Biochem. J. (1982) **207**, 637–640
Printed in Great Britain

Multimers of anionic amphiphiles mimic calmodulin stimulation of cyclic nucleotide phosphodiesterase

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(*Received 1 September 1982/Accepted 17 September 1982*)

Oleic acid, phosphatidylserine and pyrenedecanoic acid were found to activate calmodulin-deficient cyclic nucleotide phosphodiesterase at concentrations above their critical micellar concentration. In contrast with calmodulin these activators do not require the presence of Ca^{2+} for their action. It is shown that the size of phosphatidylserine vesicles is of crucial importance with respect to the activating potency of phosphatidylserine. Fluorescence measurements with the probe pyrenedecanoic acid revealed that micelles rather than monomers are the active species for stimulation of phosphodiesterase. There are indications that this result also may be applied to the other activators.

Calmodulin, the major Ca^{2+}-binding protein in non-muscle cells, stimulates a multitude of enzymes upon binding of Ca^{2+} (Cheung, 1980; Klee *et al.*, 1980). Alternatively, several calmodulin-dependent enzymes are activated in a Ca^{2+}-independent fashion by acidic phospholipids and unsaturated fatty acids (Wolff & Brostrom, 1976; Pichard & Cheung, 1977; Niggli *et al.*, 1981). However, the acidic phospholipid phosphatidylserine was reported to be a poor activator of phosphodiesterase (Wolff & Brostrom, 1976; Itano *et al.*, 1981).

It was suggested that binding of calmodulin, fatty acids or acidic phospholipids to phosphodiesterase leads to a similar conformational change of the enzyme (Wolff & Brostrom, 1976) by which the influence of an integral inhibitory peptide sequence on the catalytic domain of phosphodiesterase is suppressed (Klee, 1980; Klee *et al.*, 1980). Since calmodulin is a rather large molecule (M_r 16700) as compared with fatty acids or phospholipids, it is an open question whether these substances perform their activating effect on phosphodiesterase via their monomeric or micellar (vesicular) form.

The mechanism by which phosphodiesterase is regulated is of general interest because it may serve as a model for other calmodulin-regulated enzymes. We report here (1) that phosphatidylserine can mimic calmodulic stimulation of phosphodiesterase and (2) that multimers of the anionic amphiphiles (fatty acids, acidic phospholipids) are the activating species for this enzyme.

Abbreviation used: CMC, critical micellar concentration.

Materials and methods

All reagents were of highest purity available, Oleic acid, phosphatidylserine (product P 7769) and 5′-nucleotidase were supplied by Sigma. Pyrenedecanoic acid was synthesized according to the method of Galla & Hartmann (1981), dissolved in dimethyl sulphoxide and added to the respective assay medium with vigorous mixing. The final concentration of dimethyl sulphoxide in the assay media including the controls was always 1% (v/v).

Oleic acid and phosphatidylserine microdispersions were prepared by sonication (Branson Sonifier B12, approx. 2 min at setting 2) of the substances at room temperature under a stream of N_2 in a buffer containing 0.1 mM-EGTA and 5 mM-4-morpholine-propanesulphonic acid (Mops, pH 7.0). Stock solutions of oleic acid and phosphatidylserine (3 mg/ml) were prepared fresh daily and stored in a dark vial at room temperature.

Preparation of calmodulin and phosphodiesterase

Homogeneous calmodulin was prepared from bovine brain as described recently (Kakiuchi *et al.*, 1981). Calmodulin-sensitive phosphodiesterase was partially purified based on the method of Wang & Desai (1977). Briefly, phosphodiesterase was obtained from rat brain by homogenization in a buffer containing 10 mM-Tris/HCl (pH 7.5) and 2 mM-EDTA followed by $(NH_4)_2SO_4$ (55% satd.) precipitation of the 100000 g supernatant. The redissolved pellet of the precipitation was applied to a DEAE-cellulose column (Whatman DE52) equilibrated with a buffer consisting of 20 mM-Tris/HCl

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(pH 7.5), 1 mM-magnesium acetate, 1 mM-imidazole, 10 mM-2-mercaptoethanol and 0.1 mM-EGTA, and phosphodiesterase was eluted with the same buffer by a gradient of 50–350 mM-NaCl. In addition all buffers needed for the preparation of phosphodiesterase contained 0.1 mM-di-isopropylfluorophosphate and 0.05 μg of pepstatin A/ml.

