Comparison of the Effects of Carbisocaine and Other Local Anesthetics on ^{32}P Incorporation Into Individual and Total Phospholipids in Synaptosomes

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Abstract. The aim of the paper was to study the effect of carbisocaine, a new local anesthetic with high liposolubility on incorporation of ^{32}P into individual and total phospholipids and to compare its effect with that of other local anesthetics (procaine, lidocaine, cinchocaine, heptacaine). Carbisocaine decreased ^{32}P incorporation into neutral phospholipids and increased the incorporation into acid phospholipids, presumably by inhibiting phosphatidate phosphohydrolase, similarly as reported for other anesthetics (Brindley and Bowley 1975). The increased incorporation of ^{32}P into phosphatidylserine induced by carbisocaine suggests that this phospholipid is also synthetised from phosphatidic acid. At low concentrations, the local anesthetics studied were found to increase ^{32}P incorporation into total phospholipids, whereas at high concentrations they reduced ^{32}P incorporation. This biphasic effect is in agreement with the incorporation of ^{14}C from glucose into lipids (Laššánová et al. 1984) and with the effect of cinchocaine on glycerol incorporation into phospholipids (Allan and Michell 1975), suggesting that local anesthetics affect de novo synthesis of phosphatidic acid. Carbisocaine increased ^{32}P incorporation into phospholipids, in concentrations lower by several orders of magnitude as compared to the other local anesthetics studied. A rough correlation was observed between the concentrations at which the local anesthetics showed stimulatory effect on ^{32}P incorporation, and the average effective concentrations of the respective anesthetics. No such correlation could be found for carbisocaine.

Key words: Phospholipids — Synaptosomes — Local anesthetics — Carbisocaine

Introduction

The ability of local anesthetics to interact preferably with acidic phospholipids and thus to change the physical properties and metabolic turnover of the latters

seems to be involved in a number of membrane properties and functions, such as permeability of membranes, transport of ions, functions of receptors, as well as in inhibition of excitation of nerve membranes (Papahadjopoulos et al. 1975; Brindley et al. 1975; Salway and Hughes 1972). The interaction of local anesthetics with acidic phospholipids results in a number of biochemical changes. Increased phosphatidylinositol and phosphatidic acid labeling is an accompanying phenomenon and it correlates with the local anesthetic activity (Eichberg and Hauser 1974). The mechanism of this local anesthetics action is an increase in de novo phosphatidylinositol synthesis (Allan and Michell 1975); this mechanism is different from that underlaying the increased phosphatidylinositol and phosphatidic acid labeling induced by a physiological stimulus (e.g. acetylcholine) causing an accelerated turnover of the phosphorylinositol group in the phosphatidylinositol molecule (Michell 1975).

Phosphatidylinositol and phosphatidic acid stimulation induced by local anesthetics is based on the ability of these drugs to block the synthesis of neutral lipids in glycerolipid metabolism and to redirect it towards accumulation of acidic phospholipids (Eichberg and Hauser 1974). These changes are evoked by the ability of local anesthetics to inhibit phosphatidic acid phosphohydrolase (Brindley and Bowley 1975) and to stimulate phosphatidic acid cytidyltransferase (Sturton and Brindley 1977). The inhibition is supposed to be caused by physical interaction between positively charged local anesthetics and phosphatidic acid, which leads to neutralization of the negatively charged phosphate group (Brindley and Bowley 1975). The same complex can be a substrate for phosphatidic acid cytidyltransferase which is stimulated by these drugs and which can be inhibited only by relatively high concentrations of local anesthetics.

Acidic phospholipids play an important role in many biochemical processes in biological membranes. They bind preferably with membranes proteins (Aronstam et al. 1977), with some membrane enzymes, e.g. with Na,K-ATP-ase (Tanaka 1971) and Ca-ATP-ase (Niggli et al. 1981). These enzymes can be activated by acidic phospholipids, mainly by phosphatidylserine. Phosphatidylinositol is able to inhibit Na,K-ATP-ase (Nishikawa et al. 1985). Polyphosphoinositide metabolism plays an important role in nerve tissue (Hawthorne 1983; Agranoff and Vanrooijen 1985; Hauser and Michell 1985). Recently phosphatidylinositol 4,5-bisphosphate metabolism has gained wide interest since its hydrolysis yield two second messengers: inositol 1,4,5-trisphosphate and diacylglycerol. These two second messengers mark the beginning of a highly versatile signalling system which may have a unique role to play in modulating neural activity (Berridge 1984; Heilmeyer et al. 1985; Berridge 1986). Another acidic phospholipid, phosphatidic acid, can act as an ionophore for calcium (Harris et al. 1981; Serhan et al. 1982; Nayar et al. 1984); contrary to these

findings Holmes and Yoss (1983) demonstrated the failure of phosphatidic acid to translocate Ca^{2+} across phosphatidylcholine membranes. Interaction of local anesthetics with phospholipids can lead to changes in all the above important membrane processes.

