THE EFFECTS OF PHOSPHOLIPASE A₂ ON BETA ADRENOCEPTOR FUNCTION IN ISOLATED CARDIAC CELLS

MASAKI HATTORI, M.D., SHUICHIRO NAGAI, M.D., KOUICHI OGAWA, M.D.
TATSUO SATAKE, M.D., SATORU SUGIYAMA, M.D.*
AND TAKAYUKI OZAWA, M.D.*

The role of phospholipids in the maintenance of β-adrenoceptor function was investigated in isolated canine myocytes prepared from eight adult mongrel dogs by using collagenase. The characteristics of β-adrenoceptors were assessed by determining the number and the affinity of receptors by a radioactive ligand binding assay using 125I-iodocyanopindolol. The increase in cyclic AMP content induced by isoproterenol or forskolin was also determined by radioimmunoassay with or without pretreatment with phospholipase (PLase) A₂. The amount of free fatty acids released from isolated myocytes by PLase A₂ was measured by high-performance liquid chromatography. PLase induced a significant decrease in the number of β-adrenoceptors but did not affect their affinity. Although the isoproterenol-stimulated increase in cyclic AMP was significantly inhibited by the pretreatment with PLase A₂, the forskolin-stimulated increase was not affected. Responsive accumulation of cyclic AMP to isoproterenol was much more impaired than the decrease in β-adrenoceptor number. These results indicate that PLase A₂ deteriorates the function of the adenylate cyclase system linked-β-adrenoceptor, and suggest that PLase A₂ affects both β-adrenoceptors and the coupling of β-adrenoceptors with adenylate cyclase.

Isolated cardiac myocytes represent a unique model system in the understanding of normal cardiac function on the molecular level. Because of the relative purity of the cell type, isolated myocytes are a useful preparation for studying membrane receptor function. Receptors are believed to be protein molecules situated in membrane phospholipids, and their proper functions are considered to be dependent upon the appropriate integration with membrane phospholipids. Therefore, any change in the membrane lipid profile may affect β-adrenoceptor dynamics. Lefkowitz et al. reported that digestion of myocardial membrane with phospholipase (PLase) A deteriorated the function of the adenylate cyclase system. PLases have recently been reported to play an important role in the development of various pathogenic conditions by decomposing biomembrane. Moreover, the activation of PLases and the resulting degradation of membrane phospholipids were reported to be closely related to the development of ischemic myocardial damage.

In the present study, utilizing isolated cardiac myocytes, we investigated the effect of PLase A₂ on the increase in the content of...
cyclic AMP (c-AMP) induced by isoproterenol or forskolin and the characteristics of membrane β-adrenoceptors.

MATERIALS AND METHODS

PLase A₂ (from Naja naja venom) and isoproterenol hydrochloride were purchased from Sigma Chemical Company. Nin- anthryldiazomethane (ADAM) was purchased from Funakoshi Pharm. Co., Ltd. Collagenase (from Clostridium histolyticum) was purchased from Wako Pure Chemical Industries Ltd., and (−)-3-[125I]-iodocyanopindolol (1800 Ci/m mole) was purchased from the Radiochemical Centre, Amersham. Forskolin was purchased from Calbiochem-Behring Corp.

Preparation of isolated cardiac cells

Isolated cardiac myocytes were obtained by a modified method of Haworth et al. Eight adult mongrel dogs (8–12 kg) of either sex were anesthetized with sodium pentobarbital (50 mg/kg), given intraperitoneally. Under artificial respiration, the chest of each dog was opened with a left 4th intercostal incision. After stabilization for 30 min, the heart was removed and was perfused through a polyethylene tube cannulated to the aorta with Krebs-Henseleit solution (KH solution): 118 mM NaCl, 4.8 mM KCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂ and 11 mM glucose. The perfusate warmed to 37°C was equilibrated with 95% O₂-5% CO₂, and the pH was adjusted to 7.4. The flow rate was maintained at 100 ml/min. After 5 min of the initial perfusion, the heart was perfused with Ca-free KH solution for 5 min at 37°C. The heart was then perfused with Ca-low KH solution (50 μM Ca++) containing collagenase (0.25 mg/ml). After perfusion with this solution for 15–20 min, the left ventricle was cut out and chopped with scissors into small pieces in a glass flask containing the same solution. Myocardial cells released from the cardiac tissue by mechanical agitation of the solution at 37°C were suspended in Ca-free solution (10–15 mg/ml) after washing the cells twice by the same solution. Viability of myocytes was checked by their abilities to exclude trypan blue (0.05–0.1%). Protein concentration was determined by the biuret method.

