Calmodulin activates adenylate cyclase from rabbit heart plasma membranes

M.P. Panchenko and V.A. Tkachuk*

Institute of Experimental Cardiology, Cardiology Research Center of the USSR, Academy of Medical Sciences, 3rd Cherepkovskaya street 15A, Moscow 121552, USSR

Received 29 April 1984

It was shown that calmodulin (CM) activates the adenylate cyclase (AC) of rabbit heart light sarcolemma in the presence of micromolar free Ca^{2+} concentrations and this effect is blocked by trifluoroperazine and troponin I. GTP (in the presence of isoproterenol) and Gpp(NH)p are able to increase the CM-dependent activity of enzyme. It was concluded that there is no special CM-dependent 'form' of AC in the heart and the common catalytic component of AC can be regulated both by CM and guanine nucleotide-binding regulatory component (N-protein). In the presence of Ca^{2+} and guanine nucleotide heart AC exists as a complex: CM–catalytic component–N-protein.

Calmodulin *Adenylate cyclase* *Cardiac light sarcolemma* *Guanine nucleotide* *Ca^{2+}*

1. INTRODUCTION

Ca^{2+} inhibits the activity of adenylate cyclase (AC) from a wide variety of tissues (review [1]). However, the mammalian cerebral cortex and some other tissues contain an AC that is activated by micromolar concentrations of Ca^{2+}. It was shown that this activation is mediated by calmodulin (CM) [2–5]. There is still a view that cardiac AC is insensitive to this stimulatory Ca^{2+}-dependent action of CM, and that Ca^{2+} exerts only an inhibitory effect on cyclic AMP (cAMP) synthesis in the heart [6,7]. This paper presents evidence for the heart AC ability to be activated by Ca^{2+} through CM.

A pivotal role in AC regulation is played by the guanine nucleotide-binding regulatory protein (N-protein), which is necessary for the expression of the AC catalytic component enzymatic activity in the presence of Mg^{2+}. Hormones in the presence of GTP, synthetic poorly hydrolyzable analogues of GTP such as Gpp(NH)p, NaF and cholera toxin increase the AC activity by raising the concentration of this functionally active catalytic component–N-protein complex (review [8]). Some researchers believe that CM activates the catalytic AC component without participation of N-protein [9,10]. There are still other assumptions suggesting that CM and N-protein regulate different forms of AC [4,5].

It has been established here that guanine nucleotides, GTP (in the presence of isoproterenol) and Gpp(NH)p potentiate the effect of CM on heart AC. Our data give no reason to believe that a special CM-dependent form of AC exists in the heart. Apparently, CM activates the catalytic AC component associated with N-protein. This CM-dependent activation can be achieved through an increase in the AC catalytic turnover.

2. MATERIALS AND METHODS

Plasma membranes (light sarcolemma fraction) were isolated from the hearts of adult male rabbits according to recommendations in [11]. After

* To whom correspondence should be addressed

Abbreviation: Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate

Published by Elsevier Science Publishers B.V.

decapitation, the animals' hearts were rapidly removed, rinsed with chilled isotonic NaCl (0.9% NaCl), stripped of fat, and then the auricles were removed. All subsequent operations were performed at 4°C. The ventricles were weighed, minced with scissors and placed in 5 vols (w/v) buffer A containing 10 mM imidazole (pH 7.5) at 4°C, 1 mM EDTA, 1 mM $MgCl_2$ and 250 mM sucrose. The tissue was homogenized with a Polytron homogenizer for 1 min, then 5 vols buffer A were added to the homogenate. The homogenate was filtered through a double layer of gauze, layered on a 0.8 M sucrose solution in 10 mM imidazole (pH 7.5) at 4°C, 1 mM EDTA and 1 mM $MgCl_2$ (15 ml homogenate to 20 ml of 0.8 M sucrose solution) and then ultracentrifuged at 113000 × *g* for 90 min in an SW-27 bucket rotor. The light sarcolemma fraction, floating between 0.25 and 0.8 M sucrose solutions was collected, 2-fold diluted with buffer A, devoid of sucrose and centrifuged at 120000 × *g* for 60 min. Membranes were suspended in a solution containing 10 mM imidazole (pH 7.5) at 4°C, 1 mM EGTA and 100 mM NaCl (to a final membrane protein concentration of 1 mg/ml), and then preincubated with constant shaking for 30 min at 4°C. After preincubation, the membrane suspension was centrifuged at 120000 × *g* for 60 min. The membrane pellet was suspended in 10 mM imidazole (pH 7.5) at 4°C (to a final membrane protein concentration of 8–10 mg/ml), and was stored in liquid nitrogen.

