Alkylphosphocholines inhibit choline uptake and phosphatidylcholine biosynthesis in rat sympathetic neurons and impair axonal extension

Elena POSSE de CHAVES,*† Dennis E. VANCE,*‡ Robert B. CAMPENOT§ and Jean E. VANCE*†||

*Lipid and Lipoprotein Research Group and Departments of †Medicine, ‡Biochemistry and §Anatomy and Cell Biology, University of Alberta, Edmonton, AB, Canada T6G 2S2

At least 50 % of the major axonal membrane lipid, phosphatidylcholine, of rat sympathetic neurons is synthesized *in situ* in axons [Posse de Chaves, Vance, Campenot and Vance (1995) *J. Cell Biol.* **128**, 913–918]. In the same study we reported that, in a choline-deficient model for neuron growth, phosphatidylcholine synthesis in cell bodies is neither necessary nor sufficient for growth of distal axons. Rather, the local synthesis of phosphatidylcholine in distal axons is required for normal axon growth. We have now used three alkylphosphocholines (hexadecylphosphocholine, dodecylphosphocholine and octadecylphosphocholine) as inhibitors of PtdCho biosynthesis in a compartmented model for culture of rat sympathetic neurons. The experiments reveal that alkylphosphocholines decrease the

uptake of choline into these neurons and inhibit PtdCho synthesis, but not via an effect on the activity of the enzyme CTP: phosphocholine cytidyltransferase. We also show that when the distal axons, but not the cell bodies, are exposed to alkylphosphocholines, axonal elongation is inhibited, which is consistent with the hypothesis that phosphatidylcholine synthesis in axons, but not in cell bodies, is required for axonal elongation. The inhibitory effect of alkylphosphocholines on axon growth is most likely not mediated via a decrease in the activity of protein kinase C, since when this enzyme activity is down-regulated by treatment of the cells with phorbol ester, the alkylphosphocholines retain their ability to inhibit axonal growth.

INTRODUCTION

During axonal elongation and regeneration, newly made membrane materials must be supplied to the growing axons. It has been suggested that this process is mediated by fusion of intracellular vesicles with surface membranes [1]. Such membranous transport vesicles have been presumed to be formed from proteins and lipids synthesized exclusively in cell bodies [2–5]. However, during our investigations into the source of axonal membrane lipids in cultured rat sympathetic neurons we demonstrated, using radiolabelling experiments, that biosynthesis of the major phospholipids phosphatidylcholine (PtdCho), phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol, as well as fatty acids, but not cholesterol, occurs in pure axons [6,7].

In most of our studies we have used a compartmentalized model for culture of rat sympathetic neurons [8] (Figure 1). A major advantage of this three-compartment culture system is that metabolic events occurring in distal axons can be studied independently of those occurring in cell bodies. Using this model of neuron growth we have confirmed, by direct assay of the activity of three enzymes involved in the biosynthesis of PtdCho via the CDP-choline pathway [7], that synthesis of PtdCho occurs in axons. The specific activities of each of these enzymes in distal axons, and in the compartment containing cell bodies with proximal axons, are similar in magnitude. We have also shown that the rate-limiting step in the CDP-choline biosynthetic pathway in intact neurons, as well as in axons, is the formation of CDP-choline, catalysed by the enzyme CTP: phosphocholine cytidyltransferase [7].

Recently, we have demonstrated that local production

of PtdCho in axons contributes approx. 50 % of total axonal PtdCho [9]. Moreover, we have provided evidence that PtdCho biosynthesis is inhibited when neurons are depleted of choline, an essential precursor of PtdCho. We have also shown that a lack of choline in distal axons impairs neurite growth. In contrast, when choline is removed from the cell bodies, extension of distal neurites continues normally. These results imply that PtdCho synthesis in cell bodies alone is not sufficient for normal axonal extension, whereas PtdCho synthesis in axons is required for axonal growth.

In the present study we have used three antineoplastic alkylphosphocholines, namely dodecylphosphocholine (DoPC), hexadecylphosphocholine (HePC) and octadecylphosphocholine (OcPC), as potential inhibitors of PtdCho biosynthesis in either distal axons alone, or in the cell bodies/proximal axons, of rat sympathetic neurons. Haase et al. [10] have previously shown that these alkylphosphocholines inhibit PtdCho biosynthesis in Madine–Darby canine kidney cells by disruption of the CDP-choline pathway. These analogues have been proposed to affect the translocation of the enzyme CTP: phosphocholine cytidyltransferase from its inactive form in the cytosol to membranes where the enzyme is activated [11,12]. The alkylphosphocholines also elicit an antiproliferative effect that parallels their ability to inhibit PtdCho biosynthesis [12]. The inhibitory properties of these phosphocholine analogues depend upon the length of their alkyl chain as well as the nature of the polar head-group [12]. In Madine–Darby canine kidney cells the analogue DoPC did not decrease the incorporation of [³H]choline into PtdCho and was therefore used as a negative control.

We present evidence that the alkylphosphocholines inhibit PtdCho biosynthesis in rat sympathetic neurons, and that the

Abbreviations used: DoPC, dodecylphosphocholine; HePC, hexadecylphosphocholine; OcPC, octadecylphosphocholine; PtdCho, phosphatidylcholine.

|| To whom correspondence should be sent.

