</sup>; cortical synaptosomes contain PKC $\alpha$ , PKC $\beta$  and PKC $\gamma$ .<sup>44</sup> The relative abundance and function of the nPKC or aPKC isoforms in synaptosomes have not been determined. Activation of PKC in synaptosomes or cultured neurones by diacylglycerol or tumour-promoting phorbol esters (long-acting agents that mimic the effects of diacylglycerol, which is rapidly metabolized) results in "translocation" of cPKC from the cytosol to the membrane fraction. Persistent activation of cPKC by diacylglycerol or phorbol esters results in "down-regulation" of PKC as a result of proteolysis with loss of enzyme activity and immunoreactivity. PMA causes an increase in particulate (membrane-associated) PKC activity ("translocation") at low doses (10 nmol litre<sup>-1</sup>) followed by a dose- and time-dependent decrease in both cytosolic and particulate PKC activity ("down-regulation") in synaptosomes.<sup>34</sup>

Phorbol ester-induced translocation of cPKC in synaptosomes has been analysed previously by enzyme activity and phorbol ester binding measurements.<sup>27 34</sup> In this study we have compared the effects of phorbol ester or halothane, or both, on PKC translocation and down-regulation by three independent methods of PKC analysis: enzyme activity, phorbol ester binding and immunoblotting. Down-regulation of membrane PKC was the predominant effect of phorbol ester treatment observed for both PKC activity and [<sup>3</sup>H]PDBu binding, while both translocation and down-regulation were observed for PKC immunoreactivity. Our results for the effects of phorbol ester on PKC activity and [<sup>3</sup>H]PDBu binding generally agreed with those of previous studies.<sup>27 34</sup> Translocation of cPKC by phorbol ester has not been studied previously in synaptosomes by immunoblotting. However, comparable results have been reported by Chen and colleagues<sup>42</sup> in C<sub>6</sub> glioma cells in which 12-O-tetradecanoylphorbol 13-acetate 0.1  $\mu$ mol litre<sup>-1</sup> induced a seven-fold increase in membrane PKC $\alpha$  and significantly reduced cytosolic PKC $\alpha$ . Of the three methods used to detect PKC, measurement of PKC immunoreactivity is the most direct method and least sensitive to artefacts,<sup>28</sup> although together they provide complementary information. Possible artefacts affecting measurement of PKC activity include the presence of endogenous PKC inhibitors,<sup>35</sup> non-linear reaction conditions, sub-optimal assay conditions, substrate accessibility and inadequate concentrations of the PKC inhibitor peptide. Possible artefacts affecting measurement of [<sup>3</sup>H]PDBu binding include residual phorbol ester carried over from the preincubation with PMA (unlikely given the subcellular fraction preparation technique<sup>34</sup>) and the presence of endogenous phorbol ester binding competitors. Possible artefacts affecting measurement of PKC immunoreactivity include lack of antibody specificity and PKC degradation by proteolysis, the latter of which could also

affect the two other methods. However, no immunoreactive breakdown products were detected in the immunoblots. Together these data indicate that caution is required in comparing the results of studies of PKC subcellular distribution determined by different methods.<sup>28</sup> The discrepancy between the activity measurements and [<sup>3</sup>H]PDBu binding or immunoreactivity may reflect interactions between PKC and inhibitory regulatory protein(s) that modulate PKC activity in the membrane or cytosol, or both.<sup>35</sup>

We did not detect an effect of halothane on equilibrium [<sup>3</sup>H]PDBu binding in synaptosomes. Activation of purified rat brain PKC by PDBu *in vitro* is also insensitive to halothane (unpublished observations). Our previous studies showed that halothane increases the sensitivity of PKC to activation by diacylglycerol, an endogenous PKC activator that binds to the same site in the regulatory domain of PKC as phorbol esters.<sup>45</sup> These results suggest that the interactions with PKC of phorbol esters and diacylglycerol differ in their sensitivity to halothane.

Halothane alone had no significant effects on PKC translocation or down-regulation measured by [<sup>3</sup>H]PDBu binding or PKC immunoreactivity using the selective antibody to PKC $\gamma$ . However, significant reproducible effects of halothane were observed on cytosol PKC activity and on cytosol PKC $\alpha$  and PKC $\beta$  immunoreactivity. Overall, the effects of 2.4 vol% halothane (which is close to its EC<sub>50</sub> for activation of purified PKC and is 2.3 times the MAC at 37 °C in rat<sup>46</sup>) on PKC distribution determined by the three methods were not consistent. In contrast, the combination of 2.4 vol% halothane and PMA 0.1  $\mu$ mol litre<sup>-1</sup> consistently reduced cytosolic PKC $\alpha$ / $\beta$ / $\gamma$  immunoreactivity (increased PKC translocation) compared with treatment using PMA or halothane alone. The combination also reduced membrane PKC (increased PKC down-regulation) when measured by PKC activity, although this effect was not evident with [<sup>3</sup>H]PDBu binding or the isoform-selective PKC antibodies. The enhanced translocation of PKC induced by halothane does not appear to involve a change in the ability of PKC to bind phorbol ester. This suggests that halothane stimulates the translocation process itself, perhaps by increasing the affinity of activated PKC for its membrane receptor.

We have shown previously that halothane and propofol stimulate purified PKC activity *in vitro*<sup>14 45</sup> and endogenous synaptosomal PKC activity *in situ*.<sup>15</sup> As prolonged stimulation of endogenous PKC with phorbol esters or diacylglycerol can promote cPKC translocation and down-regulation, we have analysed the effects of halothane on the subcellular distribution and down-regulation of cPKC in synaptosomes. Our results indicated that halothane alone had no significant effects on PKC translocation and down-regulation; The combination of halothane and the phorbol ester PMA resulted in significant potentiation of both translocation and down-regulation of synaptosomal PKC activity and immunoreactivity observed with PMA alone. These findings suggest that general anaesthetics may potentiate PKC translocation and

down-regulation as a result of activation of PKC *in vivo*. Halothane thus has two distinct actions on the PKC signalling pathway in nerve terminals (synaptosomes): (1) activation of endogenous PKC activity that leads to increased phosphorylation of PKC substrates<sup>15</sup> and (2) potentiation of phorbol ester-induced cPKC translocation and down-regulation. The interactions between general anaesthetics and PKC are therefore complex; the ultimate outcome of these interactions depends on the tissue examined, PKC isoforms present, specific anaesthetic agent, dose and duration of anaesthetic treatment and possibly the coincident activation of PKC by endogenous PKC activators.

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