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EFFECTS OF DIISOPROPYL PHOSPHOFLUORIDATE, SARIN AND SOMAN ON THE ACCESSIBILITY OF PROTEINS, IN THE ELECTROPLAX MEMBRANE, TO LACTOPEROXIDASE-CATALYZED IODINATION

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Abstract—Anticholinesterases (anti-ChE) have some effects on biological properties including behavior, vision, and electroencephalograms, which are often long lasting and which do not appear to be due to cholinesterase (ChE; EC 3.1.1.7) inhibition, but which may be due to alterations in the organization and/or functioning of the cellular membrane. We assessed the effects of anti-ChE agents on the asymmetric organization of proteins in the innervated (excitable) and in the non-innervated (non-excitable) plasma membrane of the electroplax from the electric eel. Lactoperoxidase-catalyzed iodination (LCI) was carried out under impenetrable conditions in intact electroplax (where protein exposure on the external surface is monitored) and in split electroplax (where total protein labeling on both the external and internal monolayers of the plasma membrane bilayer is monitored). Labeling in split electroplax was much greater than in intact electroplax for all molecular weight groupings of proteins (30,000 to >200,000). The anti-ChE agents diisopropyl phosphofluoridate (DFP; 10^{-3} M), sarin (10^{-4} M) and soman (10^{-4} M, 10^{-6} M, 2.5×10^{-9} M) did not alter permeability, protein content or the electrophoretic pattern of the plasma membrane proteins of the electroplax. DFP, sarin and 10^{-6} M soman (but not 2.5×10^{-9} M or 10^{-4} M soman) increased labeling of some of the molecular weight fractions in the non-innervated plasma membrane as monitored by LCI in intact electroplax. Under these same conditions, DFP and 10^{-4} M soman increased labeling in the innervated plasma membrane while 10^{-6} M soman decreased labeling. When LCI was carried out in split electroplax, 10^{-4} M soman caused a decrease in labeling in both the innervated and non-innervated plasma membrane indicating a decrease of exposed proteins on the cytoplasmic surface of the plasma membrane. These concentrations of the anti-ChE agents caused almost complete ChE inhibition in the electroplax cells, except for 2.5×10^{-9} M soman which caused little or no inhibition. These results suggest that alterations in protein asymmetry, as monitored by LCI of accessible proteins, are not directly due to ChE inhibition. These changes in organization of membrane proteins could contribute to a variety of effects of anti-ChE agents which are not due to ChE inhibition.

Organophosphate anticholinesterase (anti-ChE‡) agents cause many neurological symptoms resulting from cholinesterase (ChE; EC 3.1.1.7) inhibition and subsequent hyperactivity of the cholinergic nervous system [1]. In addition, organophosphates cause effects, sometimes long lasting, which do not appear to be due to ChE inhibition. The concern about these effects has led to a multinational epidemiological study on neurotoxic effects of low-level exposure to organophosphorus pesticides by the United Nations Development Program and the World Health Organization [2]. Some of these effects, which are rather general and, in turn, must be due to more primary changes, include anxiety, restlessness, behavioral changes, insomnia, emotional instability, neurosis, taste aversion, excessive dreaming and memory impairment [3–8]. Other more specific effects by anti-ChE agents, including sarin and soman, which cannot be directly related to ChE inhibition, include alterations in the human and primate electroencephalogram which last more than one year following exposure to anti-ChE agents [4, 6, 9–12], disturbances in vision [13–15], mutagenicity induced in microorganisms [16], alterations in hormonal rhythms observed 2 weeks following exposure to a single dose of soman [17] and direct interaction with a K^+ channel in guinea pig atria [18].

The varied nature of the above effects suggests that they could be due to changes in the structure or organization of the cell membrane and its receptors and may be independent of or only triggered by the initial inhibition of ChE. Indeed, anti-ChE agents have already been shown to cause alterations in membrane constituents independent of ChE inhibition. Most of these reported changes, however, relate to the lipid component of membranes including a decrease in the cholesterol-to-phospholipid ratio in rat liver [19], increased permeability and fluidity in liposomal membranes [20, 21], increased fluidity in superior cervical ganglia [22], enhanced hydrolysis of phosphatidylcholine in rat

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‡ Abbreviations: anti-ChE, anticholinesterase; ChE, cholinesterase; DFP, diisopropyl phosphofluoridate; and LCI, lactoperoxidase-catalyzed iodination.

brain [23] and alteration of phosphatidylcholine [24] and aminophospholipid asymmetry* in the electroplax membrane. Effects of anti-ChE agents, including soman and sarin, on membrane proteins other than ChE include alterations in high-affinity choline uptake in synaptosomes [25, 26], Ca^{2+} uptake by sarcoplasmic reticulum [27], binding to muscarinic and nicotinic receptors [28–33], inhibition of protein synthesis in neuroblastoma cells [34] and decreased guanylate cyclase activity in rat brain [35]. No studies, however, have sought to identify changes in protein asymmetry in a bioelectrically excitable tissue, changes which could reflect modifications of protein organization and function.

