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Assessment of Di- and Tri-butyltin Interaction with Skeletal Muscle Membranes

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Skeletal muscle cell membranes are highly complex structures, organized in a series of structures together called 'sarcolemma'. Sarcolemma is made up of three layers, the external layer of banded collagen, a middle basement membrane consisting of collagenous and non-collagenous glycoproteins and an internal plasma membrane which envelops cell organelles(Peachey 1965).Sarcolemma plays a significant role in excitation and contraction phenomenon in muscles.Muscle cell membranes are the most exposed target sites for the interaction of environmental pollutants. Certain xenobiotics could get their entry directly through the skin absorption and affect the muscle cell membranes inadvertantly. The study on sarcolemma could provide the indepth knowledge regarding the role played by basement membrane if any, in the protection of plasma membrane and to the cell itself. Our earlier study has shown that frog muscle sarcolemma can be used as model for testing the toxicity of chemicals(Ali et al, 1988).

Organotin compounds are widely used as agricultural fungicides and miticides, industrial biocides, surface disinfectants, anthelmintic and marine antifungal agents (Smith and Smith 1975). However, organotin compounds are known to exert toxic effects in man and animals (WHO-publication,1980). Di-and tri-butyltins are known to inhibit the oxidative phosphorylation (Stockdale et al, 1970; Penninks et al,1983) and also causes hepatotoxicity (Krigman and Silverman,1984). The biological effects of di and trialkyltin compounds differ considerably (Aldridge 1976; Seinen and Penninks 1979). Some alkyltin compounds such as triethyltin and trimethyltin have been shown to influence muscle cell segments in some way or the other including muscular contractility (Tan and Ng, 1977).In the presei t

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study the binding of di- and tri-butyltins have been studied on frog skeletal muscle sarcolemma and basement membrane. The binding characteristics and the possible mechanism of interaction of organotin compounds to the membrane have been discussed.

METERIALS AND METHODS

Common Indian frog (Rana tigrina)were used throughout the study. Young adult frogs weighing 120 to 150 g were procured from local suppliers and acclimatized in the laboratory pond before use. Frogs were stunned and decapitated. Skeletal muscle from hind limbs were removed and kept in ice-cold 50 mM calcium chloride (pH 7.0). Major connective tissues, nerves and blood capillaries were dissected out. Sarcolemma and basement membrane were prepared from frog skeletal muscle according to the procedure of Ali et al (1987 a). In brief, about 50 g of muscle was minced, homogenized and processed for lithium bromide extraction to isolate the sarcolemma. After one hour extraction, suspension was centrifuged and washed with distilled water. The sediment was suspended in a solution containing 2M sodium chloride, 10 mM EDTA and 0.5% Triton X-100 and stirred for one hour at room temperature.Following one hour extraction, the contents were centrifuged and the sediment was sonicated for 30 min with 5 changes of fresh Triton-salt solution. Finally the suspension was centrifuged and washed thrice with distilled water. Basement membrane thus obtained was kept frozen until use.

Dibutyltin dichloride (Bu_2SnCl_2; 97% pure)and tributyltin chloride (Bu_3SnCl; 96% pure)were obtained from Aldrich chemical company, U.S.A. To analyse the binding of alkyltin compounds with sarcolemma membrane preparations (7-10 mg protein/ml)were incubated with alkyltin compounds (0-500 uM)for 15 min at 37°C with constant gentle shaking. After incubation contents were centrifuged at 3000 g for 10 min and supernatant/sediment separated. Sediment was washed 3-4 times in order to remove any remaining unbound compounds and subjected to alkyltin extraction. The extraction procedure was carried out according to the method described by Cremer (1957). In brief,samples were treated with equal ammount of tartaric acid(10% w/v)and allowed to stand for 5 min. Then equal volume of perchloric acid (30% v/v)was added and contents thoroughly mixed and centrifuged for 15 min at 3,000 g. Supernatant fraction containing organotin compounds was then neutralised with 5N NaOH and used for estimation of organotin componds. For scatchard analysis free

and bound organotin compounds were estimated by the dithizone assay (Aldridge and Cremer 1957). Di-and tributyltin-dithizone complex was read against a dithizone control at the optimum wavelength of 550 and 455 nm, respectively. Results were ploted according to Scatchard (1949).