In this work the effect of a new local anesthetic carbisocaine on ^{32}P incorporation into individual and total phospholipids in synaptosomes was compared with that of other local anesthetics.

Materials and Methods

Materials

Procaine. HCl and cinchocaine. HCl were obtained from Spofa (Czechoslovakia), lidocaine. HCl from Pfaltz and Bauer (England), heptacaine was prepared according to Čižmárik and Borovanský (1975), carbisocaine was prepared according to Beneš et al. (1979). Acetylcholine was obtained from BDH (England). All chemicals used in this study were of p.a. purity; solvents, also p.a., were distilled before experiments.

Preparation of synaptosomes

Synaptosomes from rat brain were prepared on sucrose gradient according to the method of Hajós (1975). Rat cerebral cortex was dispersed in 0.3 mol.l^{-1} sucrose with the aid of a Potter—Elvehjem homogenizer. The pellet obtained after centrifugation at $1500 \times g$ for 10 min was washed by resuspension in approximately the original volume of 0.3 mol.l^{-1} sucrose. The combined supernatants were centrifuged at $9000 \times g$ for 20 min. The pellet was dispersed in 5 ml of 0.3 mol.l^{-1} sucrose. The suspension was layered at the top of 20 ml of 0.8 mol.l^{-1} sucrose and centrifuged at $9000 \times g$ for 25 min. Particles dispersed in 0.8 mol.l^{-1} sucrose solution were synaptosomes. The composition of this fraction was evaluated by quantitative electron microscopy. Synaptosomes were 3 times diluted with incubation medium, centrifuged at $20{,}000 \times g$ for 10 min and resuspended in the incubation medium.

Incubation of synaptosomes

Synaptosomes were incubated according to Griffin et al. (1979). Synaptosomes were suspended in a buffered solution which contained (in mmol.l^{-1}): 124 NaCl; 5 KCl; 1.2 KH_2PO_4; 1.3 $MgCl_2$; 1.6 cytidine; 1.6 myo-inositol; 10 glucose; and 26 TRIS. The pH of this solution was adjusted with HCl to 7.4 or 6. Synaptosomes were incubated for 60 min at 37°C. The protein concentration in synaptosomes was 2—3 mg/ml, and 6 mg/ml upon phospholipid separation by thin layer chromatography. The proteins were determined by the method of Lowry et al. (1951).

^{32}P — labeling, extraction and separation of phospholipids

Synaptosomes were incubated in a medium which contained 10 μ Ci ^{32}P/ml, or 25—50 m Ci ^{32}P/ml for the chromatographic separation of phospholipids. The incubation was stopped by adding an equal volume of 20% trichloracetic acid (TCA). After leaving the samples at room temperature for 10 min, they were centrifuged at $1000 \times g$ for 5 min, the pellet was washed once with 5% TCA and once with water. The water-washed pellet was extracted with 4 ml chloroform/methanol/HCl (200 : 100 : 1), the extract was washed with 1 ml of 0.1 mol.l^{-1} HCl. The top phase was extracted with 2 ml chloroform/methanol/HCl (200 : 100 : 1). The lower phases were pooled, evaporated and restored at −20°C. Lipids were dissolved in chloroform/methanol (90 : 10) and separated by

two-dimensional chromatography on thin layers according to Rouser et al. (1970). The amount of phosphate in the phospholipid spots was estimated spectrophotometrically according to Rouser et al. (1970).

Estimation of radioactivity

^{32}P was determined using PACKARD liquid scintilation counter and Bray scintilate solution.

Results

The molecular structure of the local anesthetics tested is shown in Fig. 1. Procaine contains an ester group, lidocaine and cinchocaine have amide groups. Heptacaine (*o*-heptyloxy-[2(piperidino) ethyl]-1-carbanilate) and carbisocaine (*o*-heptyloxy-[1-methyl-2(diethylamino) ehtyl]-1-carbanilate) contain carbanilate groups.