Figure 1 Compartmented culture system for rat sympathetic neurons

Compartmented culture showing (a) an entire culture and (b) an enlargement of a single track. Dissociated neurons were plated in the centre compartment (density about one ganglion per dish). Neurites extended to the left and right compartments under silicone-grease barriers and into separate fluid environments. The tracks were formed on the collagen-coated floor of a 35-mm-diameter plastic tissue-culture dish between a series of parallel scratches from which the dried collagen substratum had been scraped. Each track was $\approx 200 \mu\text{m}$ wide, the centre compartments were $\approx 1 \text{ mm}$ wide and the barriers were about 0.5 mm wide. One culture contained up to 20 tracks occupied by neurons. Distal neurites were harvested by mechanically rinsing neurites from the left and right compartments with a jet of distilled water delivered with a syringe and a 22-gauge hypodermic needle. Cell bodies and proximal neurites were harvested by removal of the Teflon divider after the distal neurites have been removed, and all remaining cellular material was collected.

reduction of PtdCho synthesis in axons, but not in cell bodies, impairs axonal elongation.

MATERIALS AND METHODS

Materials

All radiolabelled chemicals, namely [*methyl*- ^3H]choline (specific radioactivity 15 Ci/mmol), [*methyl*- ^{14}C]choline (specific radioactivity 55 mCi/mmol) and [9,10- ^3H]palmitate (specific radioactivity 54 Ci/mmol) were purchased from Amersham Canada, Oakville, ON, Canada. Phospho[*methyl*- ^3H]choline (specific radioactivity $5\text{--}7 \text{ mCi/mmol}$), the substrate for assay of the enzyme CTP:phosphocholine cytidyltransferase, was synthesized enzymically in our laboratory from [*methyl*- ^3H]choline, ATP and choline kinase (purchased from Sigma) as described in [13]. TLC plates (silica-gel G; 0.25 mm thickness) were obtained from BDH Chemicals, Edmonton, AB, Canada. Standard phospholipids were either isolated from rat liver or were purchased from Avanti Polar Lipids, Birmingham, AL, U.S.A. Choline, phosphocholine, CDP-choline, CTP, oleate, palmitate, PtdCho and phorbol 12-myristate 13-acetate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). HePC, OcPC and DoPC were generously given by Dr. C. Geilen, Freie Universität, Berlin, Germany. All other reagents were from either Sigma or Fisher Scientific.

L15 medium without antibiotics (GIBCO Laboratories, Grand Island, NY, U.S.A.) was used for culture of neurons and was supplemented with the additives prescribed by Hawrot and Patterson [14]. Mouse 2.5 S nerve growth factor was purchased from Cedarlane Laboratories Ltd. (Hornby, ON, Canada).

Cell culture

The general culture procedure was as previously reported [15]. Briefly, neurons from superior cervical ganglia of newborn Sprague-Dawley rats were enzymically and mechanically dissociated and plated either at a density of two ganglia/well in a 24-well plate coated with collagen or into culture dishes divided into three compartments ($0.5 \text{ ganglion/dish}$). Compartmented cultures were constructed as previously described [16]. Briefly, collagen was dried on to 35-mm-diameter Falcon tissue-culture dishes, and a series of tracks was then formed between parallel scratches made in the collagen of the dish floor. A Teflon divider was seated on the dish with silicon grease. Neurons, plated in the centre compartment, settled on the substratum of the collagen, and neurites elongated on the tracks, crossing under the silicon grease barriers to enter left and right compartments (Figure 1). The L15 culture medium did not contain antibiotics, but was supplemented with the additives prescribed by Hawrot and Patterson [14], including bicarbonate and methylcellulose. The culture media contained $11 \mu\text{M}$ choline but no palmitate. Rat serum (2.5%; University of Alberta Laboratory Animal Services) and ascorbic acid (1 mg/ml) were supplied only in medium given to the centre compartment. Culture medium was routinely changed every 3–6 days. Non-neuronal cells were eliminated by exposure for the first 6 days to medium containing $10 \mu\text{M}$ cytosine arabinoside. Initially all three compartments were supplied with 2.5 S nerve growth factor (200 ng/ml) to allow cell survival and to allow neurite growth in all compartments. Nerve growth factor was discontinued in the centre compartment after 6 days, so that subsequent neurite growth was largely confined to the nerve-growth-factor-containing side compartments [8,17].

Cells were routinely cultured for 9–13 days prior to the start of each experiment. The axons elongated at a rate of approx. 1 mm/day. Each compartment contained a separate fluid environment; there was virtually no bulk flow or diffusion of small molecules or ions among the compartments [16,17].

In some experiments, cultures were neuritotomized and regeneration was monitored. Neuritotomy was performed by removal of the distal neurites in left and right compartments by washing with a jet of sterile distilled water delivered with a syringe through a 22-gauge needle. The wash was repeated twice, then fresh culture medium was added. This procedure reliably removes virtually all visible traces of neurites from the side compartments.