In order to see the effect of thiol compounds on the binding of Bu_2SnCl_2 with sarcolemma, reaction mixture containing 0.5 umol of Bu_2SnCl_2 and 5 mM phosphate buffer (pH 7.5) with varying concentrations (0-500 umol)of thiol compounds were incubated at 37°C for 15 min. Sarcolemma preparation (10 mg protein/ml)was then added to the reaction mixture and further incubated for 15 min at 37°C. At the end of incubation, reaction mixture was centrifuged at 3,000 g for 10 min. Supernatant was discarded and sediment washed three times with distilled water. Bound Bu_2SnCl_2 was estimated as described earlier.Total sulfhydryl content in sarcolemma were then estimated as described by Ellman (1959). Protein was determined according to the method of Lowry et al (1951).

RESULTS AND DISCUSSION

The binding of Bu_2SnCl_2 with sarcolemma was increased with increasing concentration. The maximum binding of Bu_2SnCl_2 was about 4.4×10^{-2} umol/mg protein with saturation at 350 uM. Further increase in the Bu_2SnCl_2 concentration did not enhance the binding. Binding of Bu_2SnCl_2 to sarcolemma was almost saturable within a period of 15 min when incubated at 37°C. However, approximately 90% binding was found within 5 min incubation (3.71×10^{-2} umol/mg protein). Further increase in incubation time did not appreciably increase the binding of Bu_2SnCl_2. No significant difference in binding of Bu_2SnCl_2 to sarcolemma at varying pH and buffers were observed. Sarcolemma preparation following incubation with Bu_2SnCl_2 at 37°C for 15 min was processed for plasma and basement membrane. It was found that about 81% of the total Bu_2SnCl_2 of sarcolemma was present in plasma membrane (Soluble fraction) removed from sarcolemma during isolation of basement membrane. Only about 19% of the total Bu_2SnCl_2 was found in basement membrane fraction. The solubilization of Bu_2SnCl_2 bound proteins was studied using sequential treatment with detergents. An initial washing of Bu_2SnCl_2 bound sarcolemma with water showed no elution of protein or Bu_2SnCl_2 from the sarcolemma. The remaining sediment when treated with 1% sodium deoxycholate did not show any significant release of Bu_2SnCl_2.However, upon increasing the concentration of sodium

deoxycholate to 3%, the remaining Bu_2SnCl_2 in the pellet was reduced to 50%. Subsequent treatment of the remaining pellet with 1% noniodet did not release any Bu_2SnCl_2. However, following 1.0% and 3.0% SDS treatment only 25 and 5% Bu_2SnCl_2/mg protein, respectively, could be detected in the remaining sediment of sarcolemma.

The higher binding of Bu_2SnCl_2 with plasma membrane compared to basement membrane can be explained on the basis of the fact that plasma membrane contains lipoprotein complexes whereas, basement membrane is devoid of these complexes. Organotin compounds being hydrophobic in nature may get bound easily with lipoproteins of the plasma membrane.The binding of Bu_2SnCl_2 with lipoprotein complex of sarcolemma was also in agreement with the fact that Bu_2SnCl_2 bound proteins could mainly be solubilized by drastic treatments with detergents. In fact, detergents generally solubilize lipoprotein complex of intrinsic proteins of membranes. Therefore,studies were extended to solubilize Bu_2SnCl_2 bound proteins of sarcolemma, using various detergents, and the effectiveness of various detergents on the solubilization of Bu_2SnCl_2 bound protein was established.

Scatchard plots relating B/F against B provided hyperbolic curves for alkyltin compounds. Results indicated the presence of more than one binding site in sarcolemma for Bu_2SnCl_2 (Fig 1) as well as for Bu_3SnCl (Fig 2). A satisfactory fit to the results may be obtained for minimum of two classes of binding sites, the high affinity and the low affinity. The high affinity binding constants for Bu_2SnCl_2 in sarcolemma were $K_1 = 1.95 \times 10^4 M^{-1}$; n_1 = 18 nmol/mg protein and the low affinity binding constants $K_2 = 1.37 \times 10^3 M^{-1}$; n_2= 120 nmol/mg protein. When a valid correction factor was employed to remove nonspecific binding as described by Chamness and McGuire (1975), the affinity constants for the major binding sites were $K_d = 4.42 \times 10^4 M^{-1}$ and n = 7 nmol/mg protein. In case of Bu_3SnCl, the high affinity constants were $K_1 = 1.64 \times 10^4 M^{-1}$ with n_1 = 33 nmol/mg protein and the low affinity constants $K_2 = 3.73 \times 10^2 M^{-1}$ with n_2= 655 nmol/mg protein. After applying correction factor, the constants were $K_d = 4.92 \times 10^4 M^{-1}$ and n = 9 nmol/mg protein. The binding site obtained following the application of correction factor could be the site responsible for the binding of most of organotin compounds.