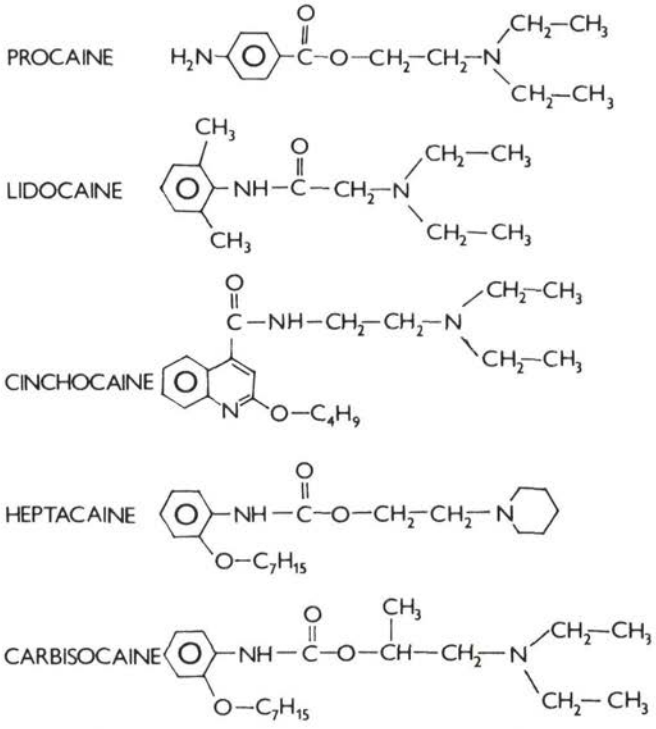

Fig. 1. The molecular structure of the local anesthetics tested.

In agreement with Yagihara et al. (1973) and Venkov et al. (1986), we found that acidic phospholipids phosphatidylinositol, phosphatidylserine and phosphatidic acid represent approximately 23%, and neutral phospholipids 77% of total phospholipide in synaptosomes, (Fig. 2).

The time course of ^{32}P incorporation into individual phospholipids (Fig. 3) shows that neutral phospholipids have a negligible specific activity compared to acidic phospholipids, and that ^{32}P incorporation is most intensive within 40 min of incubation.

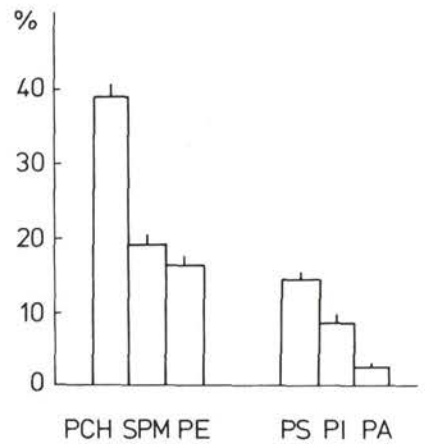

Fig. 2. Relative proportion of individual phospholipids in synaptosomes. PCH — phosphatidylcholine, SPM — sphingomyelin, PE — phosphatidylethanolamine, PS — phosphatidylserine, PI — phosphatidylinositol, PA — phosphatidic acid. Mean (± S.E.M.) of seven experiments.