Neurite extension on individual collagen tracks was measured on a Nikon Diaphot inverted microscope using a microscope digitizer (Minnesota Datametrics Corp., Minneapolis, MN, U.S.A.) of resolution 2 μm .

Experimental treatments of neurons

The three alkylphosphocholines (HePC, DoPC and OcPC) were dissolved in water to make 10 mM stock solutions which were added to medium to give the desired final concentrations. The phorbol ester phorbol 12-myristate 13-acetate was dissolved in DMSO to make a 2 mM stock solution, which was added to culture medium to give a final concentration of 2 μM . The final concentration of DMSO in culture medium was 0.1%, and media of control cultures for the experiments using phorbol ester contained 0.1% DMSO without phorbol ester.

Incorporation of radiolabelled precursors into PtdCho and Intermediates of PtdCho synthesis

Neurons in 24-well dishes were incubated with medium containing [*methyl*- ^3H]choline or [^3H]palmitate in the presence or absence of one of the alkylphosphocholines. At indicated incubation times, radiolabelled medium was aspirated and cells were washed twice with ice-cold PBS. Cellular material was collected into methanol/water (1:1, v/v). Chloroform was added to give a chloroform/methanol/water mixture of proportions 2:1:1 (by vol.). Lipids were extracted as described by Folch et al. [18] and separated by TLC in the solvent system chloroform/methanol/acetic acid/formic acid/water (35:15:6:2:1, by vol.). The bands corresponding to authentic PtdCho, phosphatidylethanolamine and phosphatidylserine were scraped from the plates and radioactivity was measured. Radioactivity was normalized to total phospholipid mass. Incorporation of [^3H]choline into water-soluble precursors of PtdCho was measured in some experiments as follows. The upper aqueous phase from the Folch extraction, which contained choline, phosphocholine and CDP-choline, was evaporated to dryness and the residue was dissolved in methanol/water (1:1, v/v). The components were separated by TLC in a solvent system consisting of ammonia/methanol/0.6% NaCl (1:10:10, by vol.). The bands corresponding to authentic standards of choline, phosphocholine and CDP-choline were scraped from the plate and incorporation of radioactivity was measured. Data are expressed as d.p.m./nmol of total phospholipid.

Choline uptake

Neurons cultured in 24-well dishes were preincubated for 24 h in medium containing or lacking 50 μM of one of the alkylphosphocholines. Cultures were washed three times with Krebs–Ringer buffer [124 mM NaCl/5.0 mM KCl/1.5 mM CaCl_2 /

1.3 mM MgCl_2 /20 mM Hepes/NaOH (pH 7.4)/10 mM glucose] as described by Rylett et al. [19]. [*methyl*- ^3H]Choline (15 $\mu\text{Ci/ml}$) in Krebs–Ringer buffer was added and cells were incubated at 37 °C for 5 min. At indicated times, cells were washed with Krebs–Ringer buffer and harvested into methanol/water (1:1, v/v). The cellular suspension was sonicated for 10 s using a probe sonicator, and an aliquot was removed for the measurement of radioactivity. Choline uptake is expressed as d.p.m. in total [^3H]choline metabolites/nmol of total phospholipid/5 min.

CTP:phosphocholine cytidyltransferase assay

Neurons that had been plated in 24-well dishes were washed twice with ice-cold PBS and harvested with 100–150 μl /well of buffer containing 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.025% sodium azide and 0.05 mM PMSF. Cells were sonicated for 10 s and the sonicated material was centrifuged at 350 000 *g* for 15 min in a Beckman TLA-100.2 fixed-angle rotor. The supernatant was designated as ‘cytosol’ and the pellet as ‘total membranes’. All fractions were either used immediately or stored at –70 °C. The cytidyltransferase activity was measured by a published procedure [20] using [*methyl*- ^3H]phosphocholine as substrate in the presence of PtdCho/oleate vesicles. The protein content of cells and subcellular fractions was measured with the BCA (bicinchoninic acid) protein assay reagent (Pierce Chemical Co., Rockford, IL, U.S.A.).

Other methods

The phospholipid content of cells was measured by lipid phosphorus determination [21].

RESULTS

Alkylphosphocholines inhibit choline uptake and PtdCho synthesis in rat sympathetic neurons

As an alternative to depriving cells of choline, PtdCho biosynthesis has also been reported to be inhibited by alkylphosphocholines such as HePC [11]. We therefore examined the effects of HePC, DoPC and OcPC, individually, on PtdCho biosynthesis in rat sympathetic neurons. In the first experiment, 13-day-old neuron cultures in 24-well dishes were incubated for 24 h with [*methyl*- ^3H]choline in the presence or absence of 50 μM of a phosphocholine analogue. The incorporation of radiolabelled precursors into phosphocholine and PtdCho was measured. The data in Table 1 show that all three alkylphosphocholines reduced the incorporation of [^3H]choline into PtdCho. For HePC, DoPC and OcPC the percentage inhibitions were 35.8, 33.1 and 59.9% respectively. The incorporation of [^3H]choline into phosphocholine was diminished by approximately the same extent as for PtdCho, i.e. by 35.0% for HePC, by 40.2% for DoPC, and by 69.3% for OcPC. We also investigated whether or not choline uptake into the neurons was influenced by alkylphosphocholines. Neurons were cultured in 24-well dishes and incubated for 24 h with 50 μM HePC, DoPC or OcPC. The cells were subsequently exposed to [*methyl*- ^3H]choline for 5 min, and choline incorporation into the cells (i.e. choline uptake) was measured. After 24 h incubation with HePC, DoPC or OcPC, choline uptake was reduced by 29, 43 and 63% respectively (Table 1).

