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INHIBITORY EFFECTS OF C-3 ON MEMBRANE-ASSOCIATED ENZYMES

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Abstract—It is reported here that 3,4,5-trimethoxybenzoyl-ε-aminocaproic acid (C-3) exhibits an inhibitory effect on adenylate cyclase, phosphodiesterase and mitochondrial ATPase. It is suggested that the mechanism of inhibition may be the same for the three enzymes; that is, a modification of the hydrophobic portion of the membrane region associated with these enzymes. The possible metabolic consequences of C-3 enzymic inhibition and its relation to the therapeutic use of this drug are also discussed.

It has been reported that 3,4,5-trimethoxybenzoyl-ε-aminocaproic acid (C-3) can be beneficially employed as a therapeutic agent in the treatment of human cardiac disorders [1]. Subsequent therapeutic regimes for treatment of coronary infarct suggest the administration of relatively large quantities of C-3 over prolonged time periods [1]. While the mode of action of C-3 remains unclear, *in vivo*, canine studies indicate that C-3 possesses alpha- and perhaps beta-blocking activity [2]. In addition, C-3 exhibits anti-arrhythmic properties which may be in part responsible for its seemingly beneficial therapeutic effects [3]. The molecular events *in vivo* mediated by alpha- or beta-blocking agents remain in large part obscure. However, epinephrine, a well-known effector of adrenergic receptors, is known to play a major role in cellular metabolism through its control of the enzyme adenylate cyclase. It may be that C-3, which also seems to interact with adrenergic receptors, possesses a similar enzymatic effect and that these effects are involved in the clinical response to C-3 therapy.

Apart from the primary events associated with cardiac infarcts, the long-term prognosis depends in large part on the ability of affected tissue to maintain a cellular energy charge [4] compatible with homeostasis under the anaerobic or near anaerobic conditions induced by the infarct. Since the energy charge of normal cardiac tissue is maintained almost exclusively by aerobic phosphorylations coupled to fatty acid oxidation, the energy charge status of infarcted cardiac tissue is severely compromised. It seems clear that in the absence of unfavorable side reactions any agent with the property of maintaining cellular energy charge, either by decreasing the rate of ATP hydrolysis or by stimulating anaerobic ATP-producing pathways, will favorably influence the course of recovery of damaged cardiac tissue.

Enzymic studies *in vitro* reported in this paper show that C-3 has marked inhibitory effects on the system of mitochondrial enzymes which maintain the aerobic cellular energy charge. In addition, C-3 is shown to be an inhibitor of adenylate cyclase and phosphodiesterase, the enzymes which effect synthesis and degradation of cyclic AMP (cAMP). Moreover, the concentration of C-3 required to observe these effects is sufficiently close to the estimated concentration *in vivo* of C-3 found in clinical settings, so that the results obtained here must be considered when employing C-3 as a human therapeutic agent.

EXPERIMENTAL

Enzyme preparation. Livers from white male Wistar rats were minced and homogenized in ice-cold 50 mM Tris–HCl, 5 mM $MgCl_2$ buffer, pH 7.5, using ten passes of a Potter–Elvehjem tissue homogenizer. The homogenate was centrifuged for 15 min at 4000 *g* in a Sorvall RC-2B centrifuge. The pellet was washed two times with the above buffer employing three passes of the tissue homogenizer followed by centrifugation at 4000 *g* for 10 min. The final pellet was resuspended in buffer using five passes of the homogenizer, and the concentration of protein was finally adjusted to 25 mg/ml. Protein was determined by the method of Murphy and Kies [5].

Phosphodiesterase assay. Either 2 or 10 ml of buffered homogenate was preincubated with drug in an Erlenmeyer flask for 5 min at 35° in a Dubnoff shaking water bath. At the end of the preincubation, cAMP (Sigma Chemical Co.), at the concentrations indicated below, was added to initiate the reaction. Aliquots (2 ml) were removed at various times and added to 0.2 ml ice-cold, 60% perchloric acid (PCA). The resulting suspension was centrifuged in a Phillips–Drucker table-top centrifuge for 5 min. A 1.0-ml aliquot of supernatant was removed and adjusted to pH 7.0 with 10 M KOH. The resulting precipitate was pelleted by centrifugation in the table-top centrifuge and the supernatant fluid was assayed for cAMP by use of high pressure liquid chromatography (HPLC).

Adenylate cyclase. Tissue homogenate was prepared as outlined under "enzyme preparation"; however, the final pellet was resuspended in 50 mM Tris–HCl, pH 7.5, containing 10 mM $MgCl_2$. Homogenate (10 ml at 20 mg protein/ml) was preincubated with 15 mM NaF and 15 mM theophylline or 15 mM C-3 at 35° for 5 min. At the end of the 5-min incubation, ATP was added to a final concentration of 5 mM. Aliquots (2 ml) were withdrawn at 0, 10 and 20 min and treated with PCA and KOH as described above. Nucleotides,

other than cAMP, were precipitated from 1.0 ml of the protein-free supernatant fluid by the addition of 0.1 ml of 0.15 M $Ba(OH)_2$ and 0.1 ml of 0.15 M $ZnSO_4$. The precipitate was pelleted by centrifugation and the Zn–Ba treatment was repeated on the supernatant fluid [6]. The theophylline-containing samples were very viscous so that all samples were frozen overnight, and after thawing, most of the precipitate could be pelleted by centrifugation. The supernatant was finally clarified by filtration through a 0.45-μm Millipore filter. Filtrates were assayed for cAMP by HPLC.

Cyclic AMP assay by HPLC. High-pressure liquid chromatography is a rapid, simple procedure used for assay of nucleotides [7]. A newly developed column, μ-Bondapak-NH_2, produced by Waters Associates (Milford, Mass.) was used for separation of cAMP from other constituents of the reaction mixture. The isocratic eluting buffer employed was 0.005 M PO_4^{-3}, pH 3.0. Absorbance at 254 nm was measured in the effluent from the column. The amount of cAMP in the sample was determined by integrating the area under the cAMP peak by triangulation and interpolating the amount of cAMP present from a standard curve constructed using pure cAMP. It was found that the retention time of cAMP in the experimental samples changed slightly from sample to sample. In order to make positive identification of the cAMP peak in each unknown sample, a mixture of authentic cAMP and sample was co-chromatographed and the cAMP peak identified by its increased area.

ATPase. Rat liver mitochondria were prepared according to the procedure of Schneider and Hogeboom [8]. Mitochondrial ghosts were prepared as described by Caplan and Greenawalt [9]. Membrane-associated ATPase activity was measured as the change in pH occurring upon addition of ATP to a weakly buffered suspension of mitochondrial ghosts [10]. Mitochondrial ghosts (0.2 ml of a 20 mg/ml suspension) were added to 4.6 ml of 2.0 mM Tris–HCl buffer, pH 7.0. C-3 at the indicated concentrations, or C-3 solvent (total vol = 0.3 ml) was added to the mixture, which was then incubated at room temperature for 1 min. Ten μl of 0.1 M ATP (0.2 mM final concentration) was added and the rate of change in pH was recorded using a Heath strip chart recorder coupled to a Corning digital 110 pH meter. The change in H^+ concentration was determined by back-titrating the reaction mixture with standard NaOH. The reaction mixture was constantly stirred and assays were at ambient temperatures.

Respiratory assay. Mitochondrial respiratory rates were measured at ambient temperature using an Interscience polarograph as previously described [11]. Succinoxidase activity (see Fig. 3) was measured in the isotonic respiratory assay medium described by Chance and Hagahara [12]. Malate + glutamate respiration was followed in a medium containing 80 mM KCl, 2 mM HEPES, and 2 mM potassium phosphate, pH 7.4.

Control experiments. Since C-3 is highly acidic and large amounts of NaOH are required to neutralize its aqueous solutions, all control experiments were run using a volume of 1.22 M NaCl equal to the volume of C-3 added. The 1.22 M NaCl approximates the ionic strength of the neutral C-3 solution. In experiments such as the respiratory experiments which require intact mitochondria, small changes in osmolarity which accompany changes in C-3 concentration were minimized by the addition of sucrose to the reaction system so that the sum of sucrose + C-3 concentrations remained constant. No significant effects were noted by the inclusion or omission of the sucrose.

RESULTS AND DISCUSSION

Since C-3 bears a structural similarity to the potent adrenergic effector epinepherine, it was initially considered that C-3 might interact with enzymes involved in cAMP metabolism. However, the results of this study clearly indicate that C-3 effects are broad-based, since C-3 inhibition has been observed with all of the membrane-localized activities which we have studied. Moreover, the broad range of C-3 activity is amply illustrated by its effect on membrane activities as diverse as plasma membrane phosphodiesterase and adenylate cyclase and mitochondrial membrane ATPase and succinoxidase.

In order to most closely approximate the *in situ* function of the plasma membrane enzymes, the effect of C-3 on their activities was studied in a cell membrane preparation. Prior studies on phosphodiesterase have shown that with the purified enzyme the widely used phosphodiesterase inhibitor theophylline effects inhibition at a concentration of approximately 200 μM [13]. The effect of theophylline on phosphodiesterase in its native environment, i.e. in the plasma membrane fraction of a cell homogenate, is, however,

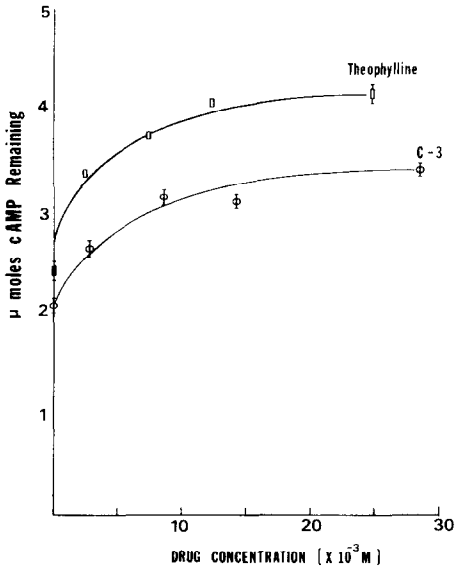

Fig. 1. Effect of theophylline and C-3 on phosphodiesterase activity. Approximately 4 μmoles cAMP was added to cell homogenates and incubated for 5 min. The amount of cAMP remaining indicates phosphodiesterase activity in the presence of the drugs. The concentration of theophylline required for 50 per cent inhibition is about 2.5 mM. For C-3, the concentration for 50 per cent inhibition appears to be 4 mM. (Note: the values plotted represent the average of two determinations. The bars represent the spread between the two values.)

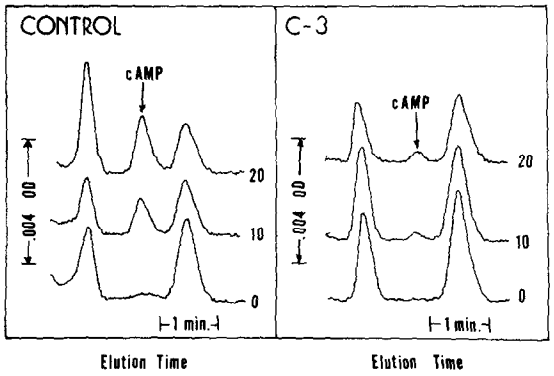

Fig. 2. Effect of C-3 on adenylate cyclase. Cell homogenates were incubated in the presence of 5 mM ATP and either 15 mM theophylline (control) or 15 mM C-3. The numbers accompanying the HPLC traces indicate incubation time.

markedly different as shown in Fig. 1. Here with the membrane preparation, theophylline inhibition (50 per cent inhibition at 2.5 mM) occurs at concentrations which are approximately one order of magnitude greater than reported in studies with the isolated enzyme [13]. Since theophylline is a lipophylic molecule with limited water solubility, the difference between the effects of theophylline on isolated and *in situ* diesterase activity is likely to be due to a complex interaction between the enzyme, the lipid phase of the membrane, and the drug. Since the pharmacological impact of drugs such as theophylline is most closely related to their effect on whole animals, assay systems which test the effects of drugs on membrane-bound enzymes such as phosphodiesterase are most appropriately studied under conditions akin to those under which the enzymes normally function. Thus, under the more physiological conditions of these assays, theophylline appears to be active at significantly higher concentrations than have been reported before.

C-3, like theophylline, is markedly hydrophobic and can be expected on the basis of this property to interact strongly with the lipid phase of membranes. As shown in Fig. 1, C-3 also inhibits phosphodiesterase activity. Although the apparent K_i of 4 mM is only slightly greater than that observed for theophylline, the fact that maximal C-3 effects yield only a 75 per cent reduction in phosphodiesterase activity indicates that the mechanism of inhibition of phosphodiesterase by C-3 is different from that of theophylline. Further, it is shown in Fig. 2 that 15 mM C-3 causes a marked reduction in adenylate cyclase activity. Since theophylline is known to be a potent inhibitor of phosphodiesterase activity [13] but has little if any effect on adenylate cyclase activity, it might be expected, as shown in Fig. 2 (control), that the concentrations of cAMP in reaction mixtures would increase with time in the presence of theophylline. However, adenylate cyclase inhibitors should prevent the latter time-dependent accumulation of cAMP. As shown in Fig. 2 (C-3), 15 mM C-3 effects a reduction of about 80 per cent in adenylate cyclase activity as estimated from the decreased quantity of cAMP produced during a 20-min incubation.

Other membrane-associated enzymes may also be affected by C-3, since its hydrophobicity would favor integration of C-3 into the lipid phase of membranes. The polarographic trace presented in Fig. 3 illustrates the effect of C-3 on the multienzyme system of oxidative phosphorylation. The most significant effect observed is a marked inhibition of ADP-stimulated (state 3) respiration, with minimal C-3 effect on the pre-ADP (state 4) respiratory rate. Virtually complete inhibition of state 3 respiration is shown in Fig. 3 with 43 mM C-3 and succinate as the respiratory substrate. Moreover, C-3 inhibition is independent of the oxidizable substrate employed in the respiratory assay as evidenced by the C-3 inhibition of malate + glutamate-supported respiration shown in Fig. 4. Again state 3 respiration is markedly inhibited by C-3 (100 per cent inhibition at about 40 mM) with little or no

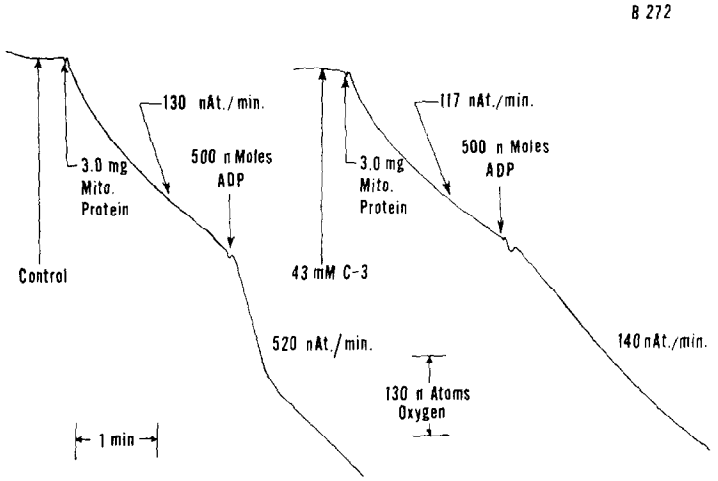

Fig. 3. Effect of C-3 on mitochondrial succinoxidase activity. Addition of C-3 to respiring mitochondria causes a slight reduction in state 4 respiration but more significantly C-3 causes a dramatic decrease in the rate of ADP-stimulated respiration. Succinate was present in both control and experimental traces at a final concentration of 8 mM. C-3 at a final concentration of 43 mM effected a 75 per cent inhibition of ADP-stimulated succinoxidase activity. All reagents were present at the indicated final concentration in a final volume of 3 ml. Succinoxidase assays were at ambient temperature.

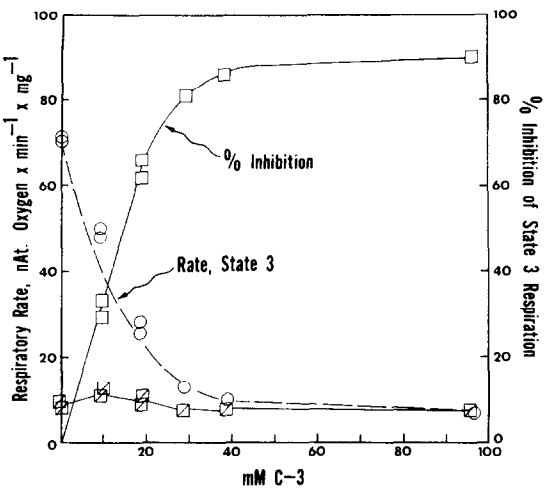

Fig. 4. Effect of C-3 on malate + glutamate-supported respiration. Addition of C-3 to mitochondria oxidizing malate + glutamate (5 mM each) effects a marked inhibition of state 3 respiration (○), with little effect on state 4 respiration (☒). Per cent inhibition was calculated using the state 3 respiratory rate obtained in the absence of C-3 as the maximal state 3 rate. ADP (500 μmoles) was added to initiate state 3 respiration. Other conditions were as described in Fig. 3.

effect on state 4 respiration. Half-maximal inhibition is observed at 12–14 mM C-3 in Fig. 4 and a like value may be obtained with succinate as the respiratory substrate.

Since C-3 inhibition of respiration is substrate independent and confined to ADP-stimulated respiration, it seemed likely that the site of inhibition might be mitochondrial ATPase. ATPase is localized in the inner mitochondrial membrane and is conveniently assayed in preparations of mitochondrial ghosts [9].

While C-3 inhibition of ATPase is not complete at concentrations which inhibit state 3 respiration, it is nonetheless clear, as shown in Fig. 5, that marked inhibition of ATPase by C-3 is observed in the same concentration range which is effective in inhibiting state 3 respiration. The difference in the extent of inhibition of state 3 respiration and ATPase activity remains unresolved, but is likely related to the fact that, while the ATPase assay is a direct measure of an individual enzymic function, the respiratory assay monitors the integrated activity of a host of individual, membrane-localized, enzymic reactions.

Figure 5 includes a comparison of the C-3 effect on ATPase with its effect on phosphodiesterase activity. The concentration of C-3 required for 50 per cent of maximum effect on ATPase is approximately 5 mM, and as can be seen in Fig. 5, this is also the concentration of C-3 required for 50 per cent effect on phosphodiesterase activity.

Based on the solution activity of C-3, it would seem that this drug is effective on both phosphodiesterase and mitochondrial ATPase. However, the membrane protein concentrations in these experiments varied markedly. In the phosphodiesterase assay, the protein concentration was 25 mg/ml, which at the point of 50 per cent inhibition of phosphodiesterase leads to a calculated C-3:protein concentration ratio of 0.2 μmole C-3/mg of protein. However, in the ATPase assay the protein concentration at the point of 50 per cent inhibition was 0.8 mg/ml leading to a C-3:protein concentration of approximately 6.25 μmoles/mg of protein. When viewed in the latter terms, these data suggest that C-3 is a much more potent inhibitor of plasma membrane-associated enzymes than of enzymes associated with mitochondrial membranes. A likely interpretation of these data is that the observed enzyme inhibition is due to a C-3-mediated change in the physical properties of the membranes and that C-3 solubility in membranes is primarily C-3 concentration dependent so that maximal or half-maximal C-3 effects are seen at the same C-3 concentration regardless of the biological membrane system studied.

As stated before, one characteristic of a useful drug for treating cardiac damage (infarct) would be that it promotes the maintenance of the cellular energy charge. Since damaged tissue is likely to experience restricted blood flow and resultant anaerobic conditions, the maintenance of cellular energy charge will, of necessity, be more dependent on anaerobic production of ATP and on a reduction of non-essential ATP-utilizing reactions. Under anaerobic conditions, mitochondrial ATPase activity may be considered an unfavorable side reaction. In view of the results reported here, it may be that, although mitochondrial ATPase is inhibited by C-3, the soluble ATP-synthesizing activities associated with glycolysis are unaffected by C-3.

C-3 also inhibits the cell membrane-associated enzymes adenylate cyclase and phosphodiesterase. The effect of inhibition of both of these enzymes would be to "freeze" cAMP concentrations at the

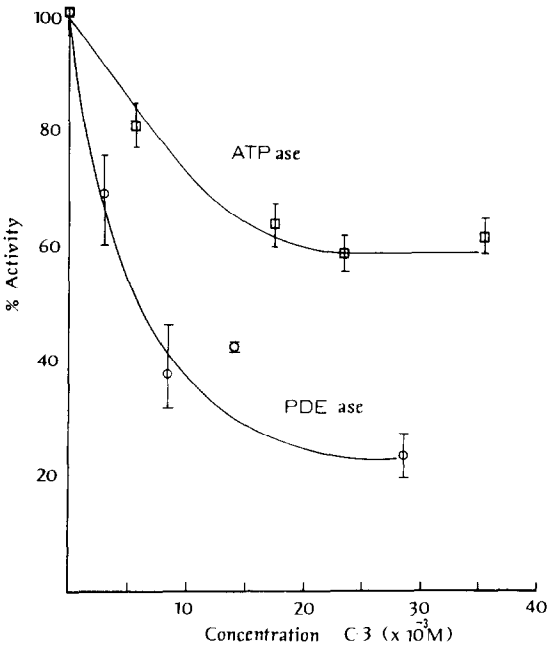

Fig. 5. Effects of C-3 on phosphodiesterase and ATPase. Per cent activity is calculated by dividing the activity in the presence of C-3 by the activity of the control. The concentration of C-3 for 50 per cent inhibition is about 5 mM for both enzymes.

levels present upon initiation of drug therapy. In a therapeutic setting, this cAMP level might be expected to be relatively high, as has been observed in instances of experimentally induced ischemia [14].

Since cAMP is known to be a positive heterotropic effector of glycogenolysis [15] and of some enzymes of the glycolytic pathway [16, 17], constant high levels of cAMP may be viewed as increasing the rate of anaerobic ATP-producing reactions, thereby maintaining cellular energy charge under ischemic conditions. In fact, it has been shown that when ischemia is experimentally produced in the heart, phosphorylase *a* is activated, glycolytic activity is increased, and end products accumulate [14]. Intravenous administration of cAMP has also been shown to decrease blood pressure, increase cardiac output, and increase contractility of the heart [18, 19]. Further, it has been shown that the phosphodiesterase inhibitors, theophylline, caffeine and papaverine, all cause positive inotropic effects [20]. Although the molecular mechanism for a positive inotropic effect is not clear, it seems likely that cAMP may be involved. The possibility exists that C-3 may also affect cardiac inotropy as the other phosphodiesterase inhibitors do, and that effects at the mitochondrial level are superimposed on the plasma membrane effects of C-3.

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