CM was isolated from bovine brain as in [12]. Troponin I, isolated from bovine heart, was generously provided by Dr N.B. Gusev (Department of Biochemistry, Moscow State University). Creatine kinase was derived from rabbit skeletal muscle as in [13] and additionally purified by chromatography on DEAE–cellulose.

The AC activity was measured in an incubation medium (final volume 50 μl) containing 50 mM Tris–HCl (pH 7.5) at 37°C, 1 mM cAMP, 5 mM $MgCl_2$, 0.5 mg/ml creatine kinase, 20 mM creatine phosphate, 0.1 mM ATP and (0.5–2.0) × 10^6 cpm [α-^{32}P]ATP. Tubes were equilibrated at the assay temperature (37°C) for 1 min prior to starting the reaction by addition of membrane preparation to a final protein concentration of 0.04–0.06 mg/ml (2–3 μg per assay tube). Reaction was carried out for 20 min. During assays the basal activity of AC was linear with time and protein concentrations, and ATP levels were maintained at 98% of the original (determined by PEI-cellulose chromatography). The reaction was terminated by adding 200 μl of 0.5 N HCl per tube. The samples were boiled in water for 5 min and then neutralized with 1.5 M imidazole (200 μl/tube). The labelled cAMP formed was purified on an alumina column as in [14]. When kinetic experiments were performed the final volume of the incubation mixture was 1.0–1.5 ml. At zero time the formation of cAMP was initiated by the addition of membrane preparation (40–90 μg protein). At distinct times the reaction was stopped by transferring 50-μl aliquots in duplicate into 200 μl of 0.5 N HCl. Each experiment was performed at least 3 times for each batch of membranes. Results quoted are the means of triplicate determinations. A standard deviation was generally less than 5%.

Different free calcium concentrations in the incubation medium were performed by 2 mM Ca–EGTA buffer, using for calculation the values of stability constants for the Ca^{2+}–EGTA, Mg^{2+}–EGTA, and H^+–EGTA complexes given in [15].

Protein was determined as in [16]. [α-^{32}P]ATP was obtained from Amersham; ATP, GTP, Gpp(NH)p from Boehringer. Tris, imidazole, EDTA, EGTA, cAMP, sucrose, isoproterenol, trifluoroperazine and bovine serum albumin were purchased from Sigma; creatine phosphate from Calbiochem.

3. RESULTS AND DISCUSSION

One of the stages in the isolation of light sarcolemma from rabbit heart included its preincubation with EGTA (see section 2). It was established that the treatment of plasma membranes from a wide variety of tissues with EGTA or EDTA leads to effective removal of the endogenous CM, and selectively eliminates the activating effect of micromolar calcium concentrations on the membrane preparation of AC without eliminating the inhibitory action of Ca^{2+} on these enzymes preparations [4,17–20]. In the EGTA-treated heart plasma membrane preparation Ca^{2+} fails to exhibit any activating effect on AC. Moreover, an increase of the free Ca^{2+} concentration from 10^{-8} to 10^{-5} M induced only a progressive inhibition of AC (fig.1). On the other hand, addition of a

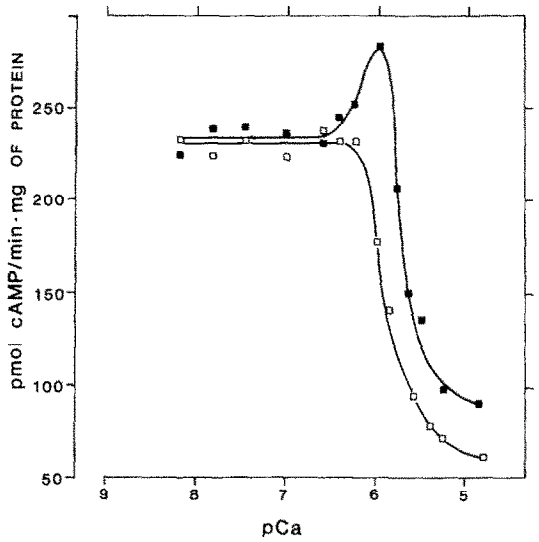

Fig.1. Effect of CM on the Ca^{2+} concentration-dependent activity of heart AC. (□—□) Activity in the absence of CM, (■—■) activity in the presence of 10^{-5} M CM.