From these results we cannot ascertain whether or not the alkylphosphocholines inhibited PtdCho synthesis, for the following reason. If the pool size of phosphocholine remained unchanged upon treatment with the analogues, the specific radioactivity of phosphocholine would be lower in cells treated

Table 1 Alkylphosphocholines inhibit choline uptake and PtdCho synthesis in neurons

Neurons that had been cultured in 24-well dishes for 13 days were treated for 24 h with 50 μ M HePC, DoPC, OcPC or no supplement as a control. The neurons were subsequently exposed to 15 μ Ci/ml [3 H]choline for 5 min and choline uptake was determined. [3 H]Choline incorporation into phosphocholine and PtdCho was measured in neurons that had been incubated with medium containing 10 μ Ci/ml [3 H]choline in the presence or absence of 50 μ M HePC, DoPC or OcPC for 24 h. Cells were collected and phosphocholine and PtdCho isolated by TLC. Results are means $\pm$ S.E.M. ($n = 10$) Statistical significance of differences compared with control samples (untreated cells) was calculated by the Student's t test ($*P < 0.05$). The experiment was repeated twice with similar results. Abbreviation: PL, phospholipid.

Treatment	Choline uptake ($10^{-3} \times$ d.p.m./ 5 min per nmol of PL)	[3 H]Choline incorporation ($10^{-3} \times$ d.p.m./nmol of PL)	
		Phosphocholine	PtdCho
None	918.8 $\pm$ 28.1	107.3 $\pm$ 8.8	708.3 $\pm$ 27.2
HePC	656.2 $\pm$ 19.5*	69.7 $\pm$ 11.4*	454.4 $\pm$ 27.8*
DoPC	523.9 $\pm$ 15.1*	64.2 $\pm$ 2.4*	473.8 $\pm$ 30.2*
OcPC	342.7 $\pm$ 11.1*	32.9 $\pm$ 5.6*	284.1 $\pm$ 15.7*

Table 2 Alkylphosphocholines inhibit palmitate incorporation into PtdCho

Neurons, 13 days old, cultured in 24-well dishes were incubated in the presence or absence of 50 μ M of one of HePC, DoPC or OcPC for 24 h. [3 H]Palmitate (5 μ Ci/ml) was present during the last 3 h of incubation. Cells were collected and lipids were extracted and isolated by TLC. Results are means $\pm$ S.D. ($n = 6$). Statistical significance of differences compared with control samples (untreated cells) was calculated by the Student's t test ($*P < 0.05$). The experiment was repeated with similar results. Abbreviation used: PL, phospholipid.

Treatment	[3 H]Palmitate incorporation ($10^{-3} \times$ d.p.m./nmol of PL)		
	PtdCho	PtdEtn	PtdSer
None	268.1 $\pm$ 24.7	35.0 $\pm$ 6.1	2.78 $\pm$ 0.3
HePC	171.2 $\pm$ 25.9*	36.4 $\pm$ 7.8	2.60 $\pm$ 0.4
DoPC	117.1 $\pm$ 27.1*	39.8 $\pm$ 1.1	2.50 $\pm$ 0.2
OcPC	67.4 $\pm$ 7.5*	30.8 $\pm$ 4.1	2.40 $\pm$ 0.3

with the inhibitor than in untreated cells. Hence, the lower incorporation of [3 H]choline into PtdCho would be the consequence of the lower specific radioactivity of phosphocholine, and PtdCho synthesis *per se* would not have been reduced by the inhibitors. Alternatively, if the alkylphosphocholines had reduced the pool size of phosphocholine, the specific radioactivity of phosphocholine might be similar to that in untreated samples. Therefore, the decreased incorporation of [3 H]choline into PtdCho would reflect a decreased rate of conversion of phosphocholine into PtdCho. Unfortunately, because only small amounts of neuronal material were available, we were unable to measure the mass of phosphocholine in cells. Therefore, as an alternative approach for determining whether or not the alkylphosphocholines inhibited PtdCho synthesis, we used [3 H]palmitate as the precursor of PtdCho. Neurons were treated with the alkylphosphocholines for 24 h as in the above experiments. [3 H]Palmitate was added for the last 3 h, and incorporation of radiolabel into PtdCho, phosphatidylethanolamine and phosphatidylserine was measured. Table 2 shows that the analogues HePC, DoPC and OcPC reduced the incorporation of [3 H]palmitate into PtdCho by 36, 56 and 75% respectively. Moreover, the inhibition was specific for PtdCho synthesis, because HePC, DoPC and OcPC did not decrease the in-

corporation of [3 H]palmitate into phosphatidylethanolamine or phosphatidylserine. The combined results from these experiments suggest that the alkylphosphocholines inhibit choline uptake by the neurons and, in addition, decrease the rate of PtdCho biosynthesis.