[125I]-iodocyanopindolol (ICYP) binding assay

For the determination of the characteristics of β-adrenoceptor, prepared myocardial cells were suspended in the Tris-buffer (10 mM MgCl₂, 50 mM Tris, pH 7.4). One ml of cell suspension (10–15 mg protein) was incubated for 5 min at 37°C in the presence or absence of 1 unit of PLase A₂. After stopping the reaction by adding 10 μl of 100 mM EGTA, the protein concentration of the samples were diluted to 0.5–0.6 mg protein/ml with the Tris-buffer. The binding assay was initiated by adding 200 μl of cell samples to 100 μl of ICYP (8.0 pM to 400 pM) with 100 μl of (D,L)-propranolol (10 μM) or with 100 μl of the Tris-buffer. At the end of incubation, the reaction mixture was immediately filtered through Whatman GF/D glass fiber filter and washed with 20 ml of the ice cold Tris-buffer. The radioactivity remaining in the protein retained by the filter was determined by Aloka Autowell Gammacounter. Specific binding was defined as the difference between in the presence and absence of (D,L)-propranolol (10 μM) expressed as f moles/mg protein. Specific binding was usually 70 to 80% of total binding. Binding data were assessed by the method of Scatchard.

Measurement of c-AMP

One ml of cell suspension (10–15 mg protein) was incubated for 5 min at 37°C with or without 1 unit of PLase A₂ in the presence of 1 mM theophylline. After stopping the reaction by adding 10 μl of 100 mM EGTA, the reaction mixture was incubated for 3 min at 37°C with
TABLE I
THE NUMBER OF MAXIMUM BINDING SITES (B_max) AND EQUILIBRIUM DISSOCIATION CONSTANT (K_d)

<table>
<thead>
<tr>
<th></th>
<th>B_max (f moles/mg protein)</th>
<th>K_d (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>52.5 ± 2.1</td>
<td>163 ± 9.3</td>
</tr>
<tr>
<td>PLa se A_2 pretreatment</td>
<td>38.7 ± 0.9**</td>
<td>159 ± 4.0</td>
</tr>
</tbody>
</table>

n = 8 (Mean ± SE)
** = p < 0.01 vs Control group;
PLa se A_2 = phospholipase A_2

TABLE II
CONTENT OF CYCLIC AMP IN ISOLATED MYOCYTES STIMULATED BY ISOPROTERENOL (p moles/mg protein)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PLa se A_2 pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoprot erenol (.-)</td>
<td>10.5 ± 1.2</td>
<td>10.3 ± 1.1</td>
</tr>
<tr>
<td>10^-8 M Isoprot erenol</td>
<td>11.5 ± 1.1</td>
<td>10.4 ± 1.1</td>
</tr>
<tr>
<td>10^-7 M Isoprot erenol</td>
<td>14.7 ± 1.5</td>
<td>10.9 ± 1.5</td>
</tr>
<tr>
<td>10^-6 M Isoprot erenol</td>
<td>18.3 ± 1.2</td>
<td>11.8 ± 1.9*</td>
</tr>
<tr>
<td>10^-5 M Isoprot erenol</td>
<td>21.6 ± 0.8</td>
<td>12.1 ± 2.2**</td>
</tr>
</tbody>
</table>

n = 8 (mean ± SE)
* = p < 0.05; ** p < 0.01 vs Control group;
PLa se A_2 = phospholipase A_2

isoprot erenol (final concentration: 10^-5-10^-8 M)
or forskolin (final concentration: 10^-5-10^-8 M)
to produce the increase in c-AMP. At the end of this reaction, 1 ml of 12% trichloroacetic acid was added to extract c-AMP from myocytes. The content of c-AMP was determined according to the method of Honma et al. using c-AMP assay kit (Yamasa Shoyu Co., Ltd.).

Measurement of free fatty acids (FFA)
Using 1 ml of isolated myocytes (10-15 mg protein), changes in the FFA content induced by 1 unit of PLa se A_2 were determined after 5 min of incubation with this enzyme at 37°C. Extraction of FFA was performed by a modified method of Folch et al. and extracted FFA were converted to fluorescent derivatives by reacting with ADAM, which is known to exclusively react with COOH radical. Determination of FFA content was performed by the quantification of respective derivatives of FFA by high performance liquid chromatography (HPLC) using Shimadzu type LC-3A system according to the method of Shimomura et al.

The significance of the results was determined by the Student’s t-test. Values of p less than 0.05 were considered statistically significant.

RESULTS
The viability of cells was 70.4 ± 3.6% (mean ± SE), and over 90% of cells which excluded trypan blue were rod-shaped.

Fig. 1 shows Scatchard plot of ICYP binding to cardiac myocytes with or without pretreatment with PLa se A_2. Table I shows the number of maximum binding sites and equilibrium...
dissociation constant (Kd) of isolated cells in each group. The number of β-adrenoceptors in the cells decreased significantly by the pretreatment with PLase A2 from 52.5 ± 2.1 fmole/mg protein (mean ± SE) for the control to 38.7 ± 0.9. Whereas, pretreatment with PLase A2 did not cause any significant change in Kd of the myocardial cells to ICYP binding.

