British Journal of Pharmacology (1996) 118, 865-870

Effects of β-adrenoceptor antagonists on Ca^{2+}-overload induced by lysophosphatidylcholine in rat isolated cardiomyocytes

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1 The effects of β-adrenoceptor antagonists including (−)- and (+)-propranolol, (−)- and (+)-penbutolol, timolol, pindolol, atenolol, acebutolol and practolol on the Ca^{2+}-overload induced by lysophosphatidylcholine (LPC) were examined in isolated cardiomyocytes of the rat.

2 Fura-2 was used for measurement of the intracellular calcium concentration ($[Ca^{2+}]_i$). LPC (15 μM) produced a rapid increase in $[Ca^{2+}]_i$ from 72±5 to 3042±431 nM which coincided with a decrease in the percentage of rod-shaped cells from 69±2 to 5±2%.

3 Preincubation with (−)-propranolol (20 μM), (+)-propranolol (50 μM), or (−)- or (+)-penbutolol (20 μM), the lipophilicity of which is higher than other β-adrenoceptor antagonists, significantly inhibited both the increase in $[Ca^{2+}]_i$ and the cell-shape change induced by 15 μM LPC. The inhibitory effects of the four drugs on the LPC-induced increase in $[Ca^{2+}]_i$ and cell-shape change were concentration-dependent. The IC_{50}s of (−)-propranolol, (+)-propranolol, (−)- and (+)-penbutolol for the increase in $[Ca^{2+}]_i$ were 1.28, 10.50, 0.67 and 0.76 μM, respectively.

4 Pretreatment with pindolol, timolol, acebutolol, practolol, atenolol or lignocaine did not inhibit the increase in $[Ca^{2+}]_i$ and the morphological change induced by LPC.

5 LPC markedly increased the release of creatine phosphokinase from 9±1 to 45±2% which could be significantly reduced by (−)- or (+)-propranolol but not by acebutolol or timolol.

6 The protective effects of (−)- and (+)-propranolol, (−)- and (+)-penbutolol against the Ca^{2+}-overload induced by LPC were not associated with the β-adrenoceptor antagonistic action, but probably with an unknown action which is related to the preservation of membrane integrity. Further studies are necessary to clarify the exact mechanisms of the protective action of these β-adrenoceptor antagonists against the Ca^{2+}-overload induced by LPC.

Keywords: Cardiomyocytes; β-adrenoceptor antagonists; lysophosphatidylcholine; Ca^{2+}-overload; creatine phosphokinase

Introduction

Lysophosphatidylcholine (LPC), an amphipathic metabolite of membrane phospholipids, has been demonstrated to accumulate in myocardial tissue during ischaemia (Corr *et al.*, 1982; Kinnaird *et al.*, 1988). Because of both hydrophilic and hydrophobic properties, LPC can easily exert deleterious effects on biological membrane systems and cause electrophysiological alterations and mechanical dysfunction (Corr *et al.*, 1984; Das *et al.*, 1986; Datorre *et al.*, 1991; Hoque *et al.*, 1995). Therefore, LPC has been suspected as one of the mediators of ischaemic injury. It has been suggested that exogenous application of LPC produces Ca^{2+}-overload in isolated or cultured ventricular myocytes (Liu E. *et al.*, 1991; Ver Donck *et al.*, 1992), resulting from the physico-chemical alteration in the membrane (Colquhoun *et al.*, 1981; Sedlis *et al.*, 1983). Since LPC may play an important role in ischaemia-reperfusion injury, it is of interest to investigate the effects of anti-ischaemic drugs on the Ca^{2+}-overload induced by LPC.

The present study aimed to examine whether β-adrenoceptor antagonists can inhibit the Ca^{2+}-overload induced by LPC in rat isolated cardiomyocytes. Our previous study (Hoque *et al.*, 1995) revealed that (+)-propranolol, which possesses weaker β-adrenoceptor antagonistic action (βAA) than (±)-propranolol (Cruickshank & Prichard, 1987), inhibited the cardiac dysfunction and metabolical derangements induced by exogenous LPC in perfused working rat hearts. It is, therefore, suggested that β-adrenoceptor antagonists protect cardiomyocytes from the Ca^{2+}-overload induced by LPC, and that βAA activity is not related to the protective mechanisms. We selected several β-adrenoceptor antagonists with different pharmacodynamic profiles in order to reveal whether βAA and membrane stabilizing activity (MSA) were involved in the protection against the Ca^{2+}-overload induced by LPC.