Geilen et al. [22] have reported that, in Madine-Darby canine kidney cells, the alkylphosphocholines inhibit PtdCho biosynthesis by blocking the translocation of CTP:phosphocholine cytidyltransferase from cytosol to membranes. We therefore evaluated whether or not the inhibition of PtdCho synthesis by the alkylphosphocholines was due to an effect on the cytidyltransferase by direct measurement of this enzyme activity in subcellular fractions of neurons that had been treated for 24 h with 50 μ M of one of the phosphocholines. The phosphocholine analogues did not significantly inhibit the activity of the cytidyltransferase either in membranes or in the cytosolic fraction. The specific activity of the cytidyltransferase in membranes was 0.90 ± 0.15 nmol/min per mg of protein for untreated cells, 0.89 ± 0.21 nmol/min per mg of protein for HePC-treated cells, 0.96 ± 0.15 nmol/min per mg of protein for DoPC-treated cells and 0.96 ± 0.20 nmol/min per mg of protein for OcPC-treated cells. In cytosol, the cytidyltransferase activities were 0.58 ± 0.19 , 0.75 ± 0.17 , 0.66 ± 0.13 and 0.89 ± 0.13 nmol/min per mg of protein for untreated cells and cells treated with HePC, DoPC and OcPC respectively.

Our conclusions from these experiments are that the alkylphosphocholines (i) decrease the uptake of choline into the neurons, and (ii) inhibit PtdCho biosynthesis by a mechanism that does not involve a reduction in activity of the cytidyltransferase.

Addition of alkylphosphocholine to axons inhibits neurite extension

The response of neurite growth to the presence of HePC, DoPC and OcPC was investigated. Rat sympathetic neurons were plated in the centre compartment of compartmented culture dishes (Figure 1) for 13 days. Distal neurites were removed from the side compartments and allowed to regenerate for 3 days, after which either the right-hand side compartment alone, the centre compartment alone, or both the centre and right-hand compartments, were supplied with medium containing 50 μ M of HePC. In all cultures, the left-hand compartment contained no HePC throughout the experiment and acted as a control. As shown in Figure 2, for cultures given HePC in the right-hand, distal axon-containing compartment alone (Ax), or in both the right and centre compartments (CB + Ax), neurite growth completely ceased after treatment with HePC. Neurites of cultures to which HePC had been added to the cell-body-containing compartment alone were unaffected and continued to elongate at the same rate as untreated cells during the 4-day treatment period. Only in those cultures to which HePC had been added to the distal axons did axonal growth cease.

Überall et al. [23] have reported that HePC binds to serum proteins, and more recently, Fleer et al. [24] have demonstrated that the rate of uptake of HePC by cells cultured in the presence of serum proteins is lower than in their absence. Therefore a possible explanation for why axonal growth was unaffected by addition of HePC to the cell-body-containing compartment was that the inhibitor bound to serum proteins supplied to the centre compartment and thereby reduced the effective concentration of available HePC. This possibility was eliminated by repetition of the experiment depicted in Figure 2, but with medium lacking serum in all compartments. As in the experiment depicted in Figure 2, when HePC was added to the cell-body-containing

Figure 2 HePC inhibits neurite growth

Eight neuron cultures were neuritotomized on day 13, and 2.8 days later (arrow) were given 50 μ M HePC in the right-hand compartment only (Ax, $\circ$), the right-hand and central compartments (CB + Ax, $\triangle$) or the central, cell-body-containing, compartment alone (CB, $\square$). In all cultures the left-hand compartment contained no HePC (Control, $\blacksquare$). Cultures given medium without HePC in all compartments throughout the experiment exhibited the same extension as controls (results not shown). Results are means $\pm$ S.E.M. from measurements of neurite extension in 60–64 tracks. The experiment was repeated three times with similar results.

Figure 3 Neurite extension in cultures incubated with 50 μ M HePC immediately after neuritotomy

Eight neuron cultures were neuritotomized after 13 days of growth, then treated with HePC-containing medium ($\downarrow$) in the side compartments containing distal axons alone (Ax, $\triangle$, $\blacktriangle$), or in the central compartment alone (CB, $\circ$). After 3 days ($\uparrow$) the medium was removed and fresh medium without HePC was given to some cultures (Ax, $\triangle$) while other cultures were incubated with HePC (Ax, $\blacktriangle$). Control cells ($\square$) were given medium without HePC in all compartments throughout the experiment. Each point represents the mean $\pm$ S.E.M. for measurement of neurite extension in 60–64 tracks. The experiment was repeated twice with similar results.

compartment alone in the absence of serum, the distal axons grew at the same rate as did axons of untreated cells (results not shown).