Table II shows the effects of PLase A2 on β-adrenoceptor function assessed by the changes in c-AMP content induced by isoproterenol. In the cells without isoproterenol treatment, PLase A2 itself did not affect a base line level of c-AMP in the myocytes. Although the stimulation of β-adrenoceptors of the myocytes induced a dose-dependent increase in c-AMP content in the intact cells, this isoproterenol-stimulated increase in c-AMP content was not observed in the cells treated with PLase A2. Pretreatment with PLase A2 induced significant inhibition of the isoproterenol-induced increase in c-AMP content when β-adrenoceptors of myocardial cells were stimulated with 10⁻²M and 10⁻⁸M of isoproterenol.

Table III shows the effects of PLase A2 on β-adrenoceptor function assessed by the changes in myocardial c-AMP content induced by forskolin. Forskolin induced a dose-dependent increase in myocardial c-AMP content in the cells. In contrast to the isoproterenol-stimulated increase in c-AMP, pretreatment with PLase A2 did not affect the dose-dependent increase in the myocardial content of c-AMP induced by forskolin.

Table IV shows the effects of PLase A2 on the content of FFA in the cell suspension. Eight kinds of FFA were detected in our cell preparation. They were myristic, palmitic, palmitoleic, stearic, oleic, linoleic, arachidonic, and docosahexaenoic acids. Incubation with PLase A2 induced a significant increase in the unsaturated FFA; ie, palmitoleic, oleic, linoleic, arachidonic, docosahexaenoic acids.

**DISCUSSION**

Phospholipids of cell membrane are known to play important roles both in the organization of membranes and in the modulation of enzyme functions within the cell membranes. In 1962, Sutherland et al. first observed that "solubilized" adenylate cyclase preparation lost their hormone responsiveness and hypothesized that this was a lipoprotein. The systemic study on lipid requirement for hormone sensitive adenylate cyclase by Pohl et al. revealed that preservation of structural component of membrane lipids was very important in the maintenance of adenylate cyclase system.

Using isolated canine cardiac cells, we observed that responsive accumulation of c-AMP to isoproterenol was significantly inhibited by the pretreatment with PLase A2. Whereas, responsive accumulation of c-AMP to forskolin (which is considered to play as a direct activator of adenylate cyclase) was not inhibited by PLase A2 pretreatment. These results seem to indicate that PLase A2 deteriorates the β-adrenoceptor function of cardiac cells without affecting the activity of adenylate cyclase. Moreover, the mechanism by which PLase A2 deteriorates the β-adrenoceptor function is suggested to be derived from the impairment of the coupling of β-adrenoceptors with adenylate cyclase or from the decrease in the binding of agonists to the receptors. Bobik et al. showed that activation of endogenous PLase A2 by melittin deteriorated the function of the β-adrenoceptors linked adenylate cyclase system in cultured cardiac cells without affecting the number of β-adrenoceptors. Although they concluded that the prime effect of PLase A2 was on the ability of β-adrenoceptors to couple with adenylate cyclase during β-agonist occupancy of the receptor, several investigators reported that digestion of membranes of erythrocytes or hepatic cells with PLases induced a decrease in the number of β-adrenoceptors.

These contradictory results might be derived from the difference in the origin of membrane and/or the preparative procedures of materials. In other words, these results have been reported by using a fundamentally different model of experiments. The use of membrane fractions in the study of β-adrenoceptor assessment can not completely eliminate the contamination of inside-out membranes, and the use of cultured cells can become an analysis of the receptors in quite a different state from adult heart cells in the characters. Therefore, it seems to be more suitable to investigate the β-adrenoceptor function using isolated cardiac cells.

Our study on β-adrenoceptor binding revealed that the impairment of responsive accumulation of c-AMP to isoproterenol induced by PLase A2 was much more impaired than the decrease in the β-adrenoceptor number by PLase A2. These results suggest that PLase A2 affects the function of the β-adrenoceptor-linked adenylate cyclase.
system in isolated cardiac cells and that PLase A₂ modulates the characteristics of β-adrenoceptors and the coupling of β-adrenoceptors with adenylate cyclase.

In conclusion, our study revealed that the preservation of membrane phospholipids plays an important role in the maintenance of β-adrenoceptor function by preserving not only binding of agonists to the receptors but also the coupling of receptors with adenylate cyclase.

Acknowledgement

We would like to express our appreciation to Mr. B.E. Virden, language consultant of our department, for reading previous draft of this paper and giving us numerous suggestions concerning language and style.

REFERENCES

7. VASDEV SC, KAKO KJ, BERO GP: Phospholipid composition of cardiac mitochondria and lysosomes in experimental myocardial ischemia in the dog. J Mol Cell Cardiol 11: 1195, 1979
9. HOWARD RB, PESCH LA: Respiratory activity of intact, isolated parenchymal cells from rat liver.


Japanese Circulation Journal Vol. 49, November 1985