Conversely, the scatchard analysis of the binding of Bu_2SnCl_2 and Bu_3SnCl with basement membrane resulted

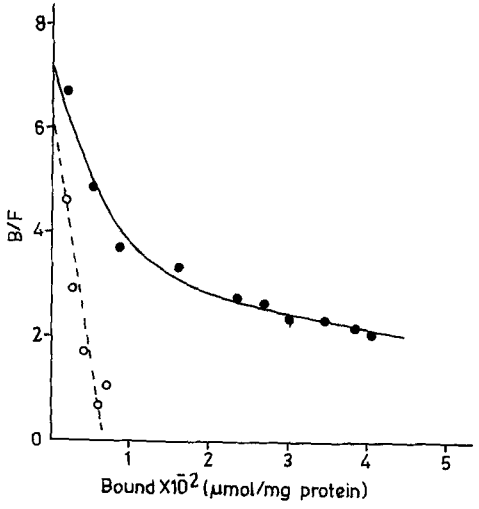

Figure 1.
Scatchard plot of the data for binding of dibutyltin dichloride to sarcolemma. B is the concentration of bound and F of free Bu_2SnCl_2 Dotted straight line was obtained after applying correction factor for non-specific binding sites showing the presence of one major binding site.

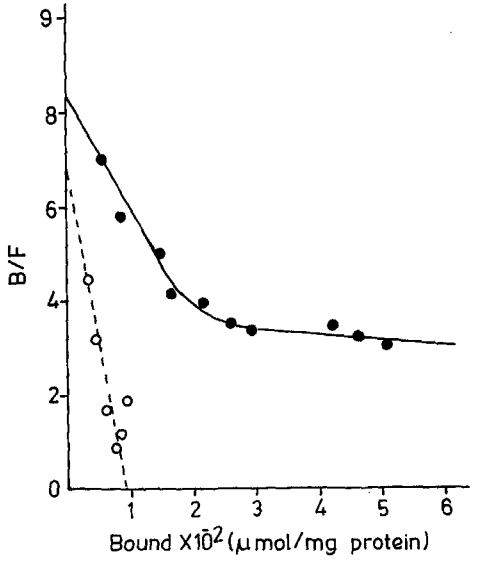

Figure 2.
Scatchard analysis of the data for binding of tributyltin chloride to sarcolemma.

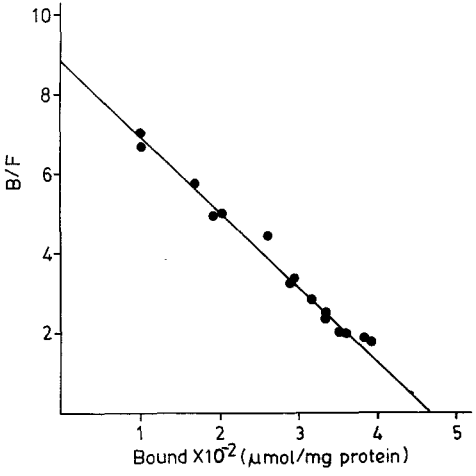

Figure 3.

Scatchard analysis of the data for binding of dibutyltin dichloride to basement membrane.

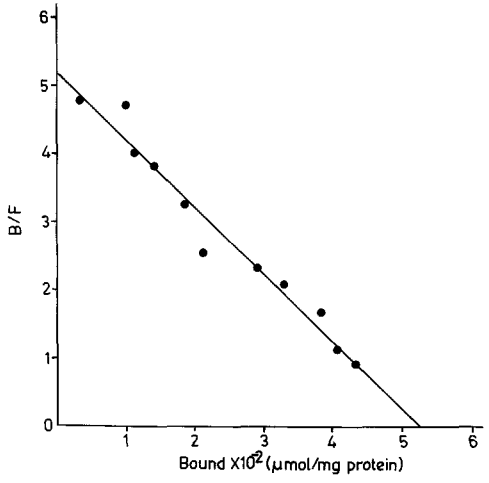

Figure 4.

