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# The Effects of Triethyltin Bromide on Red Cell and Brain Cyclic AMP-dependent Protein Kinases*

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**Triethyltin bromide activates the cyclic AMP-dependent protein kinases of human red cell membranes and of bovine brain. Additions of 25–500 μM triethyltin to red cell ghosts resulted in enhanced phosphorylation of ghost proteins. When added to partially purified cyclic AMP-dependent protein kinases from red cell ghosts or bovine brain, stimulation of the phosphorylation of calf thymus histone was observed. The enhancement of kinase activity was due to release of catalytic subunits from the intact protein kinase. Brief exposure of the partially purified enzymes to triethyltin, followed by DE52 chromatography, resulted in elution profiles for regulatory and catalytic subunits that were similar to the profile resulting after cyclic AMP activation. Triethyltin interacts with both regulatory and catalytic subunits. When it was added to the partially purified cyclic AMP-dependent protein kinases from human red cell ghosts or bovine brain, noncompetitive inhibition of cyclic AMP binding to the regulatory subunit of the enzyme was observed. It interacted with the catalytic subunit to produce slow inhibition of catalytic activity. The inhibition was noncompetitive with respect to both histone and ATP. When intact red cells were subjected to brief exposure with triethyltin, enhanced phosphorylation of certain membrane proteins occurred, suggesting that the activation of the cyclic AMP protein kinases by triethyltin may be physiologically significant.**

Administration of organotins, particularly trialkyltins (1–10 mg/kg), produces acute neurological and respiratory effects in different species of animals. Microscopic observations on the gross pathology of brains from animals intoxicated with organotin have revealed an astrocytic and axonal swelling at about 12 h after treatment with the reagent. The astrocytic enlargement occurs in both gray and white matter simultaneously; however, in the white matter, this is a transitory change (1). These morphological findings suggest that the basic defect involves faulty fluid control by these cells (2, 3) that cause progressive accumulation of fluid accompanied by a development of myelin clefts. The cleft formation, however, clearly occurs at a stage later than the swelling of axons and astrocytes (1). These observations, as well as others, suggest that structures associated with membranes may be particularly sensitive to the effects of trialkyltins. Several investigators have demonstrated selective interactions of triethyltin with myelin as well as with the brain and liver mitochondrial ATPase systems (4–6). The red cell membrane ATPase system is also sensitive to inhibition by the organotins (7) and hemolysis is a known consequence of exposure of red cells to these compounds (8).

Biochemical studies have shown that triethyltin is quite selective in its interaction with proteins. It was bound to only a few proteins among many that were investigated (5, 9). Although binding occurs with different classes of proteins, it appears to have a general effect on enzymes that use ATP as a substrate. Membrane-bound ATPase mentioned above, as well as soluble enzymes such as hexokinase, pyruvate kinase, and phosphofructokinase (10, 11) are examples. This fact, coupled with the known affinity of alkyltins for membranes, and observations on overall cellular effects that suggest membrane involvement, led us to examine the possibility that membrane protein kinases may be sensitive loci for their effects. We report here our observations on the effect of organotins on the protein kinases of red cell membranes and bovine brain.

## MATERIALS AND METHODS

Triethyltin bromide, trimethyltin bromide, tributyltin chloride, and diethyltin dichloride were purchased from the Ventron Corp. (Danvers, MA). (2-[(Dimethylamino)methyl]phenyl)diethyltin bromide was the generous gift of Dr. J. G. Noltes, Institute for Organic Chemistry TNO, Utrecht, The Netherlands. Stock solutions were prepared by dissolving the material in a small volume of absolute ethanol and then made to volume in an appropriate buffer or in distilled water. [$\gamma$-$^{32}$P]ATP (0.250 mCi in 0.5 ml of water) and cyclic [$^3$H]AMP (25–50 Ci/mmol) were obtained from ICN Inc. (Irvine, CA) and diluted with the corresponding unlabeled stock solutions to give $3 \times 10^5$ to $6 \times 10^5$ cpm/nmol of ATP and $5 \times 10^3$ to $1 \times 10^4$ cpm/pmol of cyclic AMP before use. All other reagents were of the highest possible purity obtained from commercial sources.

Fresh blood or outdated packed red cells were obtained from the Milwaukee Regional Blood Center. The red cells were washed three times in 10 mM sodium phosphate buffer, pH 7.4, containing 145 mM NaCl. Hemoglobin-free red cell ghosts were then prepared by the method of Dodge *et al.* (12).

*Assay of Protein Kinase Activity in Red Cell Ghosts*—The protein kinase activity in the ghosts was determined by measuring $^{32}$P incorporation from ATP into ghost proteins as described by Guthrow *et al.* (13). Incubations containing 250–350 μg of ghost protein in 0.2 ml were conducted for 5 min at 35 °C with or without addition of 1.0 μM cyclic AMP. When kinase activity was tested for sensitivity to triethyltin, the ghosts were preincubated with the reagent for various times at 35 °C and then assayed. The reaction was terminated by the addition of 2 ml of ice-cold 10% trichloroacetic acid to the reaction mixture. After allowing the mixture to stand on ice for 10 min, the resulting precipitate was collected on Whatman GF/A glass fiber filter and washed with seven successive 2-ml aliquots of ice-cold 10% trichloroacetic acid. The filter was transferred into vials containing 3.5 ml of 3a70B complete counting mixture (Research Products International Corp.) and the radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer.