A possible explanation for the inhibitory effect of HePC on neurite growth was that HePC was generally toxic to the cells. Therefore, as an indication of the toxicity of the alkylphosphocholines, HePC was added to the compartmented cultures immediately after neuritotomy (i.e. after 13 days of growth). When the analogue was added to the distal axon-containing compartment, essentially no further neurite growth occurred (Figure 3). However, if after 3 days of treatment HePC was removed and fresh medium containing no HePC was added,

Figure 4 Alkylphosphocholines induce retraction and degeneration of distal axons

Neurons were cultured in compartmented culture dishes for 13 days, then neuritotomized. After an additional 3 days of growth the neurons were supplied with 50 μ M HePC, DoPC or OcPC in either the distal axon-containing compartments (a) or the cell body-containing compartment (b). Neurite extension was measured just prior to (day 2.8), or one day after (day 4), addition of the alkylphosphocholine. Results are means $\pm$ S.E.M. of measurements from 52–54 tracks for each treatment. The experiment was repeated three times with similar results.

neurite growth resumed at approximately the normal rate. This result shows that, under the conditions used, the effect of HePC on neurite growth was reversible. Another indication that the inhibitors did not exert a direct cytotoxic effect was that when alkylphosphocholines were added to the cell-body-containing compartment alone, neurites extended at the normal rate (Figure 3).

Additional experiments were performed with the other two alkylphosphocholines, DoPC and OcPC (Figure 4). Cells were neuritotomized after 13 days in culture, and after an additional 3 days one of the alkylphosphocholines was added to the distal axon-containing side compartment. Neurite extension was measured both before addition of the analogue (day 2.8 in Figure 4) and after 1 day in the presence of the analogue (day 4 in Figure 4). After 1 day of treatment with HePC, axon length did not increase, whereas in the presence of DoPC, and especially OcPC, the neurites retracted and degenerated (Figure 4a). As was the case for HePC, when either DoPC or OcPC was added to the cell-body-containing compartment alone, neurite elongation was unaffected (Figure 4b). The experiments were repeated using 100 μ M alkylphosphocholine, and essentially the same results were obtained.

Inhibition of axonal elongation by alkylphosphocholines is not mediated by inactivation of protein kinase C

Alkylphosphocholines have been recognized as inhibitors of protein kinase C, as well as being inhibitors of PtdCho biosynthesis [22,23,25]. Therefore we attempted to determine if the inhibitory effects of alkylphosphocholines on neurite growth were due to inactivation of protein kinase C. Neurons were plated in the centre compartment and allowed to grow for 13 days, after which neurites were removed and allowed to re-

Figure 5 Retraction of distal neurites in response to alkylphosphocholines is not mediated by inhibition of certain protein kinases C

Neurite extension was measured in six cultures that had been neuritotomized after 13 days of growth and subsequently given $2 \mu\text{M}$ phorbol 12-myristate 13-acetate (PMA) in all compartments. Neurite extension was measured 4.0 days later. At that time, medium in the left-hand compartment, containing distal axons alone, was replaced with medium containing $2 \mu\text{M}$ PMA (■). The right-hand, distal-axon-containing, compartment was given medium containing $50 \mu\text{M}$ HePC (PMA + HePC, ●), DoPC (PMA + DoPC, □) or OcPC (PMA + OcPC, ○), in addition to $2 \mu\text{M}$ PMA. Cultures given PMA in both side compartments exhibited the same neurite extension as did untreated control cells (results not shown). Results are means $\pm$ S.E.M. of measurements from 30–32 tracks for each treatment. In some cases error bars are included within the symbols. The experiment was repeated twice with similar results.

generate for 4 days in the presence of $2 \mu\text{M}$ phorbol 12-myristate 13-acetate in all compartments. Such a treatment has previously been shown to downregulate the expression of protein kinase C in compartmented cultures of sympathetic neurons from rats [26]. We wished to determine if, under conditions for which protein kinase C activity had been downregulated, the inhibitory effect of the alkylphosphocholines on neurite growth was still apparent. After treatment of the cells for 4 days with phorbol ester, media in all compartments were removed and replaced with fresh medium containing phorbol ester in all compartments. Distal axons in the right-hand compartment were supplied with medium containing $50 \mu\text{M}$ HePC, DoPC or OcPC, and neurite extension was measured after an additional 1 and 3 days. The left-hand compartment contained no alkylphosphocholine. Figure 5 shows that even when protein kinase C activity had been down-regulated, the presence of alkylphosphocholines in the distal axon-containing compartments potentially impaired axonal growth and caused neurite retraction. The data therefore suggest that the effect of alkylphosphocholines on axonal elongation was not mediated by inhibition of protein kinase C activity. Rather, inhibition of PtdCho biosynthesis, and possibly other unidentified effects caused by the alkylphosphocholines, had inhibited axon growth.

DISCUSSION

PtdCho biosynthesis was inhibited in rat sympathetic neurons by three analogues of phosphocholine (an intermediate in the synthesis of PtdCho from choline), and the effects on axonal growth were examined. Geilen et al. [11,12] have previously shown that, in Madine–Darby canine kidney cells, HePC and OcPC, but not DoPC, inhibit PtdCho biosynthesis by blocking translocation of the enzyme CTP:phosphocholine cytidylyltransferase from cytosol to membranes. In rat sympathetic neurons we found that all three alkylphosphocholines reduced

the incorporation of [^3H]choline and [^3H]palmitate into PtdCho. The uptake of choline, the initial substrate for the CDP-choline pathway, was significantly diminished upon treatment of neurons with any of the three alkylphosphocholines. Although the biosynthesis of PtdCho was also inhibited, the mechanism of inhibition was not via a decrease in the activity of the cytidylyltransferase.

