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EFFECTS OF LOCAL ANESTHETICS ON CALMODULIN-DEPENDENT GUANYLATE CYCLASE IN THE PLASMA MEMBRANE OF *TETRAHYMENA PYRIFORMIS*

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Abstract—A highly purified preparation of *Tetrahymena* calmodulin activated a membrane-bound guanylate cyclase by more than 40-fold. This activation of guanylate cyclase by calmodulin was inhibited completely by local anesthetics such as dibucaine, tetracaine, lidocaine and procaine at concentrations that had no appreciable effect on the activities of basal guanylate cyclase (without calmodulin) and adenylate cyclase. The inhibition by dibucaine of calmodulin-mediated activation of the enzyme activity was not reversed by calcium but was partially overcome by increasing the concentration of calmodulin. Kinetic analysis of local anesthetic-induced inhibition of activation of guanylate cyclase demonstrated a mixed type of antagonism. These results suggest the possibility that the inhibition of calmodulin-dependent guanylate cyclase resulted, in part, from interaction of the drugs with calmodulin.

A Ca^{2+}-dependent regulatory protein, calmodulin, is present in a variety of eukaryotic organisms. First described as a heat-stable activator of cyclic nucleotide phosphodiesterase [1, 2], the protein has since been shown to modulate the activity of diverse enzymes and to mediate the regulatory effects of Ca^{2+} on many biological processes [3–6].

In earlier reports, we described the calmodulin in a ciliated protozoan, *Tetrahymena pyriformis* [7–9], and demonstrated that guanylate cyclase (EC 4.6.1.2.), which is entirely associated with particulate fractions, was activated by calmodulin in the presence of Ca^{2+} [7, 9–11]. No other calmodulins from the various phyla investigated thus far activated *Tetrahymena* guanylate cyclase except the calmodulin from another ciliated protozoa, *Paramecium tetraurelia* [12]. The specific interaction of guanylate cyclase with *Tetrahymena* calmodulin may reflect the amino acid sequence of this calmodulin which displays eleven substitutions and one deletion compared with bovine brain calmodulin [13].

Local anesthetics have been shown to induce some biological actions in protozoan cells. These include deciliation [14] and mucocyst discharge [15] in *Tetrahymena* and reversal of the ciliary beating direction in *Paramecium* [16]. Despite the somewhat disparate nature of the above phenomena, all have in common the involvement of calcium. Thus, it is possible that calmodulin and calmodulin-dependent enzymes may be implicated in these calcium-mediated phenomena [17]. Therefore, we have investigated the effects of local anesthetics such as dibucaine, tetracaine, lidocaine and procaine on calmodulin-dependent guanylate cyclase of *T. pyriformis* in the hope of gaining further insight into the physiological role of calmodulin in this organism.

The results obtained from this study demonstrate that the local anesthetics inhibited calmodulin-stimulated activity of membrane-bound guanylate cyclase of *Tetrahymena* at concentrations which exerted little effect on the basal guanylate cyclase activity (without calmodulin) and another membrane-bound enzyme, adenylate cyclase. They also suggest that the inhibition might have been due, in part, to a direct interaction of the drugs with calmodulin.

MATERIALS AND METHODS

Materials. [8-^{3}H]GTP and [2-^{3}H]ATP were purchased from the Radiochemical Centre, Amersham. All unlabeled nucleotides used were obtained from the Sigma Chemical Co., St. Louis, MO. Creatine phosphate and creatine kinase were purchased from Boehringer, Mannheim. 3-Isobutyl-1-methylxanthine was obtained from the Aldrich Chemical Co., Milwaukee, WI. Neutral aluminum oxide was a product of Woelm Pharma. The sources of the local anesthetics used were: dibucaine hydrochloride (Teikoku Chemical Industry, Tokyo), tetracaine hydrochloride (Kyorin Pharmaceutical Co., Tokyo), lidocaine hydrochloride (Fujisawa Pharmaceutical Co., Tokyo), and procaine hydrochloride (Iwaki Pharmaceutical Co., Tokyo).

Preparation of enzymes and calmodulin. A thermotolerant strain (NT-1) of *Tetrahymena pyriformis* was grown at 39.5° in an enriched proteose–peptone medium as described previously [18]. Cultures were harvested in the early stationary phase of cell growth. The plasma membrane fraction was isolated according to the method of Nozawa and Thompson [18] using a phosphate buffer (0.2 M K_2HPO_4/0.2 M KH_2PO_4/3 mM EDTA/0.1 M NaCl, pH 7.2), and then washed three times in 10 mM Tris–HCl (pH 7.5), containing 250 mM sucrose, 1 mM dithiothreitol and 0.5 mM EGTA† to reduce endogenous calmodulin. This preparation was contaminated with

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† Abbreviations: EGTA, ethylene glycol-bis (β-aminoethyl ether)-*N,N'*-tetraacetic acid; cyclic GMP, guanosine 3',5'-monophosphate; and cyclic AMP, adenosine 3',5'-monophosphate.

a trace amount of calmodulin [8], but this endogenous calmodulin scarcely contributes to the activation of guanylate cyclase by added calmodulin. The final pellet of plasma membrane was resuspended in the same Tris–HCl buffer without EGTA and sonicated at 9 kilocycles for 2 min in a Branson sonifier (B-12). This suspension was used for enzyme assay. Calmodulin was purified from *Tetrahymena* by the method described by Kakiuchi *et al.* [19], using fluphenazine affinity chromatography. The purified calmodulin demonstrated a single band on 15% polyacrylamide gels in the buffer system of Davis [20].

Guanylate and adenylate cyclase assays. Unless otherwise indicated, the standard assay mixture for guanylate cyclase contained 1 mM [^{3}H]GTP (5 Ci/mole), 1 mM cyclic GMP, 15 mM creatine phosphate, 20 μg creatine kinase, 1 mM dithiothreitol, 1.5 mM isobutylmethylxanthine, 3 mM $MgCl_2$, 100 μM $CaCl_2$, 25 mM Tris–maleate (pH 6.8), and 100 μg of enzyme protein in a total volume of 0.2 ml. For the adenylate cyclase assay, the same assay conditions as for guanylate cyclase were employed, except that 1 mM [^{3}H]ATP (4 Ci/mole), 0.8 mM cyclic AMP, and 10 mM $MgCl_2$ were substituted for GTP, cyclic GMP, and 3 mM $MgCl_2$ respectively. After the assay mixture was incubated at 37° for 15 min, the reaction was terminated by heating for 2 min in a boiling bath, following the addition of 1 N HCl (40 μl). The radioactive cyclic GMP or cyclic AMP was isolated by the serial use of a neutral aluminum oxide-Dowex 1-X2 column, and radioactivity was determined as described elsewhere [21]. Protein was determined by the method of Lowry *et al.* [22], with bovine serum albumin as a standard.

RESULTS

Effects of local anesthetics on the guanylate and adenylate cyclase activities of Tetrahymena. In the plasma membrane fraction, the calmodulin-dependent guanylate cyclase was maximally stimulated by 10 μg of calmodulin to about 40-fold above the basal activity in the presence of Ca^{2+}. *Tetrahymena* calmodulin did not influence the guanylate cyclase activity in the absence of Ca^{2+}. The specific activities of the basal and the maximally activated guanylate cyclase were 20–40 and 1100–1400 pmoles per min per mg protein respectively. The effects of the local anesthetics dibucaine, tetracaine, lidocaine and procaine on *Tetrahymena* guanylate cyclase with or without calmodulin are shown in Fig. 1. The reduction by the local anesthetics of the stimulation by calmodulin of guanylate cyclase was dose-dependent. The approximate concentrations of local anesthetics producing 50% inhibition of calmodulin-stimulated guanylate cyclase (IC_{50} value) were: dibucaine, 0.28 mM; tetracaine, 0.84 mM; lidocaine, 4.50 mM; and procaine, 10.1 mM. On the other hand, in the concentration range examined, the local anesthetics failed to inhibit the activity of guanylate cyclase in the absence of calmodulin.

We also examined the effects of these drugs on *Tetrahymena* adenylate cyclase activity which has been known to be localized in the plasma membrane and to be unaffected by calmodulin [10, 11]. Adenylate cyclase had a specific activity of 44.5 pmoles per min per mg protein under our assay conditions. This enzyme activity was inhibited by local anesthetics in a dose-dependent manner (Fig. 2). The IC_{50} values of dibucaine, tetracaine, lidocaine, and procaine for adenylate cyclase were 0.75, 1.80, 12.0 and >20 mM respectively. Of the drugs examined, the IC_{50} values for adenylate cyclase were several times greater than those for calmodulin-stimulated guanylate cyclase, suggesting that the local anesthetics selectively suppressed the calmodulin-dependent activation of guanylate cyclase at the low calmodulin concentration of 10 μg. In the following experiments dibucaine was employed as an example of this class of drugs.

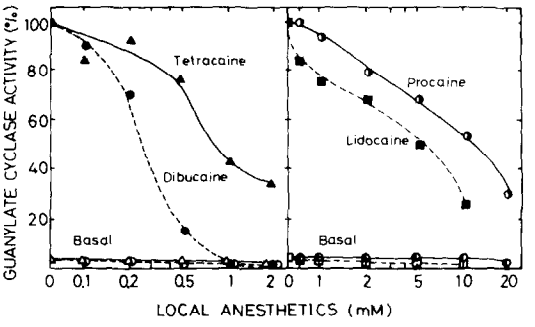

Fig. 1. Inhibition of *Tetrahymena* guanylate cyclase activity by local anesthetics in the presence and absence of calmodulin. Guanylate cyclase activity in plasma membrane of *T. pyriformis* was measured in the absence of calmodulin at various concentrations of dibucaine (○), tetracaine (△), lidocaine (□) and procaine (◐), and in the presence of 10 μg calmodulin at various concentrations of dibucaine (●), tetracaine (▲), lidocaine (■) and procaine (◑). The guanylate cyclase activity was 1100–1400 pmoles per min per mg protein in the presence of 100 μM Ca^{2+} and 10 μg calmodulin. Each point is the mean of at least two experiments.

Effects of Ca^{2+} *on dibucaine-induced inhibition of guanylate cyclase activation.* Since the activation by calmodulin of guanylate cyclase was dependent on Ca^{2+} concentration, we determined whether the inhibition of calmodulin-stimulated guanylate cyclase was due to chelation of Ca^{2+} by dibucaine. An increase of Ca^{2+} concentration in the reaction

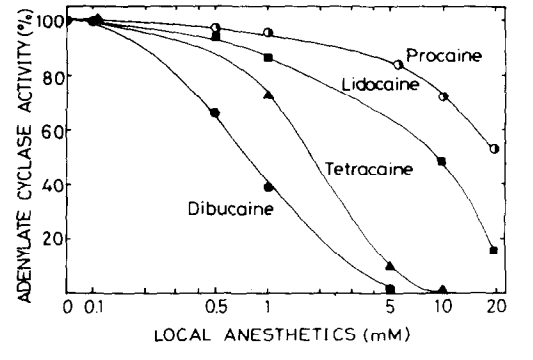

Fig. 2. Inhibition of *Tetrahymena* adenylate cyclase activity by local anesthetics. Adenylate cyclase activity in plasma membrane of *T. pyriformis* was measured at various concentrations of dibucaine, tetracaine, lidocaine and procaine. The activity of 100% was 44.5 pmoles per min per mg protein each point is the mean of ≥ two experiments.

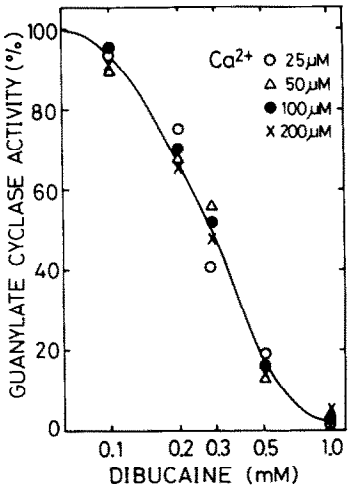

Fig. 3. Effects of Ca^{2+} on dibucaine-induced inhibition of guanylate cyclase. Stimulation of guanylate cyclase activity by 10 μg calmodulin in *Tetrahymena* plasma membrane was normalized to 100% at each Ca^{2+} concentration. The Ca^{2+} concentrations in the reaction mixture were 25, 50, 100 and 200 μM.

mixture did not affect the inhibitory effect at various concentrations of dibucaine (Fig. 3). The implication is that the inhibitory effect of dibucaine on guanylate cyclase activation was not mediated by a direct action on the Ca^{2+} that is essential for calmodulin activation of the enzyme.

Effect of substrate concentration on dibucaine-induced inhibition of guanylate cyclase. To exclude the possibility of interaction between local anesthetics and substrate, the effect of dibucaine on reaction velocity was examined as a function of GTP concentration. A Lineweaver–Burk plot of initial velocity versus GTP concentration for calmodulin-stimulated guanylate cyclase is shown in Fig. 4. The apparent K_m of GTP for guanylate cyclase in the absence and presence of 0.3 mM dibucaine was not altered and represented approximately 50 μM. Thus, the inhibition by dibucaine appeared to be noncompetitive with respect to GTP as substrate.

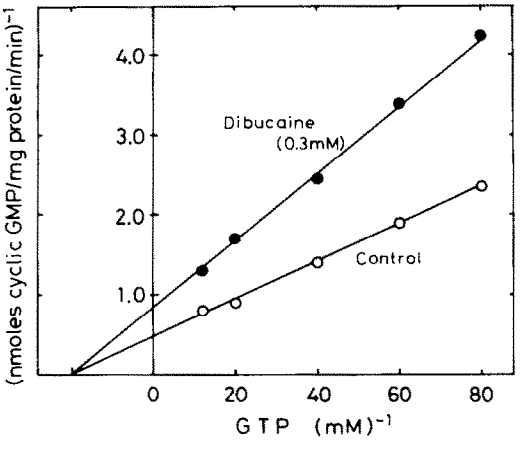

Fig. 4. Double-reciprocal plot of the effect of dibucaine on guanylate cyclase activation. The enzyme was assayed in the absence (○) or presence (●) of 0.3 mM dibucaine. GTP concentrations ranged from 12.5 to 100 μM, and 10 μg calmodulin was always present.

Restoration by excess calmodulin of dibucaine-inhibited guanylate cyclase activity. To obtain further evidence indicating that the pronounced inhibition by anesthetics of guanylate cyclase stimulation was caused by the interaction of the drug with calmodulin, we measured the dibucaine-induced inhibition of guanylate cyclase activation in various concentrations of calmodulin. As shown in Fig. 5, in the absence of the anesthetic (control samples), the added calmodulin produced a gradual increase of guanylate cyclase activity in a concentration-related fashion. In the presence of 0.5 mM EGTA, the activation of guanylate cyclase by calmodulin was almost completely abolished; on the other hand, when Ca^{2+} was present, the drug decreased the V_{max} for guanylate cyclase. Increasing the calmodulin concentration tended to reduce the dibucaine-induced inhibition of the enzyme activity but it did not restore it to maximal activity. The maximal attainable activities of guanylate cyclase in the presence of excess calmodulin up to 50 μg were approximately 77 and 52% of the control level with 0.15 and 0.3 mM dibucaine respectively. To investigate further the interactions among calmodulin, guanylate cyclase and dibucaine, double-reciprocal plots of the guanylate cyclase activity were graphed as a function of calmodulin concentration. Figure 6 shows the double-reciprocal plots of representative data in the absence or presence of dibucaine. The points at which the lines cross the y axis represent the theoretical maximum velocity (V_{max}) obtained in the presence of calmodulin. As can be seen, the V_{max} value for guanylate cyclase decreased as the concentration of dibucaine increased. On the other hand, the $K_{0.5}$ for calmodulin stimulation of guanylate cyclase was increased by the drug. These data indicate that this inhibition of guanylate cyclase activation by dibucaine was of a "mixed type", suggesting that the inhibition of calmodulin-stimulated guanylate cyclase was due not only to a competitive antagonism of calmodulin with dibucaine but also to another mechanism that was not reversible by excess calmodulin.

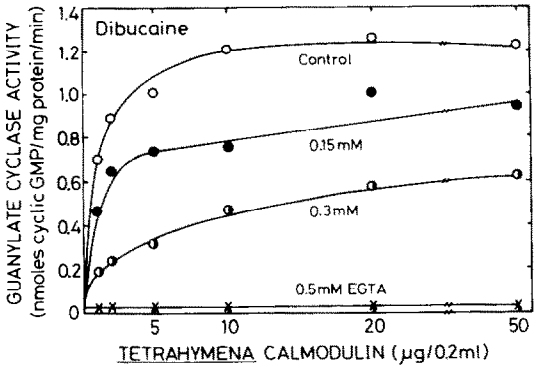

Fig. 5. Effects of calmodulin concentrations on inhibition by dibucaine or EGTA of guanylate cyclase activation. Guanylate cyclase activity in plasma membrane of *T. pyriformis* was measured in the absence of any inhibitors and in the presence of 0.15 mM dibucaine, 0.3 mM dibucaine, or 0.5 mM EGTA and various concentrations of calmodulin.

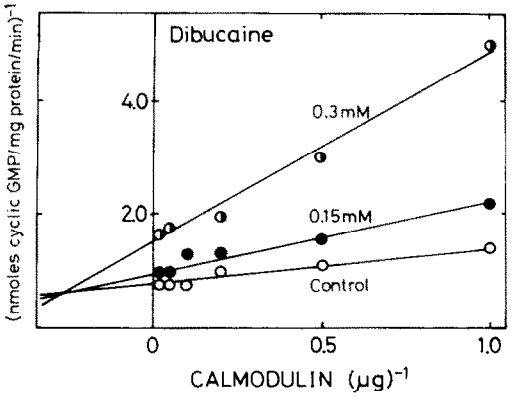

Fig. 6. Kinetic analysis of dibucaine-induced inhibition of guanylate cyclase activation. Activity of *Tetrahymena* guanylate cyclase was measured in the absence of dibucaine and in the presence of 0.15 or 0.3 mM dibucaine at various concentrations of calmodulin.

DISCUSSION

It has been reported recently that several drugs, including phenothiazines and local anesthetics, inhibit calmodulin-stimulated activities of (Ca^{2+}–Mg^{2+})ATPase, cyclic nucleotide phosphodiesterase, myosin light chain kinase, and membrane-bound calcium transport [23–26]. We previously reported that phenothiazine antipsychotic agents such as trifluoperazine inhibited the calmodulin-stimulated guanylate cyclase of *Tetrahymena* and suggested that these drugs selectively prevent Ca^{2+}-dependent activation of guanylate cyclase by competing with calmodulin [27]. The present report demonstrates that certain local anesthetics (dibucaine, tetracaine, lidocaine and procaine) exerted a strong inhibition of guanylate cyclase activation induced by calmodulin in this ciliated organism. The inhibition of calmodulin-dependent guanylate cyclase activity by local anesthetics was not completely eliminated by the addition of excess calmodulin. Simple competitive antagonism between calmodulin and these local anesthetics does not account entirely for our observations of inhibition of calmodulin-stimulated guanylate cyclase. In a recent report, Volpi *et al.* [23] demonstrated that local anesthetics inhibit calmodulin-dependent stimulation of erythrocyte Ca^{2+}-ATPase and cyclic nucleotide phosphodiesterase from brain and heart as well as the binding of ^{125}I-labeled calmodulin to the erythrocyte membrane and suggested that the enzyme inhibition was mediated through direct binding of local anesthetics on calmodulin. However, their results also demonstrated that the inhibition of membrane-bound Ca^{2+}-ATPase by local anesthetics was partially restored by the addition of excess calmodulin, while the inhibitory action on soluble phosphodiesterase was completely reversed by added calmodulin. These findings may provide an explanation for the results obtained here for the inhibition of membrane-bound guanylate cyclase which exhibited a mixed-type antagonism against calmodulin. That is, it may be that the local anesthetics affect stimulation of membrane-bound enzymes by calmodulin in a more complex way than they affect stimulation of soluble enzymes. Although the molecular mechanisms responsible for the effects of local anesthetics on calmodulin-stimulated guanylate cyclase have not been fully elucidated, one of the factors that should be considered in explaining the effects of local anesthetics on the enzymes is the interaction of local anesthetics with membrane phospholipids [28]. Indeed, in a previous paper, we have shown that dibucaine increases plasma membrane fluidity of *Tetrahymena* as estimated with 1,6-diphenyl-1,3,5-hexatriene (DPH) and also causes inhibition of ATPase activity bound to this membrane [29]. In addition, we have reported that the activity of membrane-bound adenylate cyclase of *Tetrahymena* is dependent on the physical state of plasma membrane [30]. As clearly shown in the present study, adenylate cyclase, which is unrelated to calmodulin, was also suppressed by the local anesthetics in a dose-dependent manner. Therefore, it is likely that local anesthetics at relatively high concentration may have some effects on the immediate lipid environment of adenylate cyclase and, thereby, alter their conformation and impair the enzyme activity. As these possible interactions of local anesthetics cannot be excluded, the results on the antagonism of calmodulin-dependent guanylate cyclase by local anesthetics have to be interpreted with the limitation that they may not be specific for calmodulin. Further studies on solubilized enzymes or reconstituted enzymes with different lipid environments may help to resolve the molecular mechanisms of the action of local anesthetics on membrane-bound enzyme.

Several local anesthetics are known to affect a wide range of Ca^{2+}-dependent processes, such as exocytosis [31], platelet aggregation [32], membrane transport of calcium [33] and cell spreading and motility ([34]; for review see Ref 35). We also observed that local anesthetics inhibited growth and Ca^{2+} uptake of *Tetrahymena* cells. The IC_{50} values for cell growth were found to be lower than those for both calmodulin-dependent guanylate cyclase activity and Ca^{2+} uptake, but the order of inhibitory potency of the anesthetics against cell growth was identical to that for calmodulin-dependent guanylate cyclase and Ca^{2+} uptake (unpublished data). These results suggest the possibility that the inhibition of growth by local anesthetics may result from inhibition of guanylate cyclase and also Ca^{2+} uptake, but at the present time their relative contribution is not known. On the other hand, we previously reported that both guanylate cyclase activity and calmodulin content fluctuate during cell growth and cell cycle of *Tetrahymena* and suggested that the changes in guanylate cyclase activity may reflect the intracellular level of calmodulin [36, 37]. More recently, Zwiller *et al.* [38] also suggested that the activation of guanylate cyclase might play a role in the growth of cultured cells. These data further implicate the calmodulin-guanylate cyclase system in the growth of *Tetrahymena* cells. Therefore, although we are aware that local anesthetics may interfere with the processes that are independent of calmodulin and, thus lead to deteriorated functions essential for cell growth, it is tempting to speculate that the drugs may contribute to supressed cell growth through

interaction with a Ca^{2+}–calmodulin-dependent process such as guanylate cyclase activation.

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