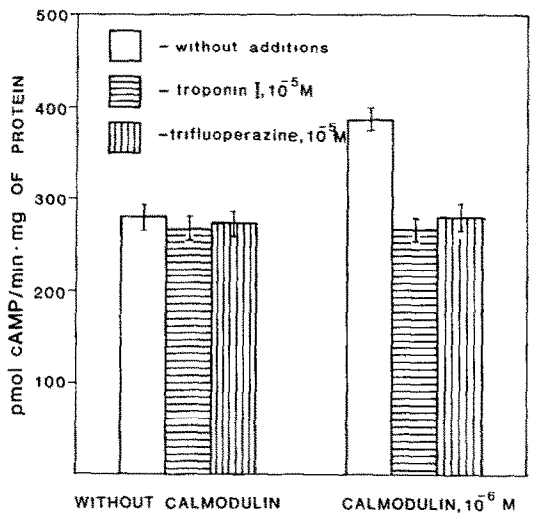

Fig.2. Elimination of the CM-induced heart AC activation by troponin I (10^{-5} M) and trifluoperazine (10^{-5} M). Concentrations of free Ca^{2+} and CM in the incubation medium were 10^{-6} M.

homogeneous CM from the brain to the incubation medium effected an alteration in the heart AC activity's dependence upon the Ca^{2+} concentration. Within the concentration range 4×10^{-7}–10^{-6} M, Ca^{2+} activated the enzyme. Ca^{2+} concentrations exceeding 10^{-6} M exerted an inhibitory effect on AC. It should be noted that the Ca^{2+}-dependent effect of CM on heart AC was retained within the range of Ca^{2+} concentrations inhibiting the enzyme (fig.1). If light sarcolemma was isolated from the heart under conditions which do not cause extraction of the endogenous CM, i.e., in the absence of EDTA and without EGTA extraction, biphasic dependence of the heart AC activity upon the Ca^{2+} concentration was also manifested in the absence of exogenous CM (in preparation).

The activating effect of CM on cardiac AC was inhibited by troponin I and trifluoperazine (fig.2). Neither troponin I (10^{-5} M) nor trifluoperazine (10^{-5} M) taken in these concentrations affected the AC activity in the absence of CM.

The Ca^{2+}-dependent AC activation by CM occurred both in the absence and in the presence of known enzyme activators: Gpp(NH)p, GTP and isoproterenol (table 1). The magnitude of CM-dependent heart AC activity increased proportionally to the stimulative action of these effectors on the CM-independent enzyme activity, which points to the participation of N-protein in the heart AC activation by CM.

Gpp(NH)p activated heart AC with a marked lag phase (fig.3). It is considered that this lag phase reflects the process of the N-protein transition to an activated state, whereas the interaction of the

Table 1

Effect of GTP + isoproterenol and Gpp(NH)p on heart AC activity in the absence and presence of CM

Additions	pmol cAMP/mg protein per min		
	Without CM	10^{-5} CM	CM-dependent activity
None	265 ± 1	431 ± 9	166 ± 9
GTP (10^{-4} M) + isoproterenol (5×10^{-5} M)	375 ± 1	585 ± 14	210 ± 14
Gpp(NH)p (10^{-4} M)	545 ± 9	953 ± 8	408 ± 9

The increments in the activities elicited by addition of 10^{-5} M CM were termed CM-dependent activity. The concentration of free Ca^{2+} in the incubation medium was 10^{-6} M

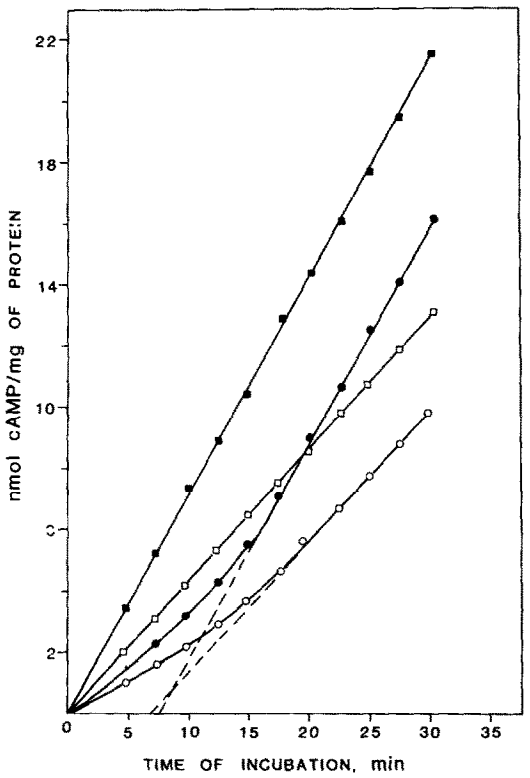

Fig.3. Time course of activation of heart AC by 10^{-5} M Gpp(NH)p (○—○), 10^{-5} M Gpp(NH)p + 5 × 10^{-5} M isoproterenol (□—□), 10^{-5} M Gpp(NH)p + 10^{-5} M CM (●—●), 10^{-5} M Gpp(NH)p + 5 × 10^{-5} M isoproterenol + 10^{-5} M CM (■—■). The apparent lag period is obtained by extrapolation (– – –). Concentration of free Ca^{2+} in the incubation medium was 10^{-6} M.