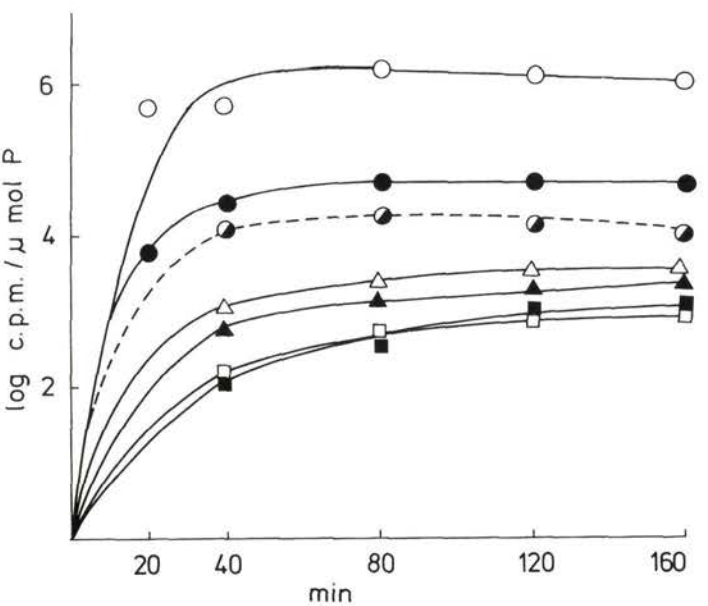

Fig. 3. Time course of ^{32}P incorporation into individual and total phospholipids. ○ — phosphatidic acid, ● — phosphatidylinositol, △ — phosphatidylserine, ▲ — phosphatidylethanolamine, ■ — phosphatidylcholine, □ — sphigomyelin, ◒ — total phospholipids. Typical example selected from three similar experiments.

Fig. 4 shows relative changes in specific radioactivities of phosphatidylinositol, phosphatidic acid and total phospholipids in the presence of acetylcholine (10^{-4} mol.l^{-1}) and carbisocaine at concentrations which stimulate (10^{-8} mol.l^{-1}) or inhibit (10^{-4} mol.l^{-1}) the incorporation of ^{32}P into phospholipids. Acetylcholine and carbisocaine at the lower concentration used, increased significantly the incorporation of ^{32}P into phosphatidylinositol, phosphatidic acid and total phospholipids. Contrary to the effect of carbisocaine, the acetylcholine-induced incorporation of ^{32}P into phosphatidylinositol was higher than that into phosphatidic acid. Carbisocaine in the higher concentration increased ^{32}P incorporation into phosphatidylinositol, but decreased ^{32}P incorporation into both phosphatidic acid and total phospholipids.

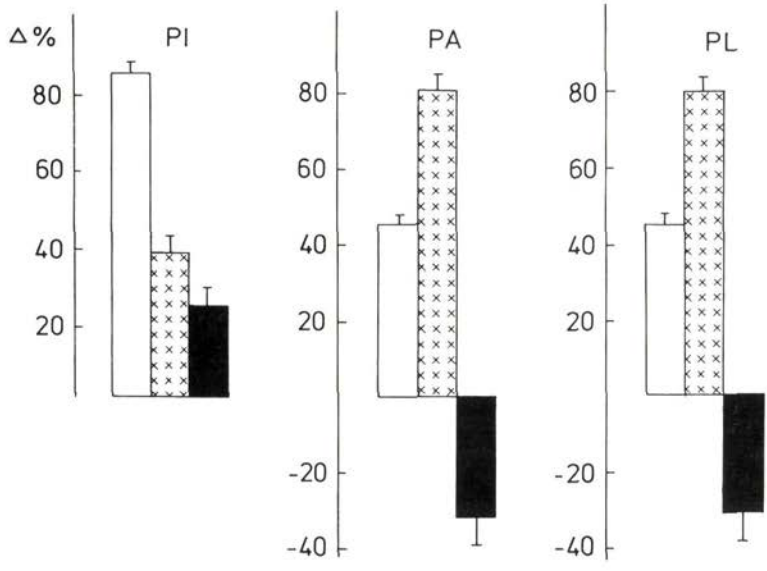

Fig. 4. Changes in relative specific activities of phosphatidylinositol (PI), phosphatidic acid (PA) and total phospholipids (PL), expressed in percentages, in synaptosomes treated by acetylcholine (10^{-4} mol.l^{-1}) □, carbisocaine (10^{-8} mol.l^{-1}) ⊠ and carbisocaine ■ (10^{-4} mol.l^{-1}). Means (± S.E.M.) of four experiments.

The effect of carbisocaine (10^{-4} mol.l^{-1}) on individual phospholipids is shown in Fig. 5. Carbisocaine inhibited the incorporation of ^{32}P into neutral phospholipids (phosphatidylethanolamine, phosphatidylcholine, sphingomyelin), and stimulated incorporation into acidic phospholipids (phosphatidylserine, phosphatidylinositol); however, the labeling of phosphatidic acid was inhibited.

Another experimental series was designed to study the incorporation of ^{32}P into total phospholipids in dependence on the concentration of highly lipid-soluble local anesthetics (carbisocaine, cinchocaine, heptacaine), (Fig. 6) and that with low lipid soluble anesthetics (lidocaine and procaine) (Fig. 7). At lower concentrations all the local anesthetics tested significantly stimulated ^{32}P incorporation into phospholipids (heptacaine insignificantly); at higher concentrations all the drugs tested, with the exception of procaine inhibited ^{32}P incorpora-

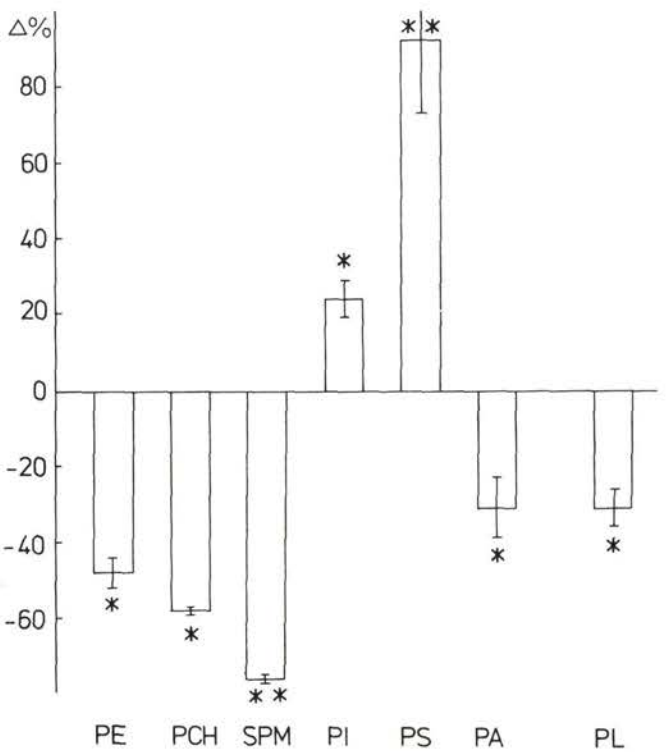

Fig. 5. Relative changes in specific radioactivities of phospholipids in synaptosomes treated by carbisocaine (10^{-4} mol.l^{-1}). For symbols see Fig. 2. Means (± S.E.M.) of four experiments. Significance vs. controls: $*P < 0.05$ $**P < 0.01$ (Student's t-test)

tion. The incorporation of ^{32}P into phospholipids was stimulated by carbisocaine (significantly at pH 7.4, insignificantly at pH 6) at concentrations lower by several orders of magnitudeas compared to other local anesthetics. The concentrations of cinchocaine, procaine and lidocaine which increased ^{32}P incorporation into phospholipids approximately correlated with the average effective concentrations of respective anesthetics on isolated nerve or axon.

The highly lipid-soluble local anesthetics (carbisocaine, cinchocaine, heptacaine) inhibited ^{32}P incorporation into phospholipids at concentrations lower by two orders of magnitude than that with low lipid solubility (lidocaine). Procaine failed to induce any significant inhibition even at the highest concentration used.