These findings in neurons differ from those in Madine–Darby canine kidney cells, in which Geilen and co-workers concluded that alkylphosphocholines did not inhibit choline uptake, but rather reduced the rate of PtdCho biosynthesis via an effect on the cytidylyltransferase [11,12]. One possible explanation for the apparent differences between our studies and those reported by Geilen and co-workers is that, in the choline-uptake experiments, we preincubated the neurons with the inhibitors for 24 h, then measured [^3H]choline uptake, whereas Geilen and co-workers exposed the cells to inhibitors for only 2 min. An alternative explanation might be that the phosphocholine analogues affected the functioning of certain membrane proteins present exclusively in neurons, for example, the Na^+ -dependent high-affinity choline transporter [27–29]. We have shown in our laboratories that, in rat sympathetic neurons, choline uptake is reduced by approx. 80% upon incubation with Na^+ -free medium (M. Bussire, R. B. Campenot, D. R. Ure, J. E. Vance and D. E. Vance, unpublished work), suggesting that the high-affinity choline transporter is active in these neurons. Alternatively, the activity of other important proteins, such as the Na^+ , K^+ -ATPase, might be affected by the alkylphosphocholines, since in purified rat brain synaptosomal membranes HePC competes with Na^+ for this protein [25].

The observation that alkylphosphocholines inhibited axonal elongation when added to distal axons, but not to cell bodies, complements our studies in which PtdCho synthesis was inhibited by choline deficiency [9]. When alkylphosphocholines were added to the distal axon-containing compartments, neurite elongation ceased, whereas when the inhibitors were added only to the compartment containing cell bodies, distal axonal growth continued normally. The inhibitory effect of the phosphocholine analogues was reversible, as shown by resumption of axonal extension upon removal of the inhibitors. Moreover, addition of alkylphosphocholines to the cell-body-containing compartment did not retard axonal growth. It should be noted, however, that after 3 days of treatment with either DoPC or OcPC in the cell-body-containing compartment, the growth rate of axons was slightly diminished (by 10–13%) compared with that in untreated cultures.

The results with alkylphosphocholines cannot be explained by selective entry of the compounds into the axons and exclusion from the cell bodies, since these compounds very readily partition into both plasma membranes and intracellular membranes [30]. Moreover, the association of HePC with membranes is not receptor-mediated, nor is a HePC-specific pump protein present, at least in Madine–Darby canine kidney cells [24].

The finding that treatment of axons with alkylphosphocholines completely blocked axonal growth, whereas when PtdCho synthesis was inhibited by choline deficiency axonal growth was inhibited by only 50% [9], suggests that alkylphosphocholines may inhibit other important processes in addition to PtdCho synthesis, especially since PtdCho synthesis was reduced to a similar extent by both perturbations. We considered the possibility that the effect of HePC and its homologues on neurite extension might be mediated by inhibition of other membrane-bound enzymes. For example, it was originally suggested that HePC blocked cell proliferation via inhibition of protein kinase C activity [22]. However, more recently Geilen et al. [12] have

suggested that the antiproliferative effect of HePC might also be mediated by inhibition of PtdCho biosynthesis. Other studies have shown that alkylphosphocholines inhibit protein kinase C activity in 3T3 fibroblasts by competing with a biological activator of protein kinase C, phosphatidylserine [23].

Protein kinase C activity in neurons can be downregulated with the phorbol ester phorbol 12-myristate 13-acetate, which is a short-term activator, but a long-term downregulator, of protein kinase C [31] in many cell types, including PC12 cells [32,33] and rat sympathetic neurons [26,32]. Our studies show that even when protein kinase C activity had been downregulated with phorbol ester [26], inhibition of neurite growth by the alkylphosphocholines persisted. The results of this type of experiment must be interpreted with caution, however, because protein kinase C consists of a family of at least eight isoenzymes which vary in their mode of regulation and substrate specificity [34]. For example, some isoenzymes [35] do not depend on Ca^{2+} for their activity. Consequently, changes in their activities would not be detected in the standard assay, such as that used by Campenot et al. [26]. In addition, some isoenzymes are not downregulated by phorbol esters [35]. In this light, we conclude that the mechanism by which alkylphosphocholines inhibit neurite growth is not via inhibition of the known phorbol-ester-downregulatable, Ca^{2+} -dependent protein kinase C isozymes.

In conclusion, our data are consistent with the hypothesis that alkylphosphocholines reduce the rate of PtdCho biosynthesis in axons and thereby inhibit axonal growth, although additional, unknown, cellular processes might also be impaired. Moreover, our data support our previous observations that choline-deficiency-induced inhibition of PtdCho biosynthesis in axons inhibits neurite extension, whereas synthesis of PtdCho in cell bodies does not appear to be essential for axonal growth.

We thank Grace Martin for preparation of neuron cultures and Dr. Christoph Geilen at the Freie Universität, Berlin, Germany, for the gift of the alkylphosphocholines. This work was supported by grants from the Rick Hansen Foundation and the Alberta Paraplegic Foundation. D.E.V. is a Medical Scientist, R.B.C. a Scholar, and E.P. de C. a Postdoctoral Fellow, of the Alberta Heritage Foundation for Medical Research.