*Autoradiography of $^{32}$P-labeled Ghost Proteins*—Sodium dodecyl

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sulfate-polyacrylamide gel electrophoresis of ghost proteins was performed by the method of Fairbanks *et al.* (14). A 5–10% continuous gradient polyacrylamide gel was employed in a Bio-Rad Model 220 Vertical Slab Electrophoresis Cell. The protein kinase assay system (40 μl) was mixed with 100 μl of sample buffer, containing 1% SDS,[1] 15% sucrose, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 40 mM β-mercaptoethanol, and 10 μg/ml of bromphenol blue, and placed in a boiling water bath for 2 min to quench the reaction. About 40–50 μg of protein in the mixture was applied to each sample well for electrophoresis. Following electrophoresis, the gels were stained with Coomasie Blue R-250, destained, and dried. Autoradiography was performed using Kodak XAR-5 x-ray film with exposure times of 2–7 days to detect phosphorylated proteins.

*Partial Purification of cAMP-dependent Protein Kinases*—The cyclic AMP-dependent protein kinase of the red blood cell membrane was partially purified by the method of Rubin (15) and that of bovine cerebral cortex membranes by a modification of the method of Rubin *et al.* (16). The Triton X-100-solubilized enzyme from 50 g of brain was loaded onto a column (2.5 × 5 cm) of DE52 previously equilibrated with 20 mM Tris-HCl, pH 7.4, 4 mM EDTA, and 20 mM benzamidine and the column was washed with 50 ml of the same buffer containing 0.025 M NaCl. Protein kinase was eluted using a linear 0.025–0.4 M NaCl gradient (300 ml total) in the same buffer at a flow rate of 50 ml/h and 3-ml fractions were collected. The specific activities of these partially purified preparations ranged from 1300–1600 pmol of $P_i$ transferred per min per mg for the brain enzyme and 1200–1500 for the red cell enzyme.

*Assay of the Partially Purified Enzymes*—Phosphorylating activity was assayed using histone or protamine (Sigma) as acceptor substrates. The standard assay mixture contained in a final volume of 0.2 ml, 0.05 M Bis-Tris-HCl, pH 6.5, 0.03 mM EGTA, 10 mM magnesium chloride, 5 mM β-mercaptoethanol, 250 μg of histone or protamine, 500 μg of bovine serum albumin and 5 μM [$\gamma$-$^{32}$P]ATP. When appropriate, 2 μM cAMP was also included. For assays of column fractions during purification, 100-μl aliquots of fractions were analyzed; otherwise, 5–25 μg of isolated enzyme protein was used in the assay. The assay mixture was incubated at 35 °C for 5 min and prepared for counting as previously described. To test for triethyltin sensitivity, the enzyme was first preincubated with the reagent at 35 °C for various periods of time, then aliquots were removed and added to the assay mixture.

*Preparation of Dissociated Catalytic and cAMP-binding Subunits*—Protein kinase subunits of the bovine brain and of the human red cell membrane were dissociated and fractionated by a modification of the methods of Rubin *et al.* (17) and Rubin (15), respectively. Typically, 1.0 mg of protein kinase was treated with either cyclic AMP (25 μM) or triethyltin (500 μM) for 2 min at 35 °C and immediately cooled to 4 °C. Each mixture was then loaded onto a column (1 × 5 cm) of Whatman DE52 which was previously equilibrated with 50 mM potassium phosphate buffer, pH 7.0. Catalytic activity was eluted from the column with 20 ml of the buffer containing 0.1 M NaCl. The cAMP-binding protein was subsequently eluted with 0.35 M NaCl. Isolated regulatory subunits dissociated by cAMP were subjected to rapid gel filtration on a column (1 × 5 cm) of Sephadex G-25 at 22 °C to remove excess cAMP and stored at −70 °C for subsequent use.

*Assay for Triethyltin*—The amount of triethyltin present in the eluted fractions was determined by precipitating the protein with 0.6 ml of 30% trichloroacetic acid to release the bound triethyltin. The samples were placed on ice for 10 min, then centrifuged at 10,000 rpm for 10 min to obtain a clear supernatant. This supernatant was then assayed by a direct spectrophotometric procedure to determine the triethyltin-dithizone complex (18).

*Assay of Cyclic AMP Binding*—Cyclic AMP binding to the red cell ghosts was performed by the method of Guthrow *et al.* (13) and to the partially purified enzyme or isolated regulatory subunit by the method of Tao (19). When tested for the effect of triethyltin, the ghosts or isolated enzymes were first preincubated with the reagent at 30 °C for the indicated times and then added to the cyclic AMP binding assay mixture.

*Effect of Triethyltin Bromide on the $^{32}$P Labeling of Membrane Proteins in Intact Red Blood Cells*—The labeling of membrane proteins in intact red blood cells was performed by a modification of the method of Plut *et al.* (20). At various times, 3 ml of the incubation mixture was removed and hemoglobin-free red cell ghosts were prepared as described previously. Small samples of the ghosts were dissolved in 3 ml of 3a70B complete counting mixture (Research Products International Corp.) and counted for $^{32}$P incorporation or prepared for SDS-PAGE and autoradiography as described above. The effect of triethyltin was determined by adding triethyltin at a final concentration of 500 μM in the incubation mixture.

*Other Methods*—Protein was determined by the method of Lowry *et al.* (21) using bovine serum albumin as standard.

## RESULTS

Phosphorylation of certain red cell membrane proteins by endogenous cyclic AMP-dependent and independent protein kinases has been clearly demonstrated by Tao and his associates (22). Because this system, including the protein components of the membrane (13, 14), is relatively well characterized, it was used initially to explore the effect of triethyltin on the phosphorylation reaction. When erythrocyte ghosts were assayed for protein kinase activity, a low basal level of phosphate incorporation into the membrane proteins was measured. Upon the addition of 1 μM cyclic AMP, the expected 2- to 5-fold stimulation of protein phosphorylation occurred. Addition of 25–500 μM triethyltin to the ghosts also caused an apparent increase in protein kinase activity (Fig. 1). Brief exposure (10–20 min) of the ghosts to the higher concentrations of the reagent resulted in rapid apparent stimulation of enzyme activity, but with prolonged incubation there was a subsequent decline in the phosphorylation activity. Resolution of the ghost components by SDS-PAGE (14) and analysis by autoradiography demonstrated that phosphorylation of certain proteins was enhanced by brief prior exposure of ghosts to triethyltin (Fig. 2). The photograph shows that when ghosts were preincubated with 500 μM triethyltin and then subjected to phosphorylation by the endogenous protein kinases, increased phosphorylation of bands 2, 2.1, 2.3, 3, 4.1, 4.2, 4.5, 4.8, and 5 occurred. In agreement with

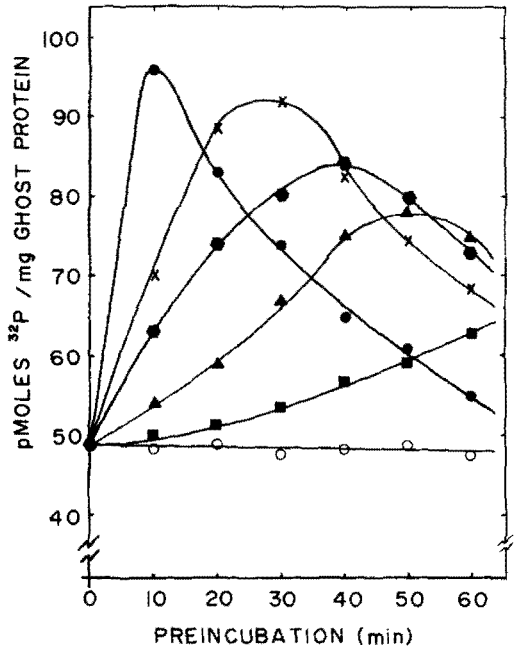


FIG. 1. **Protein kinase activity in red cell ghosts following preincubation with triethyltin bromide.** Ghosts (250 μg of protein) were preincubated with triethyltin bromide at 35 °C for the indicated times then assayed for protein kinase activity, as described in the text. No triethyltin (○), 25 μM (■), 50 μM (▲), 100 μM (●), 250 μM (×), and 500 μM (●).

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Bis-Tris, 2-[bis(α-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*-*N*,*N*′,*N*′-tetraacetic acid.

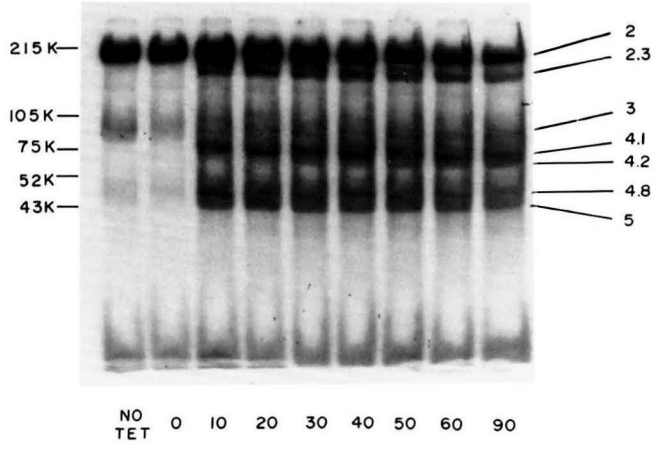


FIG. 2. **Phosphorylation of ghosts preincubated with triethyltin.** Ghosts (250 $\mu$g of protein) were preincubated with 500 $\mu$M treithyltin at 35 °C and aliquots were removed at the indicated times. [$\gamma$-$^{32}$P]ATP was added to each aliquot and the mixture was incubated for 10 min to measure the phosphorylation reaction. Samples were then prepared for SDS-PAGE and autoradiographed as described in the text.

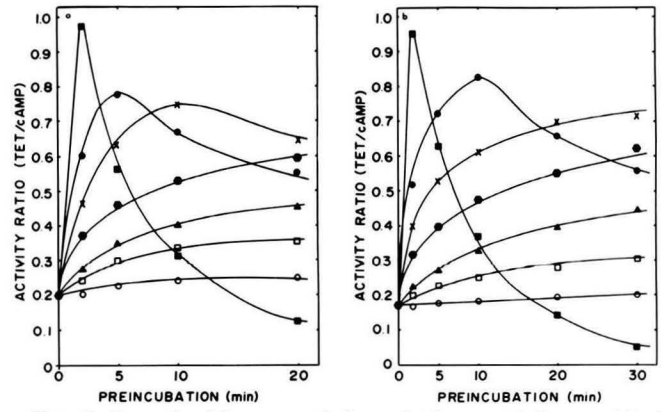


FIG. 3. **Protein kinase activity of the partially purified cyclic AMP-dependent protein kinase from red cell membranes and bovine brain following preincubation with triethyltin bromide (*TET*).** The enzyme (10 $\mu$g) was preincubated with triethyltin bromide at 35 °C for the indicated times and then assayed for protein kinase activity using histone as substrate. Activity ratios were calculated by dividing the activity in the presence of triethyltin by the activity obtained when the same enzyme was assayed in the presence of cyclic AMP. *a*, red cell membrane; *b*, bovine brain. No triethyltin (○), 10 $\mu$M (□), 25 $\mu$M (▲), 50 $\mu$M (◆), 100 $\mu$M (×), 250 $\mu$M (●), and 500 $\mu$M (■).

previous reports (22), enhanced phosphorylation of bands 2.3, 4.1, 4.5, 4.8, and 5 occurred when ghosts were exposed to cyclic AMP, indicating that proteins in these bands are acceptor substrates for phosphorylation by the cyclic AMP-dependent kinase. The cyclic AMP independent kinase normally phosphorylates proteins contained in bands 2, 2.1, and 3 (22). Band 4.2 is not normally labeled by the cyclic AMP-dependent or independent protein kinases of the red cell. Its labeling appears to be peculiar to the action of triethyltin. The results in Fig. 2 also show that prolonged preincubation with the organotin resulted in a subsequent decline in the enhancement of phosphorylation apparently due either to inactivation or inhibition of the previously stimulated enzyme.

The activation by triethyltin of protein kinase and the pattern of increased phosphorylation of membrane proteins resembled the action of cyclic AMP very closely. This suggested the possibility that triethyltin, like cyclic AMP, activates the cyclic AMP-dependent protein kinase by dissociating the catalytic subunit from the inactive holoenzyme. To explore this possibility, the effects of triethyltin on the activity of partially purified cyclic AMP-dependent kinases from red cell ghosts and from bovine brain were studied. The enzyme from brain was of interest not only because of the known sensitivity of brain to triethyltin, but also because this enzyme differs somewhat from the red cell membrane enzyme. The latter has been characterized as a type I and the former as a type II cyclic AMP-dependent protein kinase, on the basis of differences in regulatory subunit molecular weight and elution behavior from DEAE-cellulose (15, 16). When these partially purified cAMP-dependent protein kinases were preincubated with triethyltin and then assayed for kinase activity, effects similar to those observed in the phosphorylation of ghosts were noted (Fig. 3, *a* and *b*) and the two enzymes appeared to be equally sensitive to stimulation by the organotin. With the higher concentrations of triethyltin used, a rapid stimulation of activity, followed by a decline in activity was observed and with lower concentrations of the reagent, the stimulation of activity and subsequent decline in activity occurred more slowly. To determine whether the activation by triethyltin is due to dissociation of catalytic and regulatory subunits, each of the enzymes was exposed either to triethyltin or cyclic AMP and then subjected to DEAE-chromatography under conditions that are known to resolve the dissociated subunits. Fig. 4*a* shows that the regulatory (peak A) and catalytic subunits (peak B) were clearly separated by this procedure when the bovine brain enzyme was preincubated with cyclic AMP. In control experiments, the untreated holoenzyme was eluted in a position that coincided with peak A. Results identical with that with cAMP were obtained when the enzyme was first pretreated with triethyltin (Fig. 4*b*) and, as expected, catalytic activity was associated only with peak B. The enzyme isolated from red cell membranes showed identical behavior (not shown). It is therefore concluded that, as with activation by cyclic AMP, binding of triethyltin to Type I and Type II cAMP-dependent kinases causes dissociation of the catalytic and regulatory subunits. Analysis of fractions obtained from the triethyltin-treated enzyme (18) indicated that the organotin was associated with each of the two protein peaks (Fig. 4*b*). Although triethyltin was associated with the eluted catalytic subunits, protein kinase remained initially very active. Activity subsequently diminished rapidly, however, apparently because of inactivation due to the bound triethyltin. In attempts to prevent or retard the decline in activity, the effect of adding dithiothreitol to the triethyltin-activated enzyme was examined, based on the observation of Byington *et al.* (8) that dithiothreitol and similar dithiols have relatively high affinities for organotins. It was found (data not shown) that addition of dithiothreitol to the kinase immediately following activation with triethyltin lowers the rate of loss of enzyme activity. Following activation with 250 $\mu$M triethyltin, for example, 50% of the maximal activity remained after 30 min of continued incubation at 35 °C in the absence of added dithiothreitol, whereas 90% of the activity remained under the same conditions when 2.5 mM dithiothreitol was added immediately after the activation.

Although triethyltin was found to be bound to the dissociated regulatory subunit, it was not certain whether the binding occurred before or after its dissociation from the holoenzyme. The possibility remained that triethyltin binding to the regulatory subunit was unrelated to the dissociation phenomenon. To determine whether triethyltin affects the

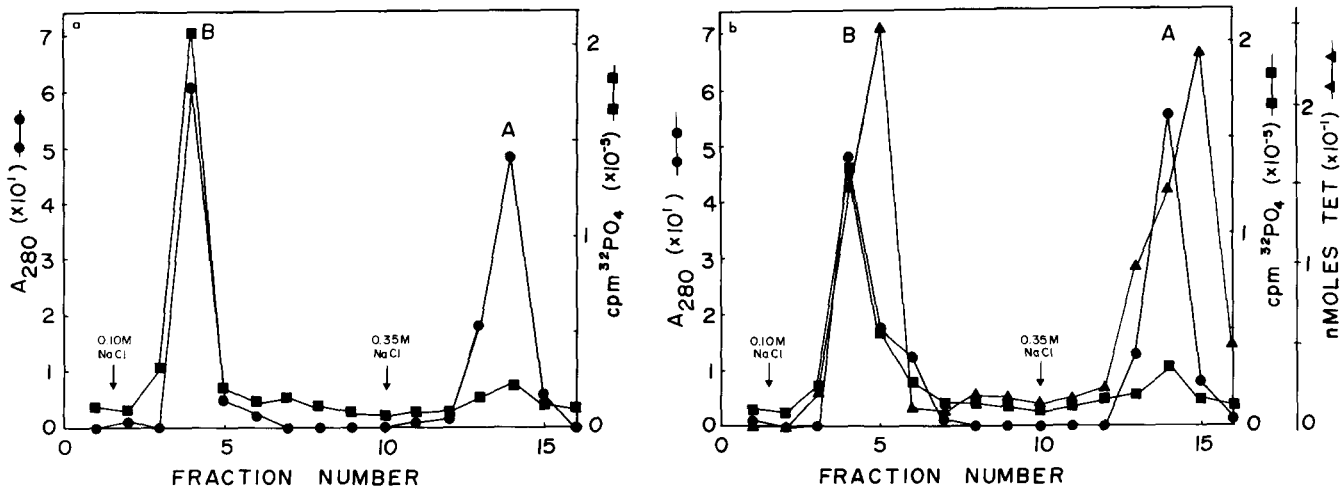


FIG. 4. **Separation of catalytic and regulatory subunits of bovine brain cyclic AMP-dependent protein kinase dissociated by cyclic AMP or triethyltin.** The enzyme (1.0 mg) was incubated with 25 μM cyclic AMP for 2 min at 35 °C, then immediately loaded onto a column of DE52 (1 × 5 cm). The catalytic subunit was eluted from the column with 0.1 M NaCl and the regulatory subunit was subsequently eluted with 0.35 M NaCl. Fractions (3 ml) were monitored at 280 nm for protein (●) and aliquots were assayed for catalytic activity (■) and for triethyltin (▲). *a*, cyclic AMP; *b*, triethyltin.

regulatory function of the R subunit, the binding of [$^3$H]cyclic AMP to red cell ghosts previously incubated with the alkyltin was examined. As shown in Fig. 5, the binding of cyclic AMP by the ghosts was strongly inhibited by the preincubation with 25–500 μM triethyltin. With 500 μM triethyltin, 50% inhibition of cAMP binding occurred within 5 min of preincubation. This effect of triethyltin on cyclic AMP binding was paralleled by a concomitant and reciprocal increase in protein kinase activity. Maximal stimulation of enzyme activity was observed in the time span in which near maximal inhibition of cAMP binding had occurred. These data suggest that the binding of triethyltin to the regulatory subunit of the holoenzyme is apparently competitive with cAMP and that it substitutes for the latter in effecting the release of the catalytic subunit. The effect of triethyltin on cyclic AMP binding was explored further using the partially purified protein kinase from bovine brain. Preincubation of the holoenzyme or its isolated regulatory subunit with triethyltin resulted in inhibition of cyclic AMP binding similar to that observed with the membrane-bound red cell enzyme. Double reciprocal plots of the data indicate that the binding of triethyltin to the regulatory subunit is noncompetitive with respect to cyclic AMP (Fig. 6) and it occurred with a $K_I$ of about 64 μM.

Triethyltin interacts with the catalytic subunit as well. It demonstrated inhibitory effects on the protein kinase activity of the holoenzyme activated by cAMP or of the isolated catalytic subunit. As can be seen from the data presented in Fig. 7, *a* and *b*, the inhibition appeared to be of a noncompetitive type, with respect to both histone and ATP, the substrates used in the analysis. The $K_m$ values of 14 μM and 16 μM for histone and ATP, respectively, obtained under these conditions are comparable to previously determined values (23, 24). The $K_I$ for the reaction of triethyltin with the catalytic subunit was 400 μM and 324 μM when measured with respect to histone and ATP, respectively. Thus, it appears that binding of triethyltin to the catalytic subunit occurred with significantly lower affinity than to the regulatory subunit.

The cascade of cellular events that can be triggered by activation of various protein kinases are well known, but the relevance of the present observations made *in vitro* to the mode of action of the organotins *in vivo* remains uncertain.

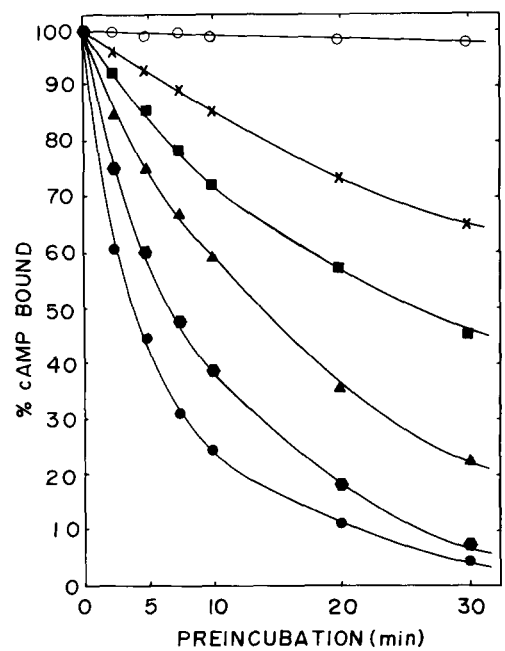


FIG. 5. **Cyclic AMP binding in red cell ghosts following preincubation with triethyltin bromide.** Ghosts (100 μg of protein) were preincubated with triethyltin bromide at 35 °C for the indicated times then assayed for cyclic AMP binding as described under "Materials and Methods." 100% binding activity corresponds to 8.2 pmol of cyclic [$^3$H]AMP bound per mg of protein. No triethyltin (○), 25 μM (×), 50 μM (■), 100 μM (▲), 250 μM (◆), and 500 μM (●).

That they may be physiologically significant is suggested by our observation that enhanced phosphorylation of certain membrane proteins occurs when intact red cells are subjected to brief exposure with triethyltin. Fig. 8, *a–c* shows that in the absence of added triethyltin or cyclic AMP, incubation of red cells with $^{32}$P-labeled inorganic phosphate resulted in a relatively large amount of $^{32}$P incorporation into components comprising bands 2 and 3, and a smaller amount in bands 4.5 and 4.8 in agreement with the report of Avruch *et al.* (25). A large amount of labeled material also occurred in the phospholipid components. When 100 μM cyclic AMP was added

to the incubation mixture, the expected change in phosphorylation pattern was observed. In addition to labeling of bands 4.5 and 4.8, enhanced incorporation of label into bands 2.1, 2.3, 4.1, 5, and 7 attributable to phosphorylation by the cyclic AMP-dependent protein kinase was found. A similar pattern of increased incorporation of $^{32}P$ was found when 500 $\mu$M triethyltin was substituted for cAMP in the reaction mixture, indicating that the organotin activated the cyclic AMP-dependent protein kinase within the intact red cell which used endogenously generated [$^{32}P$]ATP to bring about the phosphorylation of these membrane proteins. This concentration of triethyltin is at the low end of the concentration range required to cause lysis of human red cells *in vitro* (5) and it approximates the level of triethyltin found in the blood of animals injected with toxic concentrations of the compound (1). The figure also shows that a significant amount of $^{32}P$ was incorporated into the membrane protein designated band 4.2 when intact red cells were incubated in the presence of triethyltin. Phosphorylation of this component appears to be specifically associated with the effects of triethyltin since it does not occur with added cAMP and, as noted previously, phosphorylation of this band was also enhanced when red cell ghosts were exposed to organotins.

The results shown in Table I demonstrate the comparative effects of different organotin compounds on the activation of red cell membrane protein kinase. Clearly, triethyltin, (2-[(dimethylamino)methyl]phenyl)diethyltin, and tributyltin were more potent than diethyltin as activators of the kinase. With 25 $\mu$M tributyltin, results comparable to that of 250 $\mu$M triethyltin were found and, with trimethyltin, much higher concentrations were required to give similar effects. Some information regarding the nature of the binding site(s) for the organotins on the protein kinase was obtained using the compound, (2-[(dimethylamino)methyl]phenyl)diethyltin bromide. Unlike triethyltin, which is able to form pentacoor-

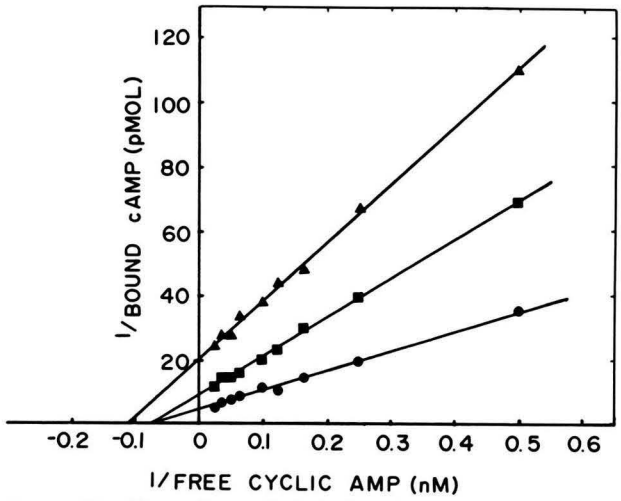


FIG. 6. **Double reciprocal plot of cyclic AMP binding to the isolated regulatory subunit of bovine brain cyclic AMP-dependent protein kinase in the presence of triethyltin bromide.** The regulatory subunit (5 $\mu$g) was incubated with triethyltin and increasing concentrations of [$^{3}H$]cyclic AMP at 35 °C for 5 min then assayed for cyclic AMP binding as described under "Materials and Methods." No treithyltin (●), 50 $\mu$M (■), and 500 $\mu$M (▲).

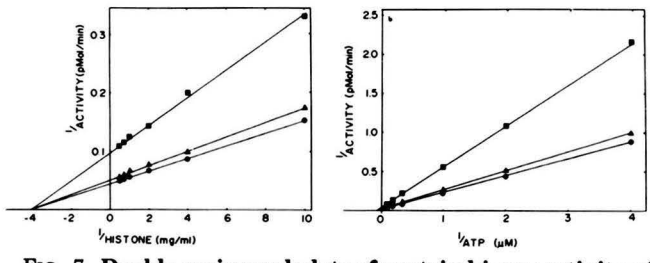


FIG. 7. **Double reciprocal plots of protein kinase activity of the isolated catalytic subunit from the bovine brain enzyme in the presence of triethyltin bromide.** The catalytic subunit (10 $\mu$g) was assayed for protein kinase activity in the presence of increasing concentrations of histone (*a*) or [$\gamma$-$^{32}P$]ATP (*b*) at 35 °C as described under "Materials and Methods." No triethyltin (●), 50 $\mu$M (■), and 500 $\mu$M (▲).

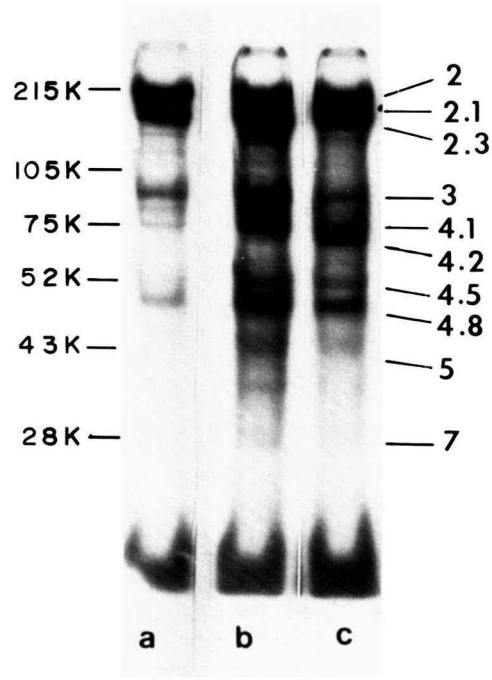


FIG. 8. **Phosphorylation of red cell membrane proteins labeled in the intact red cell with $^{32}P$.** Red cells were incubated with $^{32}P_i$ at 37 °C for 1 h, then aliquots were removed and ghosts were prepared as described under "Materials and Methods." The ghosts were then subjected to SDS-PAGE and the resulting gels were autoradiographed. Incubations were carried out in the absence of cyclic AMP (*a*), in the presence of 100 $\mu$M cyclic AMP (*b*), or in the presence of 500 $\mu$M triethyltin bromide (*c*). The resolution of bands 4.1 and 4.2 is poorly reproduced in the photograph but was distinctly seen in the autoradiograph.

TABLE I

*Activation of the partially purified red cell membrane cAMP-dependent protein kinase by various organotin compounds*

The partially purified cAMP-dependent protein kinase from red cell membrane (10 $\mu$g) was incubated with the indicated concentration of the organotins for 2 min at 35 °C, then assayed for protein kinase activity as described under "Materials and Methods." The activity ratio was calculated by dividing the activity observed in the presence of the organotin by the activity obtained when the enzyme was activated by 2 $\mu$M cAMP.

| Concentration of organotin ($\mu$M) | Activity ratio | | | | |
|---|---|---|---|---|---|
| | Diethyltin | Trimethyltin | Triethyltin | SnC[a] | Tributyltin |
| 0 | 0.20 | 0.19 | 0.20 | 0.19 | 0.20 |
| 2 | | | | | 0.22 |
| 10 | 0.22 | 0.19 | 0.22 | 0.23 | 0.27 |
| 25 | 0.24 | 0.20 | 0.26 | 0.28 | 0.49 |
| 50 | 0.28 | 0.20 | 0.37 | 0.39 | 0.75 |
| 100 | 0.30 | 0.21 | 0.40 | 0.44 | 0.98 |
| 250 | 0.46 | 0.22 | 0.55 | 0.58 | 0.84 |
| 500 | 0.67 | 0.23 | 0.97 | 0.98 | 0.41 |

[a] SnC = (2-[(dimethylamino)methyl]phenyl)diethyltin bromide.

dinate complexes with proteins by interacting with two properly positioned axial donor atoms (26), this compound has only one site on the metal available for coordinate binding to a protein (27). This internal pentacoordinate tin compound produced rapid activation of the protein kinase followed by a slow inactivation as well. Like triethyltin, it inhibited binding of cyclic AMP to the regulatory subunit and inactivated the isolated catalytic subunit (results not shown). It appears, therefore, that the binding of these organotins to the regulatory and catalytic subunits of the cAMP-dependent protein kinase involves a single donor atom for tin on each of these subunits. When the comparative effects of these compounds on the bovine brain enzyme were examined, essentially the same results were obtained.

## DISCUSSION

Conditions other than the exposure to cyclic AMP that lead to activation of the type I cyclic AMP-dependent protein kinase have been noted (15, 28, 29). Incubation of the holoenzyme with 0.5 M NaCl, 50 μg/ml of protamine, and 700 μg/ml of histone results in dissociation of the holoenzyme into regulatory and active catalytic subunits. The type II protein kinase, in contrast, is activated by cyclic AMP but not by these other treatments (16, 30). As shown in the present work, both type I and type II kinases are activated by exposure to relatively low concentrations of organotins (10–500 μM). Thus, the effect of organotins resembles that of cyclic AMP in this sense. However, while 2 μM cyclic AMP gave rapid and maximal activation of the kinases, approximately 250-fold higher concentrations of triethyltin were required for a similar effect. After 1–2 min of preincubation with 500 μM triethyltin, the activation observed was 90% of the level achieved with cyclic AMP and, with 250 μM triethyltin, it was about 50%. At lower concentrations, the activation occurred more slowly. With 50 μM triethyltin, for example, half-maximal stimulation occurred after 10 min of incubation and, with 10 μM, the activation was slower but still clearly discernible.

The holoenzymes of the type I and type II protein kinases are known to consist of two regulatory and two catalytic subunits (31). The results of the present work suggest the following scheme as a working model for the mode of action of triethyltin on these kinases.

$$R_2C_2 + x\ TET \xrightarrow{k_1} R_2\text{–}TET_x + 2C \xrightarrow[y\ TET]{k_2} 2\ (C\text{–}TET_y)$$

The scheme suggests that triethyltin (TET) binds first to the regulatory subunits of the enzyme and induces the release of the active catalytic subunits. That binding to the regulatory subunit occurs is shown by our observation that triethyltin is bound to the isolated R subunit and that triethyltin inhibits binding of cAMP to the holoenzyme and to the isolated regulatory subunit. Kinetic analysis of the data indicates that the activation step occurs at a pseudo-first order rate with respect to triethyltin concentration, with a calculated rate constant, $k_1$ of $2.40 \times 10^{-2}$ $min^{-1}$. The subsequent slow loss in activity is an apparently irreversible inactivation of the enzyme since activity cannot be regained. When the data were analyzed in terms of a triethyltin-induced irreversible inhibition of activity (32), they were consistent with that of a second order rate of decay of activity. A rate constant, $k_2$, of $5.13 \times 10^2$ liters $mol^{-1}$ $min^{-1}$ was found for the reaction. It is not known at this stage whether the reagent binds to both types of subunits within the holoenzyme or whether it binds to the catalytic subunit only after it is dissociated. However, the similarity in the second order kinetics of the triethyltin-induced loss of activity following initial activation by triethyltin or cyclic AMP suggests that triethyltin binds to the catalytic subunit after it is released from the regulatory subunit. No information is available on the stoichiometry of binding or on the affinity of the catalytic subunits for triethyltin as yet, but such information should be forthcoming when adequate quantities of appropriately purified enzymes become available. From the data presented in Fig. 6, an estimate as to the stoichiometry of triethyltin binding to the regulatory subunit can be made. Between two and three triethyltin molecules are bound per $R_2$ subunit. However, further work is required to determine the exact number.

Binding of triethyltin to subunits in certain other proteins with quaternary structures also produces major conformational and functional changes. In the case of cat hemoglobin, binding of 2 eq of triethyltin is known to occur within the α-subunits (26). The interaction results in the formation of a stable adduct with gross conformational changes, as measured by electrophoretic mobility, oxygen affinity (7, 26), and UV-Vis difference spectra.[2] In results of work to be published elsewhere, we report that the interaction of triethyltin with yeast hexokinase produces effects that are similar to those reported here with the cyclic AMP-dependent kinases, insofar as an initial rapid subunit dissociation followed by a slow inactivation of the enzyme are involved.

The triethyltin binding sites in the cAMP-dependent protein kinases and yeast hexokinase seem to be different from sites that occur in cat and rat hemoglobins (26, 33). Binding to these hemoglobins occurs with the formation of a pentacoordinate complex between the tin of the trialkyltin and the sulfur of a cysteine and a nitrogen of an imidazole group of histidine in the native globin structure (26, 33). The binding of organotins to the protein kinase(s) and hexokinase, on the other hand, appear to involve a single coordination site on the tin since the internally pentacoordinate organotin compound (2-[dimethylamino)methyl]phenyl)diethyltin bromide with only one coordination site available on the tin exert the same effects as the tetracoordinate triorganotins. Aldridge *et al.* (27) used such compounds to assess the binding of organotins to the rat liver mitochondrial ATPase system and found that they were as effective or even more so than their corresponding trialkyltins as inhibitors. They concluded from results of studies using a variety of organotin compounds with different alkyl and aryl (R) groups that in addition to the single coordination site for the tin, hydrophobic interactions of the R groups with the protein play a part in the binding. Our studies on the interaction of organotins with the cyclic AMP-dependent protein kinases and yeast hexokinase indicate s similar mode of binding. The nature and location of the donor ligand on the protein kinase have yet to be determined, but the known affinity of organotin compounds for sulfur (26, 27, 33) and the occurrence of cysteine residues in the catalytic region of the enzymes (34) suggest their possible involvement.

The phosphorylation of protein band 4.2 in red cell ghosts or intact cells is of some interest because it appears to be peculiar to the action of the tetracoordinate alkyltin compounds. It does not occur when cAMP is used to activate the protein kinases or even when the internal pentacoordinate organotin compound (2-[(dimethylamino)methyl] phenyl)diethyltin bromide is substituted for triethyltin. This difference in the effects of the tetracoordinate trialkyltin and the pentacoordinate compound is reminiscent of the difference that was noted previously when the binding of these compounds to cat hemoglobin was analyzed. The similarity

[2] K. R. Siebenlist and F. Taketa, unpublished observation.

in relative effects of these compounds on cat hemoglobin and on component band 4.2 suggests that triethyltin exerts its effect on the latter by directly interacting with it. Apparently, the appropriate donor ligands are present in the protein of band 4.2 for formation of a pentacoordinate complex with triethyltin and, as with the conformational change that results from such an interaction in cat hemoglobin (26), a change in conformation of the protein probably ensues. Its altered conformation could then make the protein become a phosphoryl acceptor in the reaction catalyzed by the endogenous protein kinases.

The enhanced phosphorylation of the membrane proteins resulting from brief exposure to triethyltin in intact red cells is consistent with the organotin-induced activation of the cyclic AMP-dependent protein kinase and suggests that this particular mode of action of organotins may be significant *in vivo*. Data on protein kinase activity in tissues of animals administered chronic or acute doses of alkyltins remain to be obtained. Such information, especially concerning nervous tissue, should be useful since the primary pathophysiological effects of alkyltins are associated with this tissue. Because organotins exert the dual effect of activation and then subsequent inhibition of cyclic AMP-dependent protein kinases, data on temporal effects of triethyltin exposure on protein kinase activity and their relationship to cellular effects are also of interest.

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