Gpp(NH)p-activated N-protein with a catalytic component occurs rapidly, without a lag phase [21]. In the presence of isoproterenol, the lag phase in the action of Gpp(NH)p disappeared but the steady-state activity of AC remained unchanged (fig.3). Thus isoproterenol accelerated the activation of N-protein without affecting the number of functionally active catalytic component–N-protein complexes formed in the presence of Gpp(NH)p. Unlike the hormone, CM did not affect the lag phase in Gpp(NH)p action, i.e., it failed to alter the velocity of the N-protein transition to the Gpp(NH)p-activated state of AC (fig.3). While discussing the mechanism by which CM affects AC, one cannot avoid mentioning the following principal fact: the magnitude of CM-dependent AC activity increased proportionally to the development of the Gpp(NH)p effect (i.e., as the N-protein–catalytic component complex is being formed), the degree of enzyme activation by CM being unchanged with an increase in the concentration of this complex. CM equally intensifies the activity of AC activated both by Gpp(NH)p and Gpp(NH)p + isoproterenol (fig.3). Since the magnitude of CM-dependent AC activity is proportional to the amount of the N-protein–catalytic component complex, one may assume that CM interacts with the N-protein–catalytic component complex 'holo AC' and increases its catalytic turnover.

ACKNOWLEDGEMENTS

The authors acknowledge with gratitude the excellent assistance of A. Khvostov and M. Skugarevskaya in preparing the manuscript.

REFERENCES

[1] Birnbaumer, L. (1973) Biochim. Biophys. Acta 300, 129–138.
[2] Brostrom, C.O., Huang, Y.C., Breckenridge, B.M. and Wolff, D.J. (1975) Proc. Natl. Acad. Sci. USA 72, 64–68.
[3] Cheung, W.Y., Bradham, L.S., Lynch, T.J., Lin, Y.M. and Tallant, E.A. (1975) Biochem. Biophys. Res. Commun. 66, 1055–1062.
[4] Brostrom, M.A., Brostrom, C.O., Breckenridge, B.M. and Wolff, D.J. (1978) Adv. Cyclic Nucleotide Res. 9, 85–99.
[5] Bradham, L.S. and Cheung, W.Y. (1980) in: Calcium and Cell Function (Cheung, W.Y. ed.) vol.1, pp.109–126, Academic Press, New York.
[6] Potter, J.D., Piascik, M.T., Nisler, P.L. and Johnson, C.L. (1981) Ann. NY Acad. Sci. 356, 220–231.
[7] Walsh, M.P., Le Peuch, C.J., Vallet, B., Cavadore, J.C. and Demaille, J.G. (1980) J. Mol. Cell. Cardiol. 12, 1091–1101.
[8] Ross, E.M. and Gilman, A.G. (1980) Annu. Rev. Biochem. 49, 533–564.
[9] Salter, R.S., Krinks, M.H., Klee, G.B. and Neer, E.J. (1981) J. Biol. Chem. 256, 9830–9833.
[10] Bitonti, A.J., Moss, J. and Vaughan, M. (1982) Biochemistry 21, 3650–3653.
[11] Kidwai, A.M., Radcliffe, M.A., Duchon, G. and Daniel, E.E. (1971) Biochem. Biophys. Res. Commun. 45, 901–910.

[12] Gopalakrishna, K. and Anderson, W.B. (1982) Biochem. Biophys. Res. Commun. 104, 830–836.
[13] Noda, L., Kuby, S. and Lardy, H. (1955) Methods Enzymol. 11, 605–610.
[14] White, A.A. (1974) Methods Enzymol. 38C, 41–46.
[15] Bartfai, T. (1979) Adv. Cyclic Nucleotide Res. 10, 219–242.
[16] Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
[17] Piascik, M.T., Wisler, P.L., Johnson, C.L. and Potter, J.D. (1980) J. Biol. Chem. 255, 4176–4181.
[18] Malnoë, A., Cox, J.A. and Stein, E.A. (1982) Biochim. Biophys. Acta 714, 84–92.
[19] Thams, P., Capito, K. and Hedeskov, C.J. (1982) Biochem. J. 206, 97–102.
[20] Pinkus, L.M., Sulimovici, S., Susser, F.I. and Roginsky, M.S. (1983) Biochim. Biophys. Acta 762, 552–559.
[21] Neer, E.J. and Salter, R.S. (1981) J. Biol. Chem. 256, 12102–12107.