Assay of phosphodiesterase activity

Phosphodiesterase activity was determined at 37°C as described by Wang & Desai (1977) based on the method of Butcher & Sutherland (1962). This procedure involved coupling of the phosphodiesterase reaction with the 5′-nucleotidase reaction and measuring the inorganic phosphate produced within 30 min. The assay mixture consisted of 40 mM-Tris/HCl (pH 7.5), 40 mM-imidazole, 3 mM-magnesium acetate, 1.2 mM-cyclic AMP and 0.1 mM-$CaCl_2$ or 0.1 mM-EGTA.

Fluorescence measurements

Fluorescence spectra were taken with a Schoeffel RRS 1000 fluorescence spectrometer. Samples were irradiated at 329 nm.

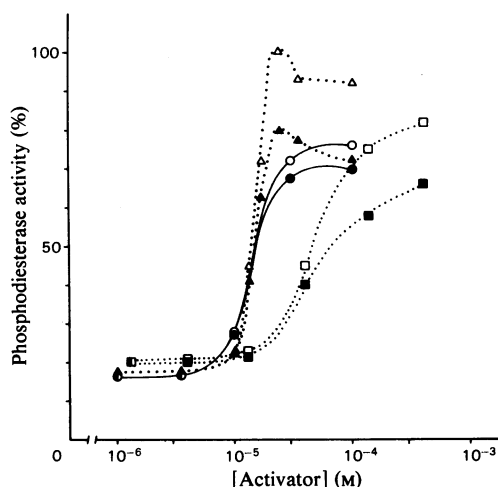

Fig. 1. *Activation of calmodulin-deficient phosphodiesterase by anionic amphiphiles*
Brain phosphodiesterase (40 μg of protein/ml) was preincubated in the assay medium with different concentrations of the respective activator (△, ▲, oleic acid; □, ■, phosphatidylserine; ○, ●, pyrenedecanoic acid) for 10 min at 37°C before the reaction was started by the addition of cyclic AMP. The assay was performed either in the presence of 0.1 mM-$CaCl_2$ (filled symbols) or in the presence of 0.1 mM-EGTA (open symbols). Phosphodiesterase activity is expressed relative to the activity of the enzyme fully stimulated by 30 mM-calmodulin (100% activity = 0.8–1 μmol/min per mg of protein). Each point is the mean of at least four independent determinations.

Results and discussion

Anionic amphiphiles like oleic acid, phosphatidylserine and the fluorescent probe pyrenedecanoic acid were found to be activators of brain phosphodiesterase (Fig. 1). Activation of the enzyme by each of these substances is accompanied by a simultaneous elimination of calmodulin stimulation to the same extent as activation by the other activator takes place (results not shown). Activation of phosphodiesterase by oleic acid, phosphatidylserine or pyrenedecanoic acid does not require the presence of Ca^{2+} (Fig. 1), in contrast with calmodulin-induced activation (Cheung, 1980). On the contrary, the presence of Ca^{2+} in the assay medium has an adverse effect on the activating efficacy of anionic amphiphiles, especially at higher concentrations of the activators (Fig. 1).

Stimulation of phosphodiesterase by oleic acid confirms results from several laboratories (Wolff & Brostrom, 1976; Pichard & Cheung, 1977). A new fact is our finding that also phosphatidylserine at concentrations of 130–400 μM (100–300 μg/ml) stimulates phosphodiesterase up to 82% of the activity of the maximally stimulated enzyme by calmodulin (Fig. 1). In contrast, Wolff & Brostrom (1976) obtained even with 2 mg of phosphatidylserine/ml only a 25% increase over the basal phosphodiesterase activity, and Itano *et al.* (1981) found no activating effect of phosphatidylserine at concentrations ⩽300 mg/ml.

In search of an explanation for this discrepancy we discovered that the preparation of phosphatidylserine vesicles is of crucial importance. Phosphatidylserine dispersed in an EGTA-containing medium (see the Materials and methods section) and stored at room temperature stimulates phosphodiesterase by a factor of 5 (Fig. 2, column 3) over the basal activity (Fig. 2, column 1) whereas calmodulin activates the enzyme by a factor of 6.6 (Fig. 2, column 2). We observed that phosphatidylserine suspensions required under the applied conditions 1.5–2 min of sonication to gain full stimulating efficacy. Sonication for longer periods did not improve the activating effect on phosphodiesterase. Storage of the phosphatidylserine vesicle suspension at 0°C for several hours results in a dramatic loss in activating efficacy (Fig. 2, column 4). Low temperature is known to induce particle aggregation and/or an increase in particle size. The formation of larger particles became obvious from an increased turbidity of the phosphatidylserine suspension. That indeed that particle size of phosphatidylserine vesicles inversely correlates with the