Scatchard analysis of the data for binding of tributyltin chloride to basement membrane.

into a straight line indicating only one class of binding (Fig 3 and 4). The affinity constant (K) for the binding of Bu_2SnCl_2 with basement membrane was $2.49 \times 10^4 M^{-1}$ having concentration of the number of binding sites n = 46.3 nmol/mg protein. The affinity constant for Bu_3SnCl was $1.29 \times 10^4 M^{-1}$ and n = 53 nmol/mg protein.

Non-specific binding of organotin compounds to basement membrane could not be detected possibly due to the lack of lipids. The single binding of site also suggested the possible involvement of a specific protein of basement membrane. Affinity constants for the binding of Bu_2SnCl_2 and Bu_3SnCl to basement were found to be similar with the high affinity constants for the binding of these compounds obtained in Sarcolemma. The possibility of the presence of high affinity binding site in sarcolemma could be due to the presence of basement membrane components external to plasma membrane. Another possibility for the high affinity binding of organotin compound to membrane might be due to its exposed location in sarcolemma. The external location of basement membrane provides easy access for interaction of foreign compounds. Our earlier studies have also shown a single class binding site for tributyltin, and more than one class of binding sites for dibutyltin with human erythrocyte membrane (Ali et al 1987 b). The binding studies of organotin compounds to sarcolemma and basement membrane will be useful for further evaluation of toxicological effects of organotins in muscle membrane. The binding of organotins with basement membrane may provide some protection to the inner enzymatically active plasma membrane and to the functional activities of the cell itself.

Four thiol compounds namely glutathione, cysteine, dithiothreitol and β-mercaptoethanol were used to antagonize the binding of Bu_2SnCl_2 with sarcolemma (Table 1). Preincubation with dithiothreitol reduced the Bu_2SnCl_2 binding with sarcolemma. The binding was almost completely abolished at a concentration of 10 umol of dithiothreitol. The effectiveness of other thiol compounds in reducing the Bu_2SnCl_2 binding was in the order of cysteine > glutathione > β-mercaptoethanol. Glutathione and cysteine concentrations higher than 200 umol also abolished the binding. Wherease, β-mercaptoethanol even at 500 umol concentration reduced only 67% of the total binding. Effect of Bu_2SnCl_2 treatment on total sulfhydryl (-SH) content of sarcolemma revealed a concentration-dependent decrease in -SH content. A maximum of 20% decrease was

Table 1. Effect of Thiol Compounds on the Binding of Bu_2SnCl_2 with Frog Skeletal Muscle Sarcolemma

Thiol Conc. (umol)	Binding of Bu_2SnCl_2 (nmol/mg protein)			
	Gluta-thione	Cysteine	Dithio-threitol	β-mercapto-ethanol
0.0	17.3	17.2	21.4	16.3
1.0	-	-	20.9	-
5.0	-	-	18.4	-
10.0	-	-	1.2	-
20.0	11.0	11.0	0.0	10.6
50.0	9.0	5.0	0.0	9.3
100.0	8.2	3.0	0.0	8.4
200.0	6.5	0.0	0.0	6.8
500.0	0.0	0.0	0.0	5.4

Reaction mixture containing 0.5 umol of Bu_2SnCl_2 was incubated with thiol compounds at 37°C for 15 min. Sarcolemmal preparation (10 mg protein/ml) was added and again incubated for 15 min.

observed at 0.5 umol of Bu_2SnCl_2.

The protective effect of sulphur compounds on the hemolysis of red blood cells caused by triphenyltin has been mainly attributed to chemical interaction between organotin and thiol compounds (Byington et al 1974). In the present study, dithiothreitol and cysteine were found to be more effective. Results have shown a possible interaction of thiol groups with organotin compounds and suggest the generation of inactive species of tin compounds which, in turn, is unable to bind with sarcolemmal proteins. Further, it has been found that the binding of Bu_2SnCl_2 to sarcolemma decreases the free sulfhydryl contents of the membrane. This suggested a possible involvement of sulfhydryl groups of the sarcolemmal protein in the binding of Bu_2SnCl_2. However, the involvement of other than sulfhydryl groups of proteins in this binding may be possible and need to be investigated.

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