As an example of a bioelectrically excitable tissue, we selected the electroplax cells from the Sachs organ of the electric eel [36]. It is possible to split the electroplax cell in half and thus obtain both an innervated bioelectrically excitable and a non-innervated, non-excitable surface from a single cell and compare anticholinesterase effects on these dissimilar plasma membranes [37–40]. The innervated conducting plasma membrane receives neural inputs (~50,000 synapses/cell) and shows sensitivities to drugs which would be expected for a cholinergic junction while the non-innervated plasma membrane is non-conducting and is enriched in Na^+,K^+-ATPase [36–40]. These cells are thus uniquely suited for studies on membrane asymmetry. From the isolated half-cell preparations (split electroplax), we can isolate partially purified innervated and non-innervated plasma membrane fractions. By exposing intact and split electroplax to an impenetrable protein membrane probe, we can look for changes in protein accessibility in the inner and outer monolayers of the plasma membrane bilayer, i.e. changes in membrane asymmetry.

To examine the effects of anti-ChE agents on membrane proteins, we decided to use lactoperoxidase-catalyzed iodination (LCI), although other protein probes available include immunological probes, cross-linking reagents, enzymatic cleavage, and labeling of proteins [41, 42]. LCI has the advantage that its properties are well known since it has been widely used for protein and asymmetry studies [41–49] and the iodination technique required for its use as an impenetrable probe is relatively simple. In addition, the amino acids (tyrosine and histidine) iodinated by lactoperoxidase are not the ones attacked by anti-ChE agents in the esteratic site of the active center of ChE.

This study is therefore of special interest since no comparable study has been carried out in a bioelectrically excitable tissue. For the first time we are able to compare protein exposure to an external probe in an electrically excitable and a non-excitable plasma membrane from the same cell. We selected soman, sarin and DFP for these studies because they are extremely potent and have been reported to produce some of the long-lasting effects noted previously. We were able to demonstrate that they altered the protein asymmetry in the electroplax membrane in a manner suggesting an effect(s) independent of ChE inhibition.

* Rosenberg P and Weber B, unpublished observations.

MATERIALS AND METHODS

Materials. Diisopropyl phosphofluoridate (DFP) was purchased from the Aldrich Chemical Co. (Milwaukee, WI) and stored at 4°. Soman (*O*-1,2,2-trimethylpropylmethyl phosphonofluoridate) and sarin (*O*-isopropylmethyl phosphonofluoridate) were provided by the United States Army Medical Research and Development Command as solutions (~2 mg/mL of isopropanol) of over 90% purity. These solutions were stored at −80° and diluted immediately prior to use. Lactoperoxidae (EC 1.11.1.7; 60–80 units/mg protein), glucose oxidase (EC 1.1.3.4; type VII, 50,000 units/0.374 mg), and acetylcholinesterase (EC 3.1.1.7; type VI S, 220 units/mg protein) were all purchased from the Sigma Chemical Co. (St. Louis, MO). $Na^{125}I$ (carrier free in 0.1 M NaOH solution) was obtained from New England Nuclear (Boston, MA). The specific activity was 17.4 Ci/mg iodine and the radioactive concentration 20 mCi/mL at the activity reference date. [U-^{14}C]Sucrose was obtained from the Amersham Co. (Arlington Heights, IL) as an aqueous solution containing 200 μCi/mL (1.53 mCi/mg) with a radiochemical purity of 99%. Prestained molecular weight standards for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were obtained from Bethesda Research Laboratories (Gaithersburg, MD) and consisted of myosin, H-chain (200,000), phosphorylase b (97,400), bovine serum albumin (68,000), ovalbumin (43,000) and α-chymotrypsinogen (25,700). Acrylamide, *N,N'*-methylenebisacrylamide, SDS, tetramethylethylenediamine (TEMED), ammonium persulfate and Coomassie brilliant blue R-250 were all purchased from Bio-Rad Laboratories (Richmond, CA). Other chemicals were of reagent grade and obtained commercially.

Dissection of electroplax cells. Live electric eels (*Electrophorus electricus*) approximately 1.5 m in length were obtained from Worldwide Scientific Animals (Apopka, FL). The eels were decapitated upon arrival and the Sachs organ was sliced into approximately 6–8 sections, each 4 cm in length. A slice of Sachs organ was placed in eel Ringer's solution (160 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 1.2 mM Na_2HPO_4, 10 mM glucose, pH 7.4) which was oxygenated throughout the dissection. A triple row of cells was removed from the slice and mounted in a dissecting chamber where it was held rigid in a frame and covered with oxygenated eel Ringer's solution. By cutting between the innervated membrane and the connective tissue compartment membrane, a row of cells was separated into intact single electroplax cells (~0.2 × 2 × 10 mm) using a Zeiss stereomicroscope and microdissecting instruments [36]. To split the cells in half, the connective tissue at the edge of each membrane surface was grasped with forceps; the innervated and non-innervated surfaces were then separated using a teasing procedure causing the cell to split in a plane of least resistance along the mid-plane of the cell [37–40].