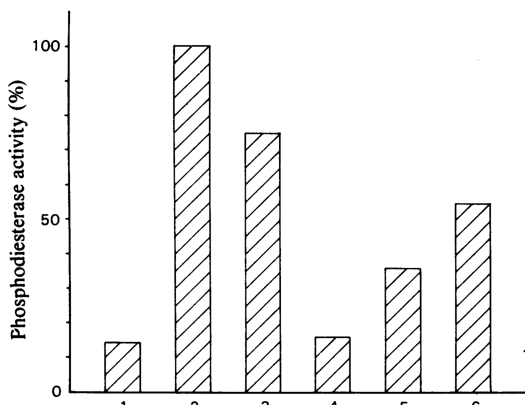

Fig. 2. *Activation of phosphodiesterase by phosphatidylserine vesicles of different size and by calmodulin*
Phosphodiesterase was assayed either in the presence of 0.1 mM-$CaCl_2$ when activated by calmodulin or in the presence of 0.1 mM-EGTA when phosphatidylserine was the activator. Column 1, basal activity in the absence of calmodulin; column 2, activity in the presence of 30 nM-calmodulin; columns 3–6, activities obtained by the addition of 130 μM-phosphatidylserine. Before use the phosphatidylserine microdispersion (prepared as described in the Materials and methods section) was subjected to the following treatments: column 3, stored for 4 h at 22°C; column 4, stored for 4 h at 0°C; column 5, stored for 4 h at 0°C, then sonicated for 45 s at setting 2; column 6, stored for 4 h at 0°C, then sonicated for 90 s at setting 2. Values are mean values obtained from two experiments.

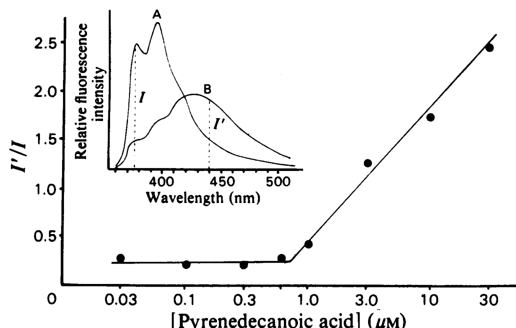

Fig. 3. *CMC of pyrenedecanoic acid*
The excimer-to-monomer fluorescence intensity (I'/I) is plotted as function of the concentration of pyrenedecanoic acid in aqueous medium (10 mM-Mops, pH 7.0) containing 1% dimethyl sulphoxide. The appearance of a strong excimer band at concentrations >1 μM is a measure of the formation of micelles. Two typical spectra at concentrations of 0.1 μM (A) and 30 μM (B) are given in the inset.

stimulating efficacy on phosphodiesterase could be clearly demonstrated by sonication of the cold-inactivated phosphatidylserine preparation, which results in restoration of its stimulating efficacy (Fig. 2, columns 5 and 6). These results are compatible with the interpretation that only vesicles of the smallest possible size are capable to activate calmodulin-depleted phosphodiesterase. This is in agreement with the finding of Itano *et al.* (1981) who found a stimulation of phosphodiesterase by phosphatidylserine only when adding 600 μg of Triton X-100/ml, which results in the formation of smaller particles of phosphatidylserine. The fact that anionic amphiphiles are less effective in activating phosphodiesterase when preincubation and assay are performed in the presence of Ca^{2+} as compared with the presence of EGTA (Fig. 1) can be interpreted in the same way. Addition of Ca^{2+} to amphiphiles in aqueous media causes formation of larger aggregates (Papahadjopoulos *et al.*, 1977).

From the experiments with the fluorescent probe pyrenedecanoic acid, which also mimics calmodulin activation, we expected to gain some further insight into the molecular mechanism of activation of phosphodiesterase by anionic amphiphiles.

The ratio I'/I of the excimer to monomer fluorescence intensity of pyrenedecanoic acid is displayed as function of probe concentration in Fig. 3. At low concentrations (<0.5 μM) the fluorescence spectrum exhibits only the monomer fluorescence (spectrum A). With increasing concentration a second component appears at long wavelength (spectrum B) which is due to the formation of excited complexes, so-called excimers (Galla & Hartmann, 1980). Here we used the appearance of the excimer peak at 440 nm as an indicator of the formation of pyrenedecanoic acid micelles (Galla *et al.*, 1979). At concentrations below the CMC probe molecules are soluble in water; at concentrations greater than the CMC probe molecules assemble to micelles. Due to the close packing in such a micelle strong excimer formation can be expected. The CMC in this buffer solution is found to be about 1 μM. Since activation of phosphodiesterase by pyrenedecanoic acid occurs at concentrations >10 μM (Fig. 1) it is clear that this stimulation is dependent on the concentration of micelles.

Upon interaction with hydrophobic sites some fluorescent dyes undergo an increase in their fluorescence intensity. Such a polarity-dependent change has been observed in many fluorophores and was used, for example, to determine lipid phase transition (Haynes & Stärk, 1974). Since pyrenedecanoic acid exhibits characteristic fluorescence spectra for its monomeric and micellar form (Fig. 3),

this probe seems to be an ideal tool to differentiate whether monomers or micelles are the activating species of phosphodiesterase. The spectrum of the species interacting with phosphodiesterase is expected to undergo a fluorescence intensity change. Fig. 4 shows fluorescence spectra of pyrenedecanoic acid interacting with different concentrations of phosphodiesterase. A concentration of 30μM of pyrenedecanoic acid was used in these experiments since this is a concentration producing pronounced stimulation of phosphodiesterase. The fluorescence is mainly determined by the strong excimer band. The monomeric form in solution superposes the excimer spectrum in a wavelike fashion at the short wavelength side. The excimer-to-monomer ratio remains constant upon addition of phosphodiesterase, which shows that binding of pyrenedecanoic acid does not change the micellar structure and that the whole micelle must activate phosphodiesterase. From the increase of total intensity with increasing phosphodiesterase concentrations we conclude that binding of the micelles to a hydrophobic site of phosphodiesterase favours the radiative transitions. This could be clearly shown by lifetime measurements of the fluorophore in the aggregated excimer state. The excimer lifetime increases from approx. 1 to 2ns after addition of 40μg of phosphodiesterase protein/ml.

The results obtained for pyrenedecanoic acid presumably can be applied to the other anionic amphiphilic activators of phosphodiesterase, in that also these compounds exert their stimulating effect via their multimeric form (micelle, vesicle). Like pyrenedecanoic acid these amphiphiles stimulate phosphodiesterase at concentrations above their respective CMC. Phospholipids are known to have a CMC in the range of 10^{-9}–10^{-10} M (Helenius & Simons, 1975) and we determined by means of light scattering the CMC of oleic acid to be in the range 10–15μM.

It was suggested by Wolff & Brostrom (1976) that calmodulin and amphiphilic activators lead to a similar conformational change of phosphidiesterase. Binding of amphiphilic activator micelles (vesicles) to phosphodiesterase possibly occurs at the same site where calmodulin binds, since anionic amphiphiles and calmodulin compete for activation of the enzyme (Wolff & Brostrom, 1976, and our results). It is tempting to speculate that the molecular mechanism of activation of phosphodiesterase by anionic amphiphiles may be applied also to other calmodulin-regulated enzymes.

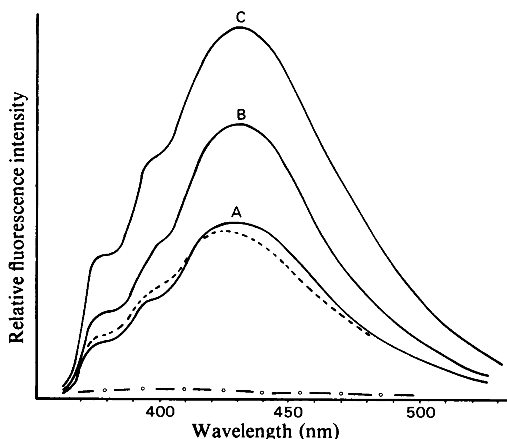

Fig. 4. *Interaction of pyrenedecanoic acid with phosphodiesterase*

Fluorescence spectra of pyrenedecanoic acid at a concentration of 30μM in the absence (broken line) and in the presence of phosphodiesterase (A, 20μg of protein/ml; B, 40μg/ml; C, 80μg/ml). Phosphodiesterase itself (–·–·–·) gives only a negligible fluorescence contribution.

We thank Miss Roswitha Brenner for her help in the preparation of the manuscript. This work was supported by grants of the Deutsche Forschungsgemeinschaft. Y.-H. X. was supported by a grant from the government of the People's Republic of China.

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