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Effect of Triton X-100 and Alamethicin on the susceptibility of brain adenylate cyclase to EGTA inhibition*

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Divalent cations are important modulators of hormone-sensitive adenylate cyclase. The presence of a metal ion is required for enzymatic activity since the substrate for the enzyme is a metal–ATP complex. Mg^{2+} · ATP is probably the physiological substrate but Mn^{2+} or Co^{2+} can substitute for Mg^{2+} *in vitro* [1, 2]. In most systems, in fact, the activity measured with Mn^{2+} is higher than that measured with Mg^{2+} [2–8]. In addition to the requirement for metal to complex with ATP, recent evidence suggests that there is a separate, regulatory metal ion-binding site on the enzyme and that binding of a metal ion to this site is required for full adenylate cyclase activity [3–5].

Adenylate cyclase from several regions of the brain is inhibited by EGTA† (0.1 to 0.5 mM), but the enzyme from other tissues is not affected [2, 6–10]. This inhibition by 0.1 to 0.5 mM EGTA is not due to chelation of the metal required to form the substrate metal · ATP complex since it occurs in the presence of 5–12 mM Mg^{2+}. Rather, it is probably due to chelation of a metal ion associated with the enzyme itself. Recent experiments from this laboratory have shown that inhibition of detergent-solubilized brain adenylate cyclase by EGTA can be almost entirely reversed by the addition of Mn^{2+} in excess of EGTA [11]. These experiments suggest that brain adenylate cyclase may have associated manganese ions which are required for activity. Co^{2+} does not reverse EGTA inhibition [11], whereas Ni^{2+}, Zn^{2+} and Fe^{2+} are inhibitors of the enzyme [2]. The EGTA inhibition can also be partially reversed by adding calcium in excess of EGTA [11]. Brostrom *et al.* [8] and Cheung *et al.* [9] have show that the effect of calcium requires the presence of calmodulin, a small protein which mediates the action of calcium in several systems. It is not clear whether Ca^{2+} and Mn^{2+} affect the same population of adenylate cyclase molecules.

Inhibition of brain adenylate cyclase by EGTA has been reported to occur both in detergent-solubilized and in particulate preparations. In the former, the degree of inhibition is between 75 and 100 per cent [2, 10, 11]. The results with particulate enzyme, however, are much more variable, with reports ranging from no inhibition to 70 per cent inhibition [7, 12, 13]. The present studies show that enzyme need not be solubilized for maximum inhibition by EGTA, but that some disruption of the lipid bilayer seems to be required. This suggests that the metal ion required for full brain adenylate cyclase activity may be located in a region that is not accessible to EGTA in the intact membrane.

Mature rats were killed by asphyxiation in CO_2 or by a blow to the head. The brains were immediately removed and chilled, and the cortices were dissected free of white matter. The tissue was homogenized in a Dounce homogenizer with a loose fitting pestle in 0.1 M Tris · Cl (pH 7.6), 0.075 M sucrose, 10 mM $MgCl_2$, and 1 mM dithiothreitol. The unfractionated homogenate was used in some experiments. For others, a membrane-enriched fraction was prepared by discontinuous sucrose density gradient centrifugation as described previously [14]. Homogenates were also prepared in hypotonic buffer, a procedure frequently used to lyse synaptosomes; cortical tissue was homogenized in 2 mM Tris · Cl (pH 7.6), the homogenate was centrifuged at 20,000 *g* for 20 min at 4°, and the pellet was washed twice more and resuspended in the same buffer.

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† Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; and ECTEOLA, epichlorhydrin triethanolamine cellulose.

Basal adenylate cyclase activity was assayed by a modification of the method of Krishna *et al.* [15] using [^{3}H]ATP as the substrate. This method has been described previously [11]. The composition of the assay mixture was: 0.8 to 1 mM [^{3}H]ATP (Amersham, Arlington Heights, IL; unlabeled ATP was from the Sigma Chemical Co., St. Louis, MO), 1 mg/ml bovine serum albumin, 0.4 mg/ml creatine kinase (Boehringer, 34 units/mg), 12 mM creatine phosphate (Boehringer), 0.05–0.1 M Tris·Cl (pH 7.6), 2 mM cyclic AMP (Sigma), and 12 mM $MgCl_2$; 5 mM $MnCl_2$ or 0.5 mM EGTA was added as indicated. Assays were performed at 37° for 10 min. The fact that the radioactive product measured was indeed cyclic AMP was verified in selected assays with or without Alamethicin by rechromatography on ECTEOLA paper (Whatman) by the method of Rao *et al.* [16].

Protein was determined by the method of Lowry *et al.* [17] as modified by Bailey [18].

Table 1 shows the effect of EGTA on basal adenylate cyclase activity in three kinds of particulate preparations: an unfractionated homogenate made in an approximately isotonic buffer, a membrane fraction prepared by flotation in a discontinuous sucrose density gradient, and a particulate fraction prepared and washed in a hypotonic buffer. Inhibition of adenylate cyclase activity by 0.5 mM EGTA ranged from 17 to 37 per cent. In all the preparations, inhibition was maximal with 0.25 mM EGTA; increasing the EGTA concentration to 2 mM did not change the degree of inhibition. From these results it is apparent that the method of tissue preparation affects the ability of EGTA to inhibit activity. EGTA inhibited least when the cells were broken in isotonic buffer and the enzyme was assayed without further manipulation. The more the membranes were manipulated and disrupted, the greater the inhibition of adenylate cyclase by EGTA.

Two explanations may be proposed for the resistance of membrane bound adenylate cyclase to inhibition by EGTA. First, the site to which EGTA must gain access might be sealed within impermeable vesicles. Second, the site might be in a hydrophobic region either within the plasma membrane or in a hydrophobic portion of the enzyme. To try to distinguish these possibilities we examined the effects of two agents that disrupt the structure of the membrane, probably in different ways: Triton X-100, a non-ionic detergent, and Alamethicin*, an ionophorous antibiotic that has been reported to reveal latent adenylate cyclase activity in cardiac sarcolemmal vesicles [19].

Figure 1 shows the effect of increasing concentrations of Triton X-100 on adenylate cyclase activity assayed with Mg^{2+}, Mg^{2+} and EGTA, or Mg^{2+} and Mn^{2+}. As we and others have shown previously, Triton X-100 increases the activity of the brain enzyme [20, 21]. Maximum activation occurred with 0.03 to 0.04% Triton X-100. Adenylate cyclase was increasingly inhibited by EGTA as the detergent increased, so that with 0.05% detergent, inhibition was similar to that found with the soluble enzyme [11]. As in the soluble enzyme [11], EGTA inhibition of particulate adenylate cyclase was reversed by adding 5 mM Mn^{2+} to the assay. Addition of as little as 0.01% Triton X-100 entirely abolished any hormone response in homogenates or membranes (data not shown).

The amount of detergent sufficient to allow maximal EGTA inhibition was not sufficient to solubilize the enzyme from the membranes. Ninety-five per cent of the activity could be recovered in the pellet when homogenates or membranes treated with 0.05% Triton X-100 were centrifuged at 100,000 *g* for 30 min. Electron micrographs of control and 0.05% detergent-treated membranes showed that both contained intact membrane vesicles.†

Figure 2 shows the effect of increasing amounts of Alamethicin on adenylate cyclase activity and on EGTA inhibition. Alamethicin produced a much greater activation of adenylate cyclase than Triton X-100 (60–80 per cent with the detergent compared to 150 per cent with the antibiotic). Activation was maximal with 0.15 to 0.25 mg Alamethicin/ml. The presence of an intact membrane seems to be required for Alamethicin activation. The ionophore will not activate adenylate cyclase in the presence of detergent. It also will not activate enzyme solubilized with 1% Triton X-100 from which the free detergent has been removed by Sepharose 6B gel filtration without Triton X-100 (see Ref. 22, Fig. 4).

Table 1. Inhibition of adenylate cyclase by EGTA in different particulate preparations of bovine cerebral cortex*

	cAMP [nmole·mg^{-1}·min^{-1}]		
	No EGTA	0.5 mM EGTA	% Inhibition
Homogenate	0.29 ± 0.02 (18)	0.24 ± 0.03 (15)	17 ± 2 (15)
"Membranes"	0.63 ± 0.07 (5)	0.44 ± 0.05 (5)	31 ± 2 (5)
Pellet washed in 2 mM Tris·Cl	0.30 ± 0.03 (7)	0.19 ± 0.02 (7)	37 ± 5 (7)

* Values are the means ±S.E.M. for the number of experiments shown in parentheses. The homogenate was made in 0.1 M Tris·Cl (pH 7.6), 0.075 M sucrose, 10 mM $MgCl_2$, and 1 mM dithiothreitol. The method of preparing the fractions is described in the text.

* Alamethicin was a gift from Dr. George Whitfield, Jr., The Upjohn Co., Kalamazoo, MI, USA.

† For electron microscopy, homogenates were incubated with 0.05% Triton X-100 or 0.15 mg/ml Alamethicin at 37° for 10 min. They were fixed at 37° with 2% paraformaldehyde–2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and centrifuged. Electron microscopy of Epon-embedded, sectioned pellets was performed by Dr. Michael Gimbrone, Peter Bent Brigham Hospital, Boston, MA, USA.

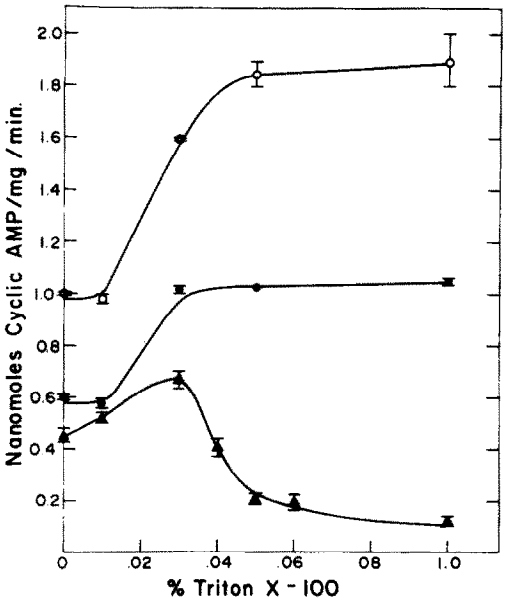

Fig. 1. Effect of Triton X-100 on adenylate cyclase activity in membranes from rat cerebral cortex. Enzyme activity was measured with 12 mM $MgCl_2$ (●——●), 12 mM $MgCl_2$ and 3 mM $MnCl_2$ (○——○), or 12 mM $MgCl_2$ and 0.5 mM EGTA (▲——▲). The protein concentration in the assay was 0.8 mg/ml. A membrane fraction prepared by discontinuous sucrose gradient centrifugation was used for the experiment shown. Data points show the mean and range of duplicate assays. The experiment was repeated three times with the same results using either a membrane fraction or crude homogenates.

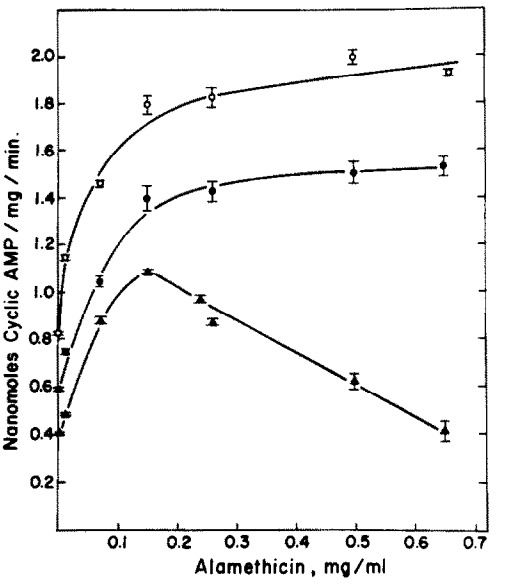

Fig. 2. Effect of Alamethicin on adenylate cyclase activity in membranes from rat cerebral cortex. Enzyme activity was measured with 12 mM $MgCl_2$ (●——●), 12 mM $MgCl_2$ and 3 mM $MnCl_2$ (○——○); or 12 mM $MgCl_2$ and 0.5 mM EGTA (▲——▲). The protein concentration in the assay was 0.8 mg/ml. Alamethicin was added as a solution in 30% ethanol. The amount of ethanol added had no effect on control enzyme activity (data not shown). A membrane fraction prepared by discontinuous sucrose density gradient centrifugation was used for the experiment shown. Data points show the mean and range of duplicate assays. The experiment is representative of three similar ones.

The mechanism by which Alamethicin activates membrane-bound adenylate cyclase is not known. It has been proposed that it acts as an ionophore allowing ATP to enter impermeable vesicles [19]. This is probably not the case since Bonnafous *et al.* [22] reported that Alamethicin can activate adenylate cyclase even in permeable membrane vesicles from lymphocytes.

A higher concentration of Alamethicin is needed to allow full EGTA inhibition than to give maximal activation of adenylate cyclase. This is similar to our observation with Triton X-100. If activation of adenylate cyclase by Alamethicin or Triton X-100 were due solely to permeabilization of sealed membrane vesicles, then the concentration of antibiotic or detergent that maximally increases enzymatic activity would be the concentration that makes the vesicles permeable to ATP. This concentration should also allow EGTA to enter. If resistance to EGTA were only due to sequestration of adenylate cyclase within sealed vesicles, the amount of detergent or antibiotic required for activation and EGTA inhibition should be the same. Furthermore, if an effect on membrane vesicle permeability were the basis of the action of both agents, the degree of enzyme activation should be the same with both agents. Neither of these predictions is supported by our data. We suggest, therefore, that Alamethicin and Triton X-100 perturb the hydrophobic environment around the enzyme and that this disruption is required for EGTA to have its effect. Solubilization of the enzyme from membranes, however, is not necessary. Thus, the metal ion which seems to be required for full adenylate cyclase activity may be located in a hydrophobic region. This is similar to the finding of Pick and Racker [23] that the calcium ion which activates the Ca^{2+}-dependent ATPase from sarcoplasmic reticulum is located in a hydrophobic region of the molecule.

In summary, we have shown that a non-ionic detergent, Triton X-100, and an amphipathic antibiotic, Alamethicin, enhance EGTA inhibition of particulate adenylate cyclase from rat cerebral cortex. Inhibition by EGTA became equal to that reported for soluble adenylate cyclase although the concentrations of detergent and Alamethicin used did not solubilize the enzyme or break up membrane vesicles seen on electron microscopy. These experiments suggest that EGTA only inhibits adenylate cyclase if the chelator gains access to a region of the enzyme that is not exposed in intact membranes and that may be buried in the hydrophobic region of the membrane bilayer.

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Leukotriene C_4 stimulates TXA_2 formation in isolated sensitized guinea pig lungs

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The leukotrienes constitute a new group of biologically active compounds derived from polyunsaturated fatty acids [1–5]. Leukotriene A_4 (LTA_4), an unstable epoxide intermediate, is formed from arachidonic acid via 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid. It can be transformed enzymatically by hydrolysis into LTB_4 and by addition of glutathione into LTC_4 (5(S)-hydroxy-6(R)-*S*-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid). LTC_4 is converted into the corresponding cysteinylglycine derivative (LTD_4) by γ-glutamyl transpeptidase [6]. Slow Reacting Substance of Anaphylaxis (SRS-A) which is an important mediator in immediate hypersensitivity reactions has been shown to be due to LTC_4 and LTD_4 [5, 7].

Crude preparations of SRS-A have been found to stimulate release of thromboxane A_2 (TXA_2) from guinea pig lungs [8, 9]. Sensitized lungs release more TXA_2 than normal lungs following stimulation by crude SRS-A [9]. It was therefore of interest to study the effect of chemically pure LTC_4 on the release of thromboxane from guinea pig lungs. The effect of an SRS-A antagonist (FPL55712) on the SRS-A stimulated release of thromboxane was also studied [10].

Methods

Lungs from actively sensitized guinea pigs [11] (300–400 g) were used. The organs were removed and perfused through the pulmonary artery with Krebs-bicarbonate solution at a flow rate of 10 ml min^{-1}. The pulmonary outflow continuously superfused two spirally cut rabbit aortas (RbA), in order to detect TXA_2-like material. The bioassay tissues were first challenged with a single dose of noradrenaline (NA, 1 nmole) in order to assess their reactivity and subsequently treated, throughout the experiment, with a mixture of antagonists [12] and with indomethacin (1 μg $ml^{-1}min^{-1}$) to increase their sensitivity. Changes in the tone of the tissues were recorded with isometric transducers (Grass model FT 03).

After passage over the assay organs, the pulmonary effluent was collected for periods of 1 min directly into excess methanol and subsequently assayed for mono-*O*-methyl-TXB_2 [13]. This compound is an indicator of TXA_2. The collection of the perfusate was started directly after administration of LTC_4 to the lungs; the maximal biological activity was registered during the collection time.

Chemically pure leukotriene C_4 was prepared at the Karolinska Institutet, Stockholm, Sweden, as previously described [2].

Experimental data were processed according to the method of factorial analysis of variance for completely randomized design with two factors at two levels. Multiple comparison according to Duncan was also performed [14].

Results

When LTC_4 (0.55–2.2 pmoles) was injected as a bolus into the isolated lungs of ovalbumin sensitized guinea pigs, a dose dependent formation of vasoactive material was observed (Fig. 1). No direct effect of LTC_4 on the isolated vessels was observed (Fig. 1) and pretreatment of the isolated lungs with eicosatetraynoic acid (1 μg ml^{-1} min^{-1}) prevented formation of the vasoactive material.