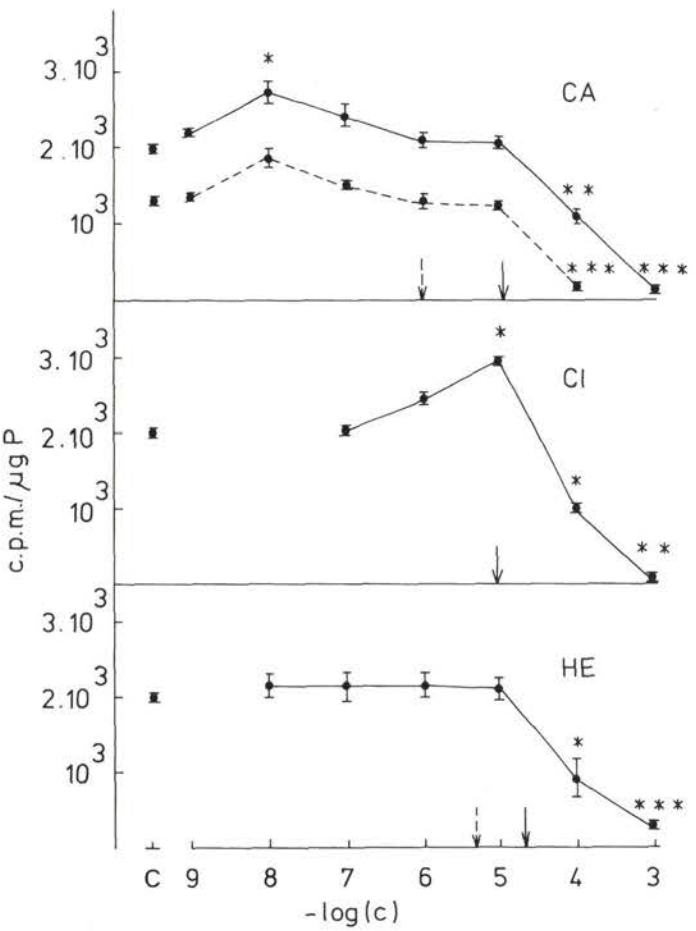

Fig. 6. Incorporation of ^{32}P into phospholipids in dependence on the concentrations of local anesthetics. ↓ average effective concentration on isolated nerve ⇣ average effective concentration on isolated axon. Mean (± S.E.M.) of triplicate determinations from a representative experiment. Significance vs. controls (C): $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Student's *t*-test), ——— pH, — — — pH6, CA — carbisocaine, CI — cinchocaine, He — heptacaine

Discussion

Relative proportions of neutral and acidic phospholipids in synaptosomes in our studies corresponded to those reported by Yagihara et al. (1973) and Venkov et al. (1986); approximately 23% acidic phospholipids and 73% neutral phospholipids.

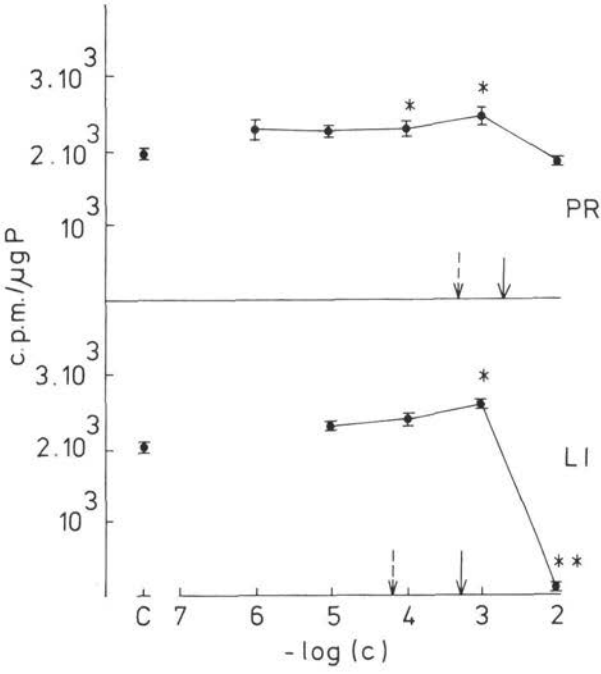

Fig. 7. Dependence of ^{32}P incorporation into phospholipids on the concentration of local anesthetics. ↓ average effective concentration on isolated nerve. ⁞ average effective concentration on isolated axon. PR — procaine, LI — lidocaine. Mean (± S.E.M.) of triplicate determinations from a representative experiment. Significance vs. controls (C): $* P < 0.05$, $** P < 0.01$, $*** P < 0.001$ (Student's *t*-test).

First, we studied the influence of acetylcholine as a physiological stimulus for the metabolic turnover of phospholipids. The observed relative changes in specific radioactivity induced by acetylcholine showed a stimulatory effect of acetylcholine on the turnover of phosphatidylinositol and phosphatidic acid. Yagihara and Hawthorne (1972) observed a higher rate of ^{32}P incorporation into phosphatidic acid in synaptosomes, whereas our results were similar to those reported by Schacht and Agranoff (1972) who showed that acetylcholine increased mainly the labeling of synaptosomal phosphatidylinositol. Yagihara et al. (1973) explained these differences by differences in synaptosome preparation.

The stimulation of phosphatidic acid and phosphatidylinositol induced by low concentration (10^{-8} mol.l^{-1}) of carbisocaine corresponded with a similar increase in ^{32}P incorporation into these phospholipids induced by some local anesthetics (Eichberg and Hauser 1974). The underlying mechanism may be inhibition of phosphatidate phosphohydrolase (Brindley and Bowley 1975) and thus divertion of the synthesis of neutral phospholipids and its direction to the synthesis of acidic phospholipids. The disproportion between the stimulation of phosphatidylinositol synthesis and the inhibition of phosphatidic acid synthesis by high carbisocaine concentration (10^{-4} mol.l^{-1}) was obviously due to profound derangement of phospholipid metabolism. Carbisocaine in such a concentration can inhibit also phosphatidate cytidyltransferase and possibly also phosphatidate glyceroltransferase which catalyses phosphatidic acid synthesis as reported by Sturton and Brindely (1977). We agree with the opinion of these authors that the effect of amphiphilic cations (including local anesthetics) on the metabolic turnover of acidic phospholipids probably depends on which enzyme reaction is the rate determining one. This may be different in various tissues and under different physiological conditions. The high carbisocaine concentration applied, significantly decreased the turnover of neutral phospholipids, which was in agreement with the assumption of the inhibition of phosphatidate phosphohydrolase. The increased synthesis of phosphatidylserine at the high carbisocaine concentration is in agreement with the results of Novotný (1982) who found that amphiphilic agents increased the incorporation of ^{32}P into phosphatidylserine. In some cases phosphatidylserine might be synthesed not only via replacement interaction of serine with ethanolamine but also via phosphatidic acid, as shown by Pullakart et al. (1981) in rat brain microsomes.

At low concentrations, the local anesthetics studied were found to increase ^{32}P incorporation into total phospholipids, whereas at high concentrations they reduced ^{32}P incorporation. This biphasic effect is similar to incorporation of ^{14}C from glucose into lipids (Laššánová et al. 1984) and to the effect of cinchocaine on glycerol incorporation into phospholipids (Allan and Michell 1975), suggesting that local anesthetics affect de novo synthesis of phospholipids. The concentrations of cinchocaine, lidocaine and procaine which stimulated incorporation of ^{32}P into phospholipids roughly corresponded to the average effective concentrations of the respective anesthetic on isolated nerve or axon. The effectivity stimulation of ^{32}P incorporation in our experiments paralleled the anesthetic potency (De Jong 1970) and decreased in the order: cinchocaine, lidocaine, procaine. These results are in agreement with those of Eichberg and Hauser (1974) who presented evidence that several tertiary local anesthetics increased the labeling of pineal phospholipids to an extent which paralleled their anesthetic potency.

Carbisocaine stimulated the incorporation of ^{32}P into phospholipids at far

lower concentrations than its average effective concentration on isolated nerve or axon (Stankovičová and Štolc 1981). The observation that carbisocaine — induced incorporation of ^{32}P into phospholipids is slower at pH 6 than at pH 7.4, although the drug has a higher biological effect at pH 6 (Stankovičová and Štolc 1980), may also be associated with the fact that the anesthetic effect of this agent is not related to stimulation of ^{32}P incorporation into phospholipids. The lower stimulation of ^{32}P incorporation into phospholipids at pH 6 can be explained by the fact that more acidic pH is less favorable than physiological pH for phospholipid metabolism enzymes. Highly lipid soluble local anesthetics (carbisocaine, cinchocaine, heptacaine) significantly inhibited incorporation of ^{32}P incorporation into phospholipids at lower concentrations (10^{-4} mol.l^{-1}) than did anesthetics with low solubility, lidocaine (10^{-2} mol.l^{-1}) or procaine. The latter did not inhibit incorporation of ^{32}P into phospholipids even at the highest concentration used. This suggests that the solubility of local anesthetics in lipids is important for their effect on the incorporation of ^{32}P into phospholipids. Our results are in agreement with the findings of other authors (Eichberg and Hauser 1974) who showed that low concentrations of local anesthetics supported electric stabilization of nerve membranes while higher concentrations damaged the membrane structure; the degree of the damage corresponded to lipid solubility of the respective drugs.

The effect of local anesthetics on ^{32}P incorporation into phospholipids depends on the solubility of these agents in lipids, as well as on their structure. In contrast to other local anesthetics, there is no correlation between the effect on ^{32}P incorporation into total phospholipids and the local anesthetic activity in carbisocaine. Carbisocaine affects ^{32}P incorporation into phospholipids in far lower concentrations as compared to other local anesthetics.

Acknowledgements. We gratefully acknowledge the valuable advice by Prof. Strunecká concerning the separation methods of phospholipids. We also thank Prof. Čižmárik for his providing us with heptacaine, and Prof. Beneš for his kindly supply of carbisocaine.

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Final version accepted May 21, 1987