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Methods

Isolation of myocytes

Calcium-tolerant cardiomyocytes were isolated from male Sprague-Dawley rats (about 250 g body weight) according to the method described by Piper *et al.* (1982) with minor modifications (Hashizume *et al.*, 1994). The isolated cardiomyocytes were suspended in Krebs-Ringer bicarbonate (KRB) buffer containing (mM): NaCl 119, $NaHCO_3$ 15, KCl 2.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 11 and $CaCl_2$ 1.

Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

The suspension of myocytes was diluted to a concentration of 5% (v/v) with the KRB buffer and then incubated with 5 μM Fura-2/acetoxymethyl ester (AM) for 1 h at room temperature and continuously gassed with 5% CO_2/95% O_2. Thereafter, 1 ml cell-suspension was taken and washed twice with fresh KRB buffer before use. The fluorescence intensities were measured using a fluorescence spectrometer (model CAF-110, Japan Spectroscopic Co. Ltd., Tokyo, Japan). The excitation wavelengths were 340 and 380 nm, and the emission wavelength was 510 nm. The fluorescence ratio was defined as the fluorescence intensity at 340 nm divided by the fluorescence intensity at 380 nm. Calibration of the fluorescence intensity was done at the end of each experiment; digitonin (100 μM)

and ethyleneglycol-*bis*-(β-aminoethyl)-*N,N,N',N'*-tetraacetic acid (EGTA) (20 mM) were added to the cell-suspension to obtain the maximal/minimum fluorescence levels, respectively. The $[Ca^{2+}]_i$ was estimated on the basis of both fluorescence and the calibration according to the method described by Grynkiewicz *et al.* (1985).

Observation of morphological change

For the observation of the cell-shape change, the myocyte samples were fixed in 2.5% glutaraldehyde. About 150 myocytes were counted under a light microscope, and the percentage of rod-shaped cells to the total cells was calculated and used as an indicator of the morphological change.

Measurement of creatine phosphokinase (CPK)

The CPK activity was measured by means of a kit purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). The cell-samples were centrifuged, and CPK was extracted from pellets (cells) with 1% Triton X-100. Both the supernatant and the cellular extract were measured. The activity of CPK released into the supernatant was expressed as a percentage of the total CPK activity (supernatant plus pellets).

Experimental protocol

We selected nine β-adrenoceptor antagonists ((−)-propranolol, (+)-propranolol, (−)-penbutolol, (+)-penbutolol, acebutolol, pindolol, timolol, atenolol and practolol) with different potency of βAA and having or not having MSA. The characteristics of each β-adrenoceptor antagonist are summarized in Table 1. Myocyte-suspensions were preincubated with one of the tested drugs for 5 min, and then LPC 15 μM (the final concentration) was added. The fluorescence intensity of fura-2 was monitored from the beginning of incubation to 5 min after the addition of LPC. Samples for determination of morphological change and CPK release were taken before and 5 min after the addition of LPC. During the experiment, the temperature was maintained at 37°C.

Materials

LPC (L-α-lysophosphatidylcholine, palmitoyl) was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Propranolol [(±), (−) and (+)-propranolol)] was obtained from ICI-Pharma Manufacturing (Osaka, Japan), (−)- and (+)-penbutolol from Nippon Hoechst Co. (Tokyo, Japan), timolol and acebutolol from Chugai Pharmaceutical Co. (Tokyo, Japan), practolol from Sumitomo Chemical Co. (Osaka, Japan), and pindolol and atenolol from Sigma Chemical Company (St. Louis, MO, U.S.A.). All the drugs were dissolved in distilled water except for pindolol and practolol which were dissolved in 99.5% ethanol and further diluted with distilled water so that the final concentration of ethanol was 0.1% (this concentration of ethanol did not affect $[Ca^{2+}]_i$ and cell-shape). All the drugs were prepared immediately before use. Fura-2/AM was purchased from Dojindo Laboratory (Kumamoto, Japan).

Statistical analysis

Results are given as means ± s.e.mean. In all experiments, n indicates the number of experiments which have been done in cell-samples obