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THE EFFECT OF SLOW FREEZING AND HYPERTONIC NaCl ON THE HYDROLYTIC ACTIVITY OF RAT ERYTHROCYTE CYCLIC AMP PHOSPHODIESTERASE AND ITS SENSITIVITY TO THE INHIBITOR, D,L-4-(3-BUTOXY-4-METHOXYBENZYL)-2-IMIDAZOLIDINONE*

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SUMMARY

Slow, in contrast to rapid, freezing of a rat erythrocyte hemolysate irreversibly activated the cyclic AMP phosphodiesterase (3′:5′-cyclic-AMP 5′-nucleotidohydrolase, EC 3.1.4.17) and reduced its sensitivity to the inhibitor, BMI (D,L-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone) regardless of the presence of dithiothreitol. These effects could be mimicked by a 24-h exposure to 2 M NaCl at 4–5 °C. Brief exposures (< 1 min) to this concentration of salt resulted in reversible activation of the enzyme and increased sensitivity to the inhibitor. Kinetic analyses demonstrated two K_m values for the enzyme of approximately 4.3 μM and 5.0 mM. The 2-M NaCl had no effect on either K_m but increased the V in the low substrate range analogous to the removal of a non-competitive inhibitor. The irreversibility of the salt effect seemed to be correlated with the reduction in potency of BMI rather than the kinetic aspects of the activation. It is proposed that the high salt concentration dissociates an enzyme aggregate and/or exposes certain groups to hydrolytic cleavage.

INTRODUCTION

The rat erythrocyte cyclic AMP phosphodiesterase (3′:5′-cyclic-AMP 5′-nucleotido-hydrolase, EC 3.1.4.17) has a sensitivity to the inhibitor, BMI (D,L-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone) which is not shared by the enzyme preparations from a variety of tissues [1]. It was found, however, that placement of a ghost-free hemolysate in the −20 °C freezer resulted in an increase in hydrolytic activity and a marked decrease in the sensitivity of the enzyme to the above mentioned inhibitor. This change in inhibitor sensitivity in the direction of the enzymes from other tissues suggested the possibility that in the preparation of most other phospho-

Abbreviation: BMI, D,L-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidone.

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diesterases some alteration of inhibitor sensitivity was occurring. It was essential, therefore, that a study be initiated to determine the factors responsible for these changes and the results of such an investigation are outlined below.

METHODS

Preparation of hemolysates

The collection of the blood, the preparation of the ghost-free hemolysates and the assay were carried out as described previously [2, 3]. Briefly, rat blood collected by heart puncture was hemolyzed with 13 vol. of 20 mosM buffer, pH 7.4 (a 15.5-fold dilution of a solution containing 5 vol. of 0.103 M Na_2HPO_4 and 0.94 vol. of 0.155 M NaH_2PO_4). After centrifugation at 20 000 $\times$ g at 4 °C the supernatant was stored in an ice-bath before use. For the experiments with salt, hemolysates were prepared with 6 vol. of buffer and diluted 1:1 with the buffered salt solution under study.

Assay

Aliquots of the supernatant were incubated with 5 μM cyclic [8-^{3}H]AMP (1 μCi) in 40 mM Tris buffer containing 2 mM $MgCl_2$ for 15 min or as indicated in the text and the reaction was stopped by boiling. After centrifugation at 1000 $\times$ g, aliquots (10 μl) were chromatographed on Whatman No. 1 filter paper with carrier (0.02 μmoles) AMP, cyclic AMP and adenosine, using ethanol–1 M ammonium acetate–water (5:1:1, by vol.) as the developing solvent. The ultraviolet absorbing areas were cut out and counted in a liquid scintillation counter with butyl–PBD (0.4 %) phosphor in toluene.

The analysis of the cyclic AMP, 5'-AMP and adenosine areas in boiled enzyme controls demonstrated that up to 5 % of the ^{3}H appeared in the adenosine area, that more than half of this frequently disappeared in 15 min and that this disappearance could be inhibited by the high salt concentrations. The amount of ^{3}H which disappeared from the adenosine area could be accounted for in the 5'-AMP areas as an increment over the amount of cyclic AMP hydrolyzed. Thus, the disappearance of cyclic AMP or the accumulation of ^{3}H in the 5'-AMP minus the loss from the adenosine areas could be used as a measure of hydrolytic activity after correction by the boiled enzyme control.

Chemicals

Sodium salt of adenosine 3':5'-monophosphate (cyclic AMP), adenosine 5'-monophosphate (Type II) and adenosine were from Sigma Chemical Co., St. Louis, Mo.; Tris, Ultra Pure, and cyclic [8-^{3}H] AMP from Schwarz-Mann, Orangeburg, N.Y.; dithiothreitol (Cleland's reagent) from Calbiochem., La Jolla, Calif.; Butyl–PBD from New England Nuclear, Boston, Mass. and DL 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) from Hoffmann-La Roche Inc., Nutley, N.J.

RESULTS

Effect of freezing

An example of the effect of storage of the hemolysate in a −20 °C freezer on the activity and sensitivity to 0.1 μM inhibitor (BMI) is seen in Table 1. Storage at

TABLE I

The increase in hydrolytic activity and decrease in sensitivity to 0.1 μM inhibitor (BMI) resulting from freezing and thawing a rat erythrocyte phosphodiesterase preparation in the presence and absence of 3.75 mM dithiothreitol.

Storage		Percentage hydrolysis		Percentage inhibition	
4–5 °C	−20 °C	without dithiothreitol	with dithiothreitol	without dithiothreitol	with dithiothreitol
0	—	32.5	31.0	53.7	47.2
4 h	—	29.8	31.1	53.1	49.2
24 h	—	18.7	23.2	47.0	49.7
24 h	18 h	69.8	68.5	4.3	2.7

4 °C for 4 h had little effect on the hydrolytic activity while a significant reduction was noted after 24 h. No loss in sensitivity to BMI was observed. If however, the preparation was now placed in the −20 °C freezer for an additional day the activity increased several fold to a value greater than what was observed initially. The magnitude but not the direction of some of these changes was actually greater than observed since the utilization of more than 50% of the substrate caused significant deviations from linearity of hydrolysis. At the same time the percent inhibition fell to almost insignificant values. Dithiothreitol at 3.75 mM failed to alter either the activation or loss of sensitivity. This response to storage in the freezer could be observed by simply placing a fresh ghost-free hemolysate in a −20 °C freezer for a period of 24 h.

No such change in activity or inhibitor sensitivity could be obtained if the preparation was quick frozen in a solid CO_2–acetone mixture and thawed shortly thereafter. It appeared, therefore, that the increasing concentration of solutes which occurs during a slow freezing process could be responsible for the effects being considered. Since no activation of brain or platelet preparations could be effected by slow-freezing our attention was drawn to the possibility that the high concentration of 2,3-diphosphoglyceric acid present in erythrocytes was responsible. However, up to 10^{-4} M of 2,3-diphosphoglyceric acid failed to increase the hydrolytic activity. The chelating agent, EGTA ,also had no effect when used at the same concentration.

The possibility that production of a heat-stable, protein-like activator, desscribed by Cheung [4], had occurred was discarded when it was found that the addition of boiled extracts of an activated preparation failed to increase the activity of a fresh hemolysate. The removal of a heat-stable inhibitor was likewise discounted when the addition of a boiled extract of the fresh enzyme failed to inhibit an activated preparation. In this regard the addition of an activator fraction, prepared from dog brain by boiling as described by Cheung [4], also failed to affect the hydrolytic activity.

Ionic strength

The possibility was considered that a simple increase in ionic strength during the freezing process could lead to the changes under study. The effect of 0.9 M NaCl*

* It should be pointed out that the concentration of salt referred to in the text is that of the enzyme solution. In measuring the hydrolytic activity 0.1 ml or less of the enzyme solution was brought to a total volume of 0.25 ml, thereby reducing the salt concentration during the incubation by 60% or more.

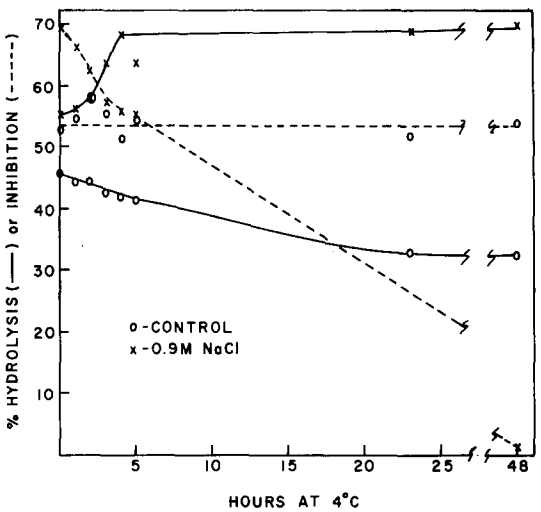

Fig. 1. Effect of 0.9 M NaCl on the activity of the rat erythrocyte phosphodiesterase stored for up to 48 h at 4–5 °C. Inhibition was determined in the presence of 10^{-7} M BMI.

for various periods of time in an ice-bath are shown in Fig. 1. There was an initial increase in hydrolytic activity which continued to rise to a maximum value over a 4-h period. As alluded to before (Table I), the control sample decreased in activity during storage in the ice-bath. The inhibition by BMI (10^{-7}M) increased initially and then fell such that no inhibition was detectable after 48 h. The hydrolytic capacity measured within a few minutes after the addition of various amounts of NaCl to the preparation is demonstrated in Fig. 2. The activity decreased after the brief exposure to 0.15 M

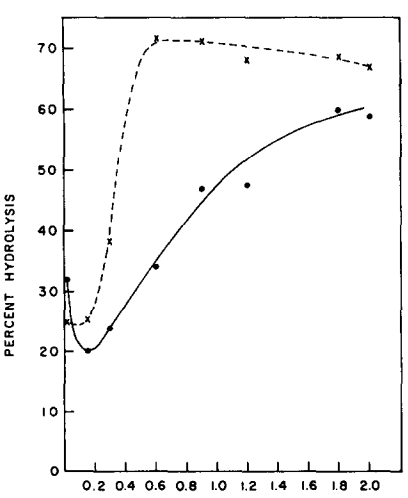

Fig. 2. The effect of various concentrations of NaCl on the hydrolytic activity of fresh enzyme preparations. Within 1 min (○—○) and 24 h (×---×) after addition of the salt, 0.1 ml was transferred to the incubation flask containing 0.15 ml of medium plus substrate and incubated for 15 min.

NaCl and then increased reaching a maximum value with a concentration of 1.8 M. The samples exposed to the salt for 24 h achieved the maximum in 0.6 M NaCl which was greater than that obtained with the samples assayed within 1 min.

The reversibility of the salt effect was studied by a simple dilution technique. After increasing the salt concentration to 2 M, the samples were diluted 4-fold immediately and after 24 h storage in the cold. It can be seen in Table II that immediate dilution to 0.5 M restored the hydrolytic capacity to what would be seen if the preparation was directly brought to 0.5 M NaCl. This increase in rate of hydrolysis was no longer reversible after storage for 24 h but, surprisingly, rose further after dilution. The same degree of increase in hydrolytic capacity was not seen if the 24 h. control sample was brought directly to 0.5 M by the addition of NaCl.

TABLE II

The effect of dilution of the enzyme preparation on the alteration of hydrolytic activity and inhibitor sensitivity produced by prior exposure to 2 M NaCl for less than 1 min and 24 h. A value of $P < 0.05$ is designated by an underline or vertical arrow for comparison with control and immediately overlying samples, respectively.

Treatment	Percentage hydrolysis		Percentage inhibition*	
	<1 min	24 h	<1 min	24 h
Control	19.8	16.7	28.7	34.7
2 M NaCl	43.7	67.4	68.5	10.2
2 M→0.5 M NaCl	↑25.4	↑74.1	↑57.1	↑ 2.8
0.5 M NaCl	27.8	↑41.5	59.4	↑37.1

* BMI was present at concentrations of 0.1 μM.

The inhibition by BMI was elevated immediately after making the solution 2 M to NaCl. This effect was also reversible by dilution to 0.5 M NaCl. After 24 h in 2 M NaCl the inhibition was reduced and this was not reversed by dilution to 0.5 M. More extensive analysis of inhibitor potency demonstrated that exposure to 2 M NaCl for up to 1 min and 24 h resulted in 50% inhibition (I_{50}) at concentrations of 0.025 and 8 μM, respectively.

In general, the effects of KCl were very much like that of NaCl except that the inhibitor potency decreased very much more slowly and on many occasions was only slightly less than what was obtained with control samples stored for 24 h in the cold.

Kinetic studies

After preliminary studies it became apparent that two K_m values existed and that in order to perform an adequate kinetic study a concentration range of 10 000 had to be covered. In addition it was necessary to vary the times of incubation to ensure that, with very low concentrations, initial velocities were being maintained and that with very high concentrations measurable hydrolysis was occurring. This necessitated 5–15 min incubations and amounts of enzyme were chosen to give linear rates of hydrolysis over these time periods.

Analysis over the substrate concentration range of 10^{-4}–10^{-2} M are presented in Fig. 3. At the lower substrate concentrations in this range the velocity of hydrolysis

for the 24 h NaCl preparation was significantly greater than that of the 24 h control but the lack of linearity of the $1/v$ versus $1/s$ plot prevented extrapolation and determination of kinetic constants in that range. At the higher substrate concentrations the curves converged and the data for the control and NaCl preparations did not differ statistically. The best-fit analysis yielded a curve whose K_m and V were found to be 5 mM and 29.4 nmoles/15 min per 0.2 ml of enzyme, respectively.

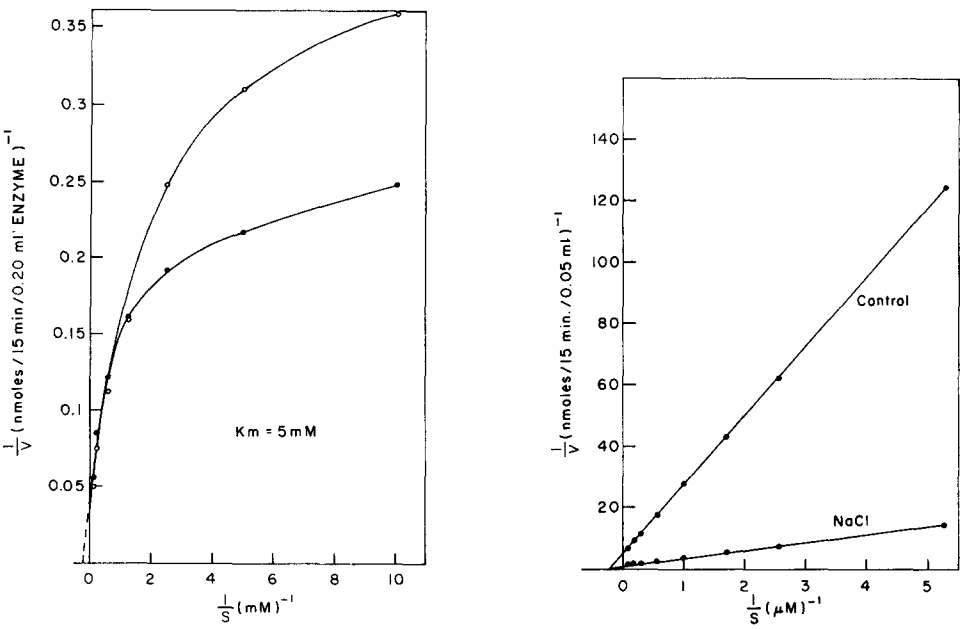

Fig. 3. A plot of $1/v$ versus $1/s$ for control and 2 M NaCl preparations, which had stood at 4–5 °C for 24 h. The concentration range covered is 0.1–12.8 mM. The control and 2 M NaCl preparations are designated by ○—○ and ●—●, respectively. Each point in this and Fig. 4 represents the mean of six determinations. In order to obtain a measurable amount of hydrolysis twice the normal amount of enzyme was used. The incubations were carried out for the usual 15 min and to increase the sensitivity of the method 4 μCi of cyclic AMP was used per incubation. The hydrolysis for the control and 2 M NaCl samples ranged from 0.9 to 11% and from 0.6 to 16%, respectively.

Fig. 4. A plot of $1/v$ versus $1/s$ for the same preparation used in Fig. 3 but over the concentration range of 0.2–13 μM. Each point represents the mean of six determinations. After 24 h in the cold, the solutions were diluted with 1 vol. of the appropriate buffer and 0.05 ml was used for the incubations. This represents one-fourth of the enzyme solution used in Fig. 3. The control samples were incubated for 15 min and yielded 5–17% hydrolysis. For the 2 M NaCl samples, the incubation times were 5 min for 0.2–3.4 μM, 10 min for 6.6 μM and 15 min for 13 μM. A range of 21–48% hydrolysis was achieved with the NaCl samples and the results were expressed on the basis of a 15 min incubation.

The Lineweaver–Burke plot covering the substrate range from 0.2–13 μM can be seen in Fig. 4. The control and salt lines intersect at the x axis with a common K_m of 4.3 μM and V values of 0.18 and 1.25 nmoles/15 min per 0.05 ml of enzyme, respectively. The same kinetic behavior was found with samples analyzed immediately after the addition of 2 M NaCl.

DISCUSSION

The results presented here demonstrate that the effects of slow freezing on both the rates of hydrolysis of cyclic AMP and its inhibition by BMI can be mimicked by a 24-h exposure to 2 M NaCl at 4–5 °C. Both of these effects are irreversible. Very brief exposure to 2 M NaCl reversibly increases the hydrolytic rate and surprisingly the sensitivity to BMI as well. In fact, an I_{50} of $2.5 \cdot 10^{-8}$ M has been recorded for BMI immediately after the addition of the 2 M NaCl, which represents an affinity over two orders of magnitude greater than that of the substrate.

Kinetic analysis of the erythrocyte phosphodiesterase has demonstrated the presence of two K_m values which are very much more separated than those found for other tissues. While the low K_m of 4.3 μM approximates the values reported for other tissues the high K_m (5 mM) value is greater than any encountered to date. However, the values obtained by Lineweaver–Burke plots for mixtures of enzymes can only be approximations lying somewhere between the real values as limits and will be influenced by the relative abundance of each type [5]. It is difficult to understand the physiological significance of such a high K_m enzyme since one would not expect to encounter such concentrations of cyclic AMP under ordinary conditions. The concept of negative cooperativity has been invoked to explain the high K_m portion of a low K_m preparation from an agarose fractionation of a rat brain preparation [5, 6]. Until the enzymes are highly purified, however, the existence of two enzymes cannot be eliminated. It is interesting to note in this regard that low and high K_m forms of the phosphodiesterase of the slime mold have been separated on an agarose column, that the high K_m was 2 mM and that conversion to a lower molecular weight low K_m form was effected with dithiothreitol [7]. Preliminary results in our laboratory could not detect the existence of a separate high K_m enzyme in the Sephadex G-200 column eluates of the rat erythrocyte preparation.

In any case, the addition of 2 M NaCl did not alter the high K_m form but appeared to increase the V of the low K_m form without affecting the K_m. This could be interpreted as the removal of a non-competive inhibitor. The presence of an inhibitor, probably a protein, has also been proposed for the enzyme of rat brain [8]. If activation was related to removal of a proteinaceous inhibitor it would suggest that in the erythrocyte this enzyme, like the protein kinases of other tissues, might be associated with a regulatory subunit which is inhibitory. On the other hand, the enzyme might readily form inhibited complexes with a variety of proteins in its environment as has been suggested for estrogen binding proteins [9]. The use of high ionic strength would be expected to promote the dissociation of such aggregates in a fashion similar to that found for a number of protein systems including the estrogen binding complexes [8]. In the case of the erythrocyte phosphodiesterase, however, such a dissociation would have to proceed in two steps, one which is reversible and the other not. Perhaps the initial phase is simply a loosening of the coupling while the second phase involves an actual separation.

It should not be overlooked, however, that similar effects could be obtained by a salt-activated hydrolytic cleavage of a phosphate, sulfate, hexosyl or polypeptide moiety. The activation by salt could occur by a direct action on the hydrolytic enzyme or by the exposure of the groups to be cleaved. With the latter mechanism one can more easily explain the initial reversible phase of the activation.

Whatever differentiates these two phases of activation is apparently not reflected in the kinetics of the salt effect but rather in the changes in the sensitivity to the inhibitor, BMI. This correlation suggests that in the process leading to the irreversible state some binding site for the inhibitor is removed or weakened. Since the inhibition by papavarine is not weakened by the salt [10] one might suggest that the binding site for the imidazolidinone moiety is the one which is altered by the salt. Preliminary evidence, however, suggests that it is not that simple.

It would appear that the combined use of hypertonic salt and the inhibitor, BMI, has provided some insights into the possible state of organization of the rat erythrocyte phosphodiesterase. It is apparent that these studies need to be extended to include purification steps involving molecular sieving with the aim of determining whether alterations in size, charge and kinetic constants have occurred.

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