Exposure of electroplax cells to organophosphorus compounds. Groups of 30 intact electroplax cells (~3.5 mg protein) were exposed in 10 mL of eel

Ringer's solution to 10^{-3} M DFP, 10^{-4} M sarin, 10^{-4} M, 10^{-6} M or 2.5×10^{-9} M soman or to control eel Ringer's solution for 30 min at room temperature. Following incubation the cells were washed extensively with eel Ringer's solution. All incubations were performed in a hood using special safety precautions.*

Lactoperoxidase-catalyzed iodination (LCI) of electroplax. LCI of exposed proteins, under impenetrable conditions, was performed using methods described in the literature for iodination of cells and proteins [50–53], with the final best conditions being similar to those used for labeling the acetylcholine receptor in electric tissue [54]. Groups of 30 control or anti-ChE-treated intact cells or separated innervated or non-innervated surfaces (split cell preparations) were incubated in scintillation vials at room temperature in a 5 mL final volume of eel Ringer's solution containing 39.5 units of lactoperoxidase, 55 μCi of $Na^{125}I$, 25 μmol of glucose and 1.34 units of glucose oxidase. The glucose oxidase was added last, followed by immediate closing of the vial to generate H_2O_2 and to start the reaction. After 15 min of incubation with shaking, the reaction was stopped by addition of 5 mL of a termination solution composed of (mM) $Na_2S_2O_3$, 0.2; NaI, 20; iodoacetamide, 10; sodium azide, 10; EDTA, 10; phenylmethyl sulfonylfluoride (PMSF), 1; pH 7.4. The cells were then washed six times, each wash being 10 mL of cold eel Ringer's solution containing (mM): iodoacetamide, 5; sodium azide, 5; EDTA, 5; PMSF, 0.5; pH 7.4. The intact cells or separated surfaces were then collected immediately by filtration or centrifugation, respectively. The intact cells were separated into innervated and non-innervated surfaces and all tissues were stored at −80° until isolation of the plasma membrane-enriched fraction. Any changes in membrane integrity induced by this freezing would not be critical since the separation into surfaces and the LCI of the proteins was carried out prior to this freezing.

Preparation of plasma membrane fraction. Plasma membrane-enriched fractions were isolated from the separated innervated and non-innervated surfaces of the electroplax using a differential centrifugation procedure. Briefly, the separated surfaces were homogenized in 5 vol. of ice-cold 0.25 M sucrose and protease inhibitor buffer (5.0 mM Tris, pH 7.4, 5.0 mM iodoacetamide, 5.0 mM EDTA, 5.0 mM sodium azide and 0.5 mM PMSF) using a Polytron homogenizer (3 × 10 sec bursts at 20-sec intervals and half-maximal speed). Plasma membrane sheets were then isolated by centrifugation of the homogenate at 600 *g* for 10 min at 4°. The pellet was resuspended with a glass tissue homogenizer in ~0.5 mL of protease inhibitor buffer without sucrose and stored at −80°. A description of the purity of the plasma membrane fraction has been published [24]. As judged by measurements of Na^+,K^+-ATPase (EC 3.6.1.3) as a marker for plasma membrane and cytochrome oxidase (EC 1.9.3.1) as a marker for mitochondria, the plasma membrane fractions of the innervated and non-innervated surfaces were enriched in plasma membranes about 5- and 6-fold, respectively, over that in the crude homogenate and the plasma membrane fraction was contaminated by less than 20% with mitochondrial membranes. Contamination by other cellular membranes is not considered significant since quantitative morphometric analysis of 16 electron micrographs of the innervated and non-innervated half-cell preparations indicated that all membranes other than plasma and mitochondrial account together for 10% or less of the total membrane surface of the electroplax [24]. Electron microscopic studies also showed that the plasma membrane fraction was enriched in sheets of plasma membrane with relatively few vesicles, mitochondria and collagen filaments being present. The plasma membrane fraction was also contaminated by small amounts of extracellular material (nerve endings, connective tissue, fibroblasts). All results with the plasma membrane were corrected for the percentage of mitochondrial cross-contamination [24] (knowing that mitochondria under the impenetrable conditions used for LCI are not labeled) and for the labeling of extracellular material (connective tissue, fibroblasts, and nerve endings) which co-sedimented in the plasma membrane fraction [24].

Gel electrophoresis. SDS–PAGE followed the procedure of Laemmli [55] using 80 × 80 mm slab gels of 2.5 mm thickness. The separation gel and the stacking gel contained 8 and 5% acrylamide, respectively. Prestained molecular weight marker proteins were applied on each slab. Electrophoresis was carried out for 8–10 hr at 50 mA constant current. Following an overnight fixation, Coomassie brilliant blue staining and destaining, each slab was cut along the lanes and each gel-lane was scanned (using a Gilford gel scanner; Gilford Instrument Laboratories, Inc., Oberlin, OH) at 550 nm and then sliced into 2-mm sections that were counted for gamma emission using an Auto Gamma Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, IL).

Data evaluation. In every slab gel we estimated (a) the molecular weights of the proteins, (b) the protein and radioactivity values along the gel, and (c) the specific labeling of the proteins. (a) The line relating best molecular weight of marker proteins and their migration distance along the gel was computed and used for the estimation of molecular weights of membrane proteins, according to their migration distance. (b) The amount of protein distributed along the gel was estimated at each 2 mm along the scan (representing 0.4 mm along the original 75-mm gel). The estimation was based upon the heights of the scan from baseline, at each particular distance. The readings of each scan were normalized to full scale, which represented one absorbance unit. The heights were used to express protein content in arbitrary protein units (APU). One APU is defined as 1 mm height (0.0041

* Standard operating procedure guidelines and safety checklist as provided by the U.S. Army Medical Research and Development Command and as described in the Ph.D. thesis of Haim Schupper (University of Connecticut, 1989, Effects of DFP, sarin and soman on the accessibility of proteins in the electroplax membrane to lactoperoxidase-catalyzed iodination).

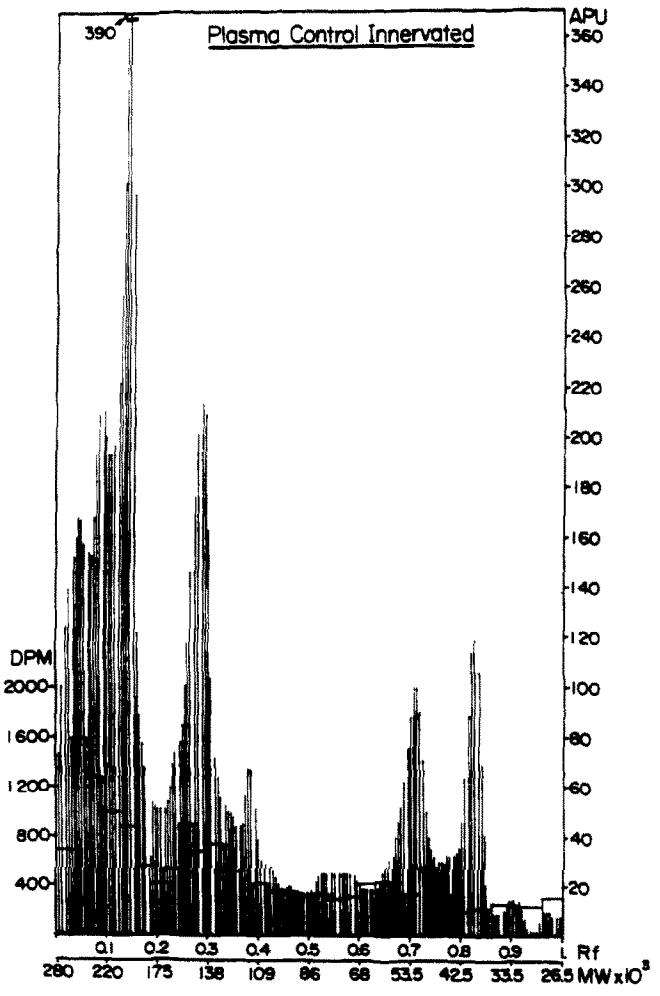

Fig. 1. Electrophoretic mobility of radiolabeled proteins from the innervated plasma membrane fraction. Vertical lines represent protein values in terms of arbitrary protein units (APU) in 0.4 mm of original gel as determined by scanning densitometry. Bars represent radioactivity values in each 2-mm gel section in terms of dpm. MW = molecular weight.

absorbance unit) measured on the ordinate from the baseline of the recording. The radioactive cpm in each 2-mm gel section from the original 75-mm gel were corrected for background radioactivity, counter efficiency and non-specific radioactivity associated with the empty gel matrix, and expressed as dpm for each 2-mm gel section. (c) In each experiment the protein content, expressed as APU, and the disintegrations, expressed as dpm, were then used to calculate the ratio of dpm per APU (specific labeling) for every 2-mm gel section. Examples of the types of results obtained by these calculations are shown in Figs. 1 and 2.

Permeability studies. These studies were performed in order to determine whether the anti-ChE agents or the LCI medium increased the permeability of the electroplax membrane to normally impenetrable compounds. Isolated single electroplax were mounted in special chambers [56, 57] over a small window (1.5 × 5 mm) separating two pools of fluid (3.5 mL and 1.5 mL) with the innervated surface facing the larger pool. [^{14}C]Sucrose or Na^{125}I was added to the larger pool and could pass to the small pool primarily by going through the cell. The effects of the anti-ChE agents on the permeability of [^{14}C]sucrose were tested by adding the anti-ChE solutions to both pools and withdrawing 0.1-mL samples from each pool at various times and replacing the volume removed by solutions identical to that originally present in the pools. The effects of the LCI medium were tested on the penetration of [^{14}C]sucrose and Na^{125}I, during the course of an iodination reaction in both pools.

Cholinesterase and protein determinations. Cholinesterase activity was determined [58] on electroplax crude homogenates and plasma membrane-enriched fractions in both untreated and organophosphate-treated electroplax cells. This spectrophotometric assay uses acetylthiocholine iodide as substrate. The percent reduction in cholinesterase activity relative to controls was calculated. Protein was determined according to a modification [59] of the method of

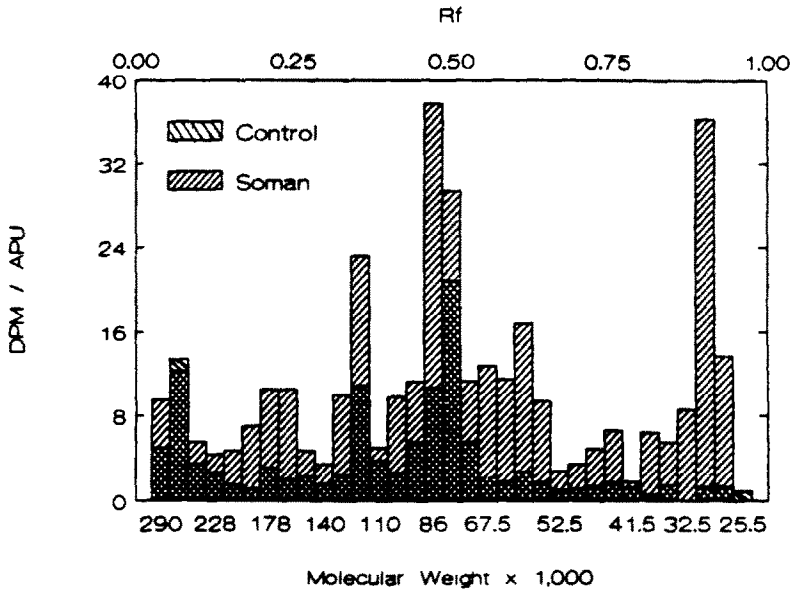

Fig. 2. Specific labeling (dpm/APU) in 2-mm pieces along the gels of untreated and soman (10^{-4} M)-treated innervated plasma membrane fractions derived from iodinated intact cells. Both the control and soman-treated values are shown for each 2-mm section. The soman values were higher for all of the sections except the second from the left. Key: (▨) indicates the superimposed control and soman diagonal lines.