REFERENCES

- Pfenninger, K. H. (1982) in *Axoplasmic Transport in Physiology and Pathology* (Weiss, D. G. and Gorio, A., eds.), pp. 52–61, Springer-Verlag, Heidelberg
- Pfenninger, K. H. and Johnson, M. P. (1983) *J. Cell Biol.* **97**, 1038–1042
- Leeden, R. W. (1985) in *Phospholipids in Nervous Tissues* (Eichberg, J., ed.), pp. 135–172, John Wiley and Sons, New York
- Toews, A. D. and Morell, P. (1985) in *Phospholipids in the Nervous System*, vol. 2: *Physiological Roles* (Horrocks, L. A., Kanfer, J. N. and Porcellati, G., eds.), pp. 299–314, Raven Press, New York
- Alberts, B., Bray, D., Lewis, J., Roberts, K. and Watson, J. D. (1989) in *Molecular Biology of the Cell*, 2nd edn., pp. 1059–1136, Garland Press, New York
- Vance, J. E., Pan, D., Vance, D. E. and Campenot, R. B. (1991) *J. Cell Biol.* **115**, 1061–1068
- Vance, J. E., Pan, D., Campenot, R. B., Bussire, M. and Vance, D. E. (1994) *J. Neurochem.* **62**, 329–337
- Campenot, R. B. (1982) *Dev. Biol.* **93**, 1–12
- Posse de Chaves, E., Vance, D. E., Campenot, R. B. and Vance, J. E. (1995) *J. Cell Biol.* **128**, 913–918
- Haase, R., Wieder, T., Geilen, C. and Reutter, W. (1991) *FEBS Lett.* **288**, 129–132
- Geilen, C. C., Weider, T. and Reutter, W. (1991) *J. Biol. Chem.* **267**, 6719–6724
- Geilen, C. C., Haase, A., Wieder, T., Arndt, D., Zeisig, R. and Reutter, W. (1994) *J. Lipid Res.* **35**, 625–632
- Vance, D. E., Pelech, S. D. and Choy, P. C. (1981) *Methods Enzymol.* **71**, 576–581
- Hawrot, E. and Patterson, P. H. (1979) *Methods Enzymol.* **28**, 574–584
- Campenot, R. B., Walji, A. H. and Draker, D. D. (1991) *J. Neurosci.* **11**, 1126–1139
- Campenot, R. B. (1979) *Methods Enzymol.* **28**, 302–307
- Campenot, R. B. and Draker, D. D. (1989) *Neuron* **3**, 733–743
- Folch, J., Lees, M. and Sloane-Stanley, G. H. (1959) *J. Biol. Chem.* **226**, 497–509
- Rylett, R. J., Goddard, S. and Lambros, A. (1993) *J. Neurochem.* **61**, 1388–1397
- Houweling, M., Jamil, H., Hatch, G. M. and Vance, D. E. (1994) *J. Biol. Chem.* **269**, 7544–7551
- Chalvardjian, A. and Rudnicki, E. (1970) *Anal. Chem.* **36**, 225–226
- Geilen, C. C., Haase, R., Buchner, K., Weider, T., Hucho, F. and Reutter, W. (1991) *Eur. J. Cancer* **27**, 1650–1653
- berall, F., Oberhuber, H., Maly, K., Zaknun, J., Demuth, L. and Gunicke, H. H. (1991) *Cancer Res.* **51**, 807–812
- Fleer, E. A. M., Bercovick, D., Eibl, H. and Unger, C. (1993) *Lipids* **28**, 731–736
- Zheng, B., Oishi, K., Shoji, M. et al. (1990) *Cancer Res.* **50**, 3025–3031
- Campenot, R. B., Draker, D. D. and Senger, D. L. (1994) *J. Neurochem.* **63**, 868–878
- Guyenet, P., Lefresne, P., Rossier, J., Beaujouan, J. C. and Glowinski, J. (1973) *Mol. Pharmacol.* **9**, 630–639
- Suszkiw, J. B. and Pilar, G. (1976) *J. Neurochem.* **26**, 1133–1138
- Kuhar, M. J. and Murrin, L. C. (1978) *J. Neurochem.* **30**, 15–21
- Geilen, C. C., Wieder, T., Haase, A., Reutter, W., Morr, D. M. and Morr, D. J. (1994) *Biochim. Biophys. Acta* **121**, 14–22
- Ballester, R. and Rosen, O. M. (1985) *J. Biol. Chem.* **260**, 15194–15199
- Matthies, H. J. G., Palfrey, H. C., Hirning, L. D. and Miller, R. J. (1987) *J. Neurosci.* **7**, 1198–1206
- Heasley, L. E. and Johnson, G. L. (1989) *J. Biol. Chem.* **264**, 8646–8652
- Nishizuka, Y. (1992) *Science* **258**, 607–614
- Parker, P. J., Kour, G., Marais, R. M. et al. (1989) *Mol. Cell. Endocrinol.* **65**, 1–11