Lowry *et al.* [60] using bovine serum albumin as standard.

Statistical analysis. For each control membrane and the corresponding organophosphate-treated membrane, electrophoresed on the same slab, dpm per APU values (specific labeling) were compared for each molecular weight range by a two-tailed *t*-test. The molecular weight ranges analyzed in the gels were as follows: (1) $30{,}000 < M_r \leq 50{,}000$, (2) $50{,}000 < M_r \leq 75{,}000$, (3) $75{,}000 < M_r \leq 100{,}000$, (4) $100{,}000 < M_r \leq 150{,}000$, (5) $150{,}000 < M_r \leq 200{,}000$, (6) $M_r > 200{,}000$. The "N" values shown in Tables 1–3 represent the number of determinations of dpm/APU values that were used in calculating the mean dpm/APU values for each of the molecular weight ranges. The values for each of the different treatments (and the matched control values) are based upon experiments using two or three different eels. The *t*-test could be used since each experimental mean value was compared to its own particular control mean value. In addition, the *t*-test has the advantage that the means being compared need not have homogeneous variances. The *t*-test was also used to compare cholinesterase activities in untreated homogenized cells and plasma membranes, and in the corresponding organophosphate-treated cells. A difference was considered significant if $P < 0.05$.

RESULTS

Impermeability of electroplax cells. The basis for all the planned experiments was that the electroplax membrane is impermeable and that LCI could reach only the external proteins on the outer leaflet of the plasma membrane. Therefore, it was necessary to prove that the organophosphorus compounds and the incubation medium used during LCI did not change the permeability of electroplax to normally impenetrable compounds such as [^{14}C]sucrose and Na^{125}I which were selected for use in these studies. After exposure for 120 min to 1 mM DFP, 0.1 mM sarin or 0.1 mM soman, less than 0.5% of [^{14}C]sucrose penetrated across the cell (three determinations). This very low level of penetration was apparently not different from that observed in the control cell (0.42% in 120 min). Therefore, it was concluded that the organophosphorus compounds did not increase the permeability of the electroplax to [^{14}C]sucrose. The low level of apparent penetration observed, even in control cells, probably represents, at least in part, diffusion around the cell because of the thin layer of gelatinous tissue in front of the innervated surface which cannot be completely removed and which is pressed against the window in the chamber. Also, the effects of LCI medium on the permeability of the electroplax were tested by using [^{14}C]sucrose and Na^{125}I, as was described in Materials and Methods. After exposure of electroplax to the LCI medium, the penetration of [^{14}C]sucrose was only 0.22% in 140 min, which is even less than that observed in control electroplax cells (0.42% in 120 min). The percent penetration of Na^{125}I was slightly higher, about 0.6% within 80 min from starting the iodination, followed by an increase to about 1.2% after 140 min. Since in the usual iodination procedure the reactants are removed at approximately 20 min after starting the reaction, both Na^{125}I and [^{14}C]sucrose can be regarded as non-penetraing molecules. In comparison, the penetration of a relatively lipid-soluble compound

Table 1. Lactoperoxidase-catalyzed iodination in plasma membrane proteins of intact and split electroplax cells

	Specific labeling (dpm/APU)		
$M_r \times 10^3$	Split cells Inner + outer surface	Intact cells Outer surface	Split minus intact Inner surface
Innervated plasma membrane fraction			
30–50	7.70 ± 2.32* (13)	2.68 ± 0.44 (104)	5.02
50–75	4.49 ± 0.87* (12)	1.89 ± 0.19 (87)	3.10
75–100	5.91 ± 0.75* (8)	3.72 ± 0.65 (59)	2.19
100–150	4.76 ± 0.66* (12)	2.42 ± 0.24 (87)	2.34
150–200	4.46 ± 0.71* (8)	1.77 ± 0.20 (62)	2.69
>200	6.58 ± 0.59* (5)	3.10 ± 0.36 (67)	3.48
Non-innervated plasma membrane fraction			
30–50	11.23 ± 3.26* (13)	2.99 ± 0.66 (92)	8.24
50–75	7.05 ± 1.88* (11)	1.96 ± 0.43 (88)	5.09
75–100	7.90 ± 1.85* (10)	2.93 ± 0.58 (60)	4.97
100–150	4.91 ± 1.16* (11)	2.20 ± 0.36 (88)	2.71
150–200	5.32 ± 1.16* (9)	3.41 ± 0.77 (62)	1.91
>200	11.19 ± 2.87* (4)	4.90 ± 1.17 (68)	6.29

Labeling in split cells represents total labeling in both the external plus internal monolayers of the plasma membrane bilayer while labeling in intact cells represents labeling in only the external monolayer. Split minus intact equals labeling in internal monolayer of plasma membrane bilayer. Abbreviations: APU, arbitrary protein unit; and M_r, molecular weight. Results are means ± SEM; N values are shown in parentheses.

* Significantly larger than corresponding intact value, based on *t*-test ($P < 0.05$).

such as dimethylaminoethanol is 4% in 40 min and 9% in 60 min [57]. In addition, electron microscopic studies showed that electroplax treated with the LCI medium were undamaged.

Labeling of electroplax membrane proteins by lactoperoxidase-catalyzed iodination. Iodination in the absence of lactoperoxidase (non-specific iodination) was only 4.2 to 9.2% of LCI observed in intact cells and only 1.2 to 2.9% of LCI observed in homogenized cells (two determinations for each of the three different conditions). The LCI label incorporated into intact cells was about 30% of the total iodination observed in homogenized cells (two determinations for each of the two different conditions), indicating that in intact cells the idoination did not extend beyond the exposed surface proteins. The finding that iodination was mostly lactoperoxidase catalyzed and that LCI in intact cells was lower than in homogenized cells is in agreement with previous data using other biological preparations [50, 51].

The extent of external labeling was determined by using intact cells while the external plus internal labeling (total labeling) was determined by using split half-cell preparations. The extent of labeling of internal proteins was obtained by subtraction of external labeling from the total labeling. The distribution of labeling (according to molecular weight classes along the gel) in plasma membrane proteins of intact cells and split cells is summarized in Table 1. Labeling was much greater in split cells than in intact cells for every molecular weight grouping, in agreement with the fact that many of the membrane proteins are exposed on the internal surface of the plasma membrane [42].

In vitro *effects of soman, sarin and DFP on labeling of proteins in the inner and outer monolayers of the plasma membrane bilayer.* DFP (10^{-3} M), sarin (10^{-4} M) and soman (10^{-4} M, 10^{-6} M, and 2.5×10^{-9} M) pretreatment of electroplax did not cause changes in the total plasma membrane protein content, as determined by the modified Lowry protein assay [59], or in the electrophoretic pattern of the plasma membrane fraction (data not presented; nine determinations with soman, three with sarin and three with DFP).

The specific labeling values (dpm/APU) of membrane proteins as percentages of control values (according to molecular weight groupings from 30,000 to above 200,000) in the innervated and non-innervated plasma membranes, following organophosphate exposure, are summarized in Tables 2 and 3. Soman at 10^{-6} M decreased the labeling (accessibility) of proteins in four molecular weight groupings in the innervated plasma membrane fractions, while it increased the labeling in three molecular weight groupings in the non-innervated plasma membrane fractions (Table 2). These results show alterations in the exposure of proteins in the external monolayer of the plasma membrane bilayer to lactoperoxidase, suggesting alterations in the asymmetric protein distribution in the plasma membrane. In contrast, soman at 10^{-4} M increased the labeling of proteins in four molecular weight groupings in the innervated plasma membrane, whereas it had no effect on the labeling of proteins in the non-innervated plasma membrane fractions (Table 2). Soman at 2.5×10^{-9} M only altered labeling in the highest molecular weight grouping of proteins in the innervated plasma membrane fraction (Table 2). Sarin at 10^{-4} M had no effect on labeling of proteins in the innervated plasma membrane fractions, while it increased labeling in two of the six molecular weight groupings in the non-innervated

Table 2. Lactoperoxidase-catalyzed iodination in plasma membrane proteins obtained from cells that were exposed while intact to the anticholinesterases and then to the iodination reaction

	Specific labeling (% of control)				
	Soman			Sarin	DFP
$M_r \times 10^3$	2.5×10^{-9} M	10^{-6} M	10^{-4} M	10^{-4} M	10^{-3} M
Innervated plasma membrane fraction					
30–50	154 ± 57 (25)	39 ± 7* (14)	436 ± 162 (16)	194 ± 63 (17)	98 ± 17 (32)
50–75	113 ± 28 (21)	32 ± 8* (12)	291 ± 64* (17)	156 ± 37 (16)	168 ± 39 (21)
75–100	107 ± 26 (16)	63 ± 5 (7)	190 ± 20 (11)	97 ± 31 (11)	153 ± 33 (14)
100–150	104 ± 19 (22)	78 ± 6 (12)	186 ± 61* (16)	100 ± 23 (15)	181 ± 36* (22)
150–200	117 ± 20 (16)	72 ± 4* (9)	265 ± 72* (11)	169 ± 49 (11)	256 ± 61* (15)
>200	141 ± 24* (17)	60 ± 6* (6)	143 ± 29* (13)	113 ± 17 (16)	330 ± 104* (15)
Non-innervated plasma membrane fraction					
30–50	110 ± 25 (27)	150 ± 31 (12)	150 ± 51 (10)	109 ± 22 (16)	99 ± 25 (27)
50–75	152 ± 31 (21)	326 ± 69* (12)	124 ± 23 (17)	121 ± 13 (16)	179 ± 68 (22)
75–100	93 ± 16 (16)	344 ± 69* (9)	151 ± 38 (11)	108 ± 27 (9)	183 ± 58 (15)
100–150	85 ± 6 (22)	196 ± 18* (12)	124 ± 33 (17)	146 ± 19* (17)	239 ± 77* (20)
150–200	78 ± 13 (16)	139 ± 18 (8)	296 ± 166 (11)	114 ± 15 (11)	200 ± 65* (16)
>200	77 ± 15 (15)	121 ± 19 (5)	145 ± 28 (12)	165 ± 28* (17)	171 ± 50* (19)

Intact cells, untreated or exposed to the anti-ChE, were iodinated while intact and then split into innervated and non-innervated surfaces and plasma membrane-enriched fraction isolated from the separated surfaces (see Materials and Methods for further details) and labeling in the outer monolayer thereby determined. Results are means ± SEM; N values are shown in parentheses. Abbreviations: APU, arbitrary protein unit; and M_r, molecular weight.

* Significantly different from control (untreated) values by the *t*-test ($P < 0.05$). Each treated value (dpm/APU) was compared with its matched control value. The actual control values in these particular experiments for each anti-ChE are not shown; however, the summed control values (dpm/APU) in the plasma membrane fractions are shown in Table 1 (intact cells).

Table 3. Lactoperoxidase-catalyzed iodination in plasma membrane proteins obtained from cells exposed to 10^{-4} M soman while intact and then to iodination after being split into innervated and non-innervated surfaces

$M_r \times 10^3$	Specific labeling (% of control)
Innervated plasma membrane fraction	
30–50	38 ± 17* (13)
50–75	20 ± 3* (12)
75–100	51 ± 15* (8)
100–150	40 ± 8* (12)
150–200	33 ± 11* (8)
>200	49 ± 18* (5)
Non-innervated plasma membrane fraction	
30–50	26 ± 8* (13)
50–75	16 ± 3* (11)
75–100	24 ± 4* (10)
100–150	40 ± 7* (11)
150–200	35 ± 5* (9)
<200	35 ± 9* (4)

In contrast to Table 2, cells were iodinated after being split into separated innervated and non-innervated surfaces. Results are means ± SEM; N values are shown in parentheses. Abbreviations: APU, arbitrary protein unit; and M_r, molecular weight.

* Significantly less than control (untreated) values by the *t*-test ($P < 0.05$). Each treated value was compared with its matched control value (dpm/APU). The control values for these particular experiments (dpm/APU), in the plasma membrane fractions, are shown in Table 1 (split cells).

plasma membrane fractions (Table 2). DFP at 10^{-3} M increased the labeling of the three highest molecular weight groupings of proteins in both the innervated and non-innervated plasma membrane fractions. All of the results in Table 2 show changes in labeling of proteins on the outer surface of the plasma membrane. In contrast, Table 3 shows changes in total labeling (internal plus external) of the plasma membrane proteins induced by 10^{-4} M soman. There was a marked decrease in the total labeling of all of the molecular weight groupings of proteins from both the innervated and non-innervated plasma membrane fractions (Table 3). Since, as shown in Table 2, 10^{-4} M soman had either no effect on labeling of external proteins (non-innervated plasma membrane fractions) or increased labeling of some of the external proteins (innervated plasma membrane fractions), the labeling (accessibility) of the internal proteins (exposed to cytoplasm) must have been decreased.

Effects of organophosphates on cholinesterase activity. Cholinesterase activity was determined in every experiment in which cells were pretreated with organophosphorus compound or control buffer. The control ChE activity in the innervated plasma membrane fraction ranged from 3 to 7.5 μmol/min/mg protein while the activity in the non-innervated plasma membrane fraction ranged from 1 to 3 μmol/min/mg protein. This is in agreement with the known enrichment of ChE in the innervated, as compared to the non-innervated, plasma membrane [36–40]. Soman at 10^{-4} and 10^{-6} M caused 100% inhibition

of ChE in the innervated and in the non-innervated plasma membrane fraction while 2.5×10^{-9} M caused 0 and 17% inhibition, respectively. DFP (10^{-3} M) and sarin (10^{-4} M) caused 95–96% inhibition of ChE in these two plasma membrane fractions.

DISCUSSION

The electroplax of the electric eel would seem to be an ideal preparation on which to test the effects of neurotoxicants (especially those acting on the cholinergic system) on membrane organization and asymmetry (see introduction). All vectorial labeling techniques require the use of a membrane preparation in which the labeling agent has access to only one side (monolayer) of the membrane bilayer [41, 42]. We have shown, for example, in this study, in a recently published report [24] and in unpublished observations (Rosenberg P and Weber B) that the intact electroplax cell is relatively impermeable to membrane probes such as LCI, a phospholipid exchange protein, and trinitrobenzenesulfonate, respectively. Specifically, in this study we have shown that the intact cell is relatively impermeable to [^{14}C]sucrose and Na^{125}I. We used a method previously found suitable for studying protein asymmetry with LCI under impenetrable conditions [50–54]. We found that labeling by LCI of the externally exposed proteins using intact cells (Table 1) is less than that of the externally plus internally exposed proteins using split cells (Table 1). We were also able to make our measurements upon fractions enriched in plasma membranes from the innervated (bioelectrically excitable) or non-innervated (inexcitable) surfaces of the electroplax. Since the anti-ChE agents did not increase the permeability of the electroplax, we were confident that any changes in protein labeling would be due to changes in protein organization and exposure, not simply due to greater penetration of lactoperoxidase. By measuring changes in specific labeling of proteins in different molecular weight groupings, we were able to see whether there was any specificity in the changes induced by the anti-ChE agents. Since sarin, soman and DFP did not alter the total protein content of the plasma membrane fraction or their electrophoretic patterns, changes in specific labeling represent changes in protein organization as reflected in alterations of asymmetry or surface exposure.

In control cells (Table 1), specific labeling of proteins in the inner monolayer (split − intact) appeared greater than in the outer monolayer (intact) in most of the molecular weight groupings in the innervated and non-innervated plasma membrane fractions. Specific labeling in the inner plus outer monolayer (split cells) which represents total accessible labeling, was higher in the non-innervated plasma membrane fractions than in the innervated fractions (Table 1). Similarly, labeling in the inner monolayer (split − intact) also appeared greater in the non-innervated plasma membrane, whereas labeling of the outer monolayer proteins (intact cells) was similar in the innervated and non-innervated plasma membrane fractions (Table 1). The greater exposure of inner monolayer proteins to the cytoplasm in the non-innervated membrane suggests a more hydrophilic cyoplasmic surface in the non-innervated plasma membrane which may be related to the much lower electrical resistance of the non-innervated plasma membrane as compared to the innervated plasma membrane [36]. Even in split cells some protein remains inaccessible to the probe and cannot be labeled with LCI unless the cells are homogenized (see Results), suggesting that these proteins are embedded within the hydrophobic core of the membrane.

The effects of the anticholinesterases on labeling of proteins in the outer monolayer of the plasma membrane bilayer, as judged by carrying out the LCI reaction on intact cells, are shown in Table 2 and the specific detailed changes were noted in Results. Overall, in both the innervated and non-innervated plasma membrane, the higher molecular weight fractions were most consistently affected by the anti-ChE agents. In the non-innervated plasma membrane, the anti-ChE agents either had no effect or increased labeling while this was also true in the innervated plasma membrane except for 10^{-6} M soman which decreased labeling. The anticholinesterase agents induced significant changes in 20 of the 60 molecular weight fractions shown in Table 2, which is much greater than the 3 which would be expected due to chance at the $P = 0.05$ level of significance. Changes in cholesterol content and membrane fluidity, induced by the anti-ChE agents [19–22, 24, 61, 62], show no relationship to the changes we have noted in protein accessibility. For example, in electroplax we found that 10^{-4} M soman increases cholesterol content in the innervated plasma membrane, while 10^{-4} M sarin decreases cholesterol in the non-innervated plasma membrane and 10^{-6} M soman has no effect [63]. These results do not correlate with our present findings on membrane proteins.

We also carried out the LCI reaction on split cells following exposure of the intact cells to 10^{-4} M soman (Table 3), and this labeling represents the total labeling of proteins exposed on both surfaces of the plasma membrane to the iodination medium. In control cells, the total labeling of proteins (measured in split cells) was, of course, much greater than the labeling on the external surface (measured in intact cells) or the labeling on the internal surface which was the difference between total and external labeling (Table 1). Soman (10^{-4} M) significantly decreased the total labeling in all of the molecular weight fractions both in the innervated and non-innervated plasma membrane (Table 3). As noted previously, soman (10^{-4} M) either had no effect (non-innervated plasma membrane) or increased the labeling (innervated plasma membrane) of some of the proteins exposed on the external surface of the plasma membrane (Table 2). This suggests that there was a marked decrease of protein exposure (labeling) on the inner (cytoplasmic) surface of the plasma membrane. This increase in exposure of proteins on the external surface of the innervated plasma membrane and decrease in exposure on the internal surface suggest a marked change in protein asymmetry induced by 10^{-4} M soman.

Cholinesterase activity of the cells was measured

in the experiments shown in Tables 1–3 in order to determine whether a correlation existed between the extent of ChE inhibition and the alteration in protein iodination following exposure to the anti-ChE agents. It is not certain how inhibition of ChE could modify protein asymmetry or exposure; however, it is especially pertinent that ChE may act as a protease and regulate cell growth and development [64]. Inhibition of protease activity could modify protein organization within the membrane. In agreement with previous findings on electroplax [24, 28], we found that ChE inhibition was irreversible with no spontaneous reactivation being observed, despite the removal of the anti-ChE agent from the incubation medium, washing of the cells, homogenization, subcellular fractionation and storage of the membranes at −80° for several days. Our results indicate that alteration in labeling of external proteins by organophosphorus compounds is not directly related to cholinesterase inhibition. For example, 10^{-6} M and 10^{-4} M soman, 10^{-4} M sarin and 10^{-3} M DFP all produce about equal and almost complete cholinesterase inhibition, yet the effects of these four treatments on LCI labeling differed markedly (Table 2). It is interesting that in recent studies [63,*] we have found that marked changes, induced by anti-ChE agents, on the asymmetry of phospholipids in the electroplax membrane were also not related to cholinesterase inhibition.

The present study had two major aims: to determine whether anti-ChE compounds alter the asymmetry of proteins in the plasma membrane and to determine whether any alterations observed are dependent on ChE inhibition. Both increases and decreases in protein labeling (exposure) on the external monolayer of the plasma membrane bilayer were caused by the anti-ChE agents. Four different treatments, all of which caused almost complete inhibition of ChE, caused a variety of different effects on exposure of proteins of the various molecular weight fractions (Table 2), suggesting, thus, that these agents are modifying membrane organization of proteins by a mechanism not directly due to ChE inhibition. At this time, however, we cannot relate these changes in protein asymmetry to any specific effect of the anti-ChE agents which is not due to cholinesterase inhibition and which may be of either short- or long-term duration (see Introduction). The varied nature of the effects caused by DFP, sarin and soman suggests a non-specific alteration in membrane functioning, rather than a specific action on any particular receptor or enzyme system. The changes in protein organization, caused by the organophosphate anti-ChE agents which were observed in this study, could be associated with changes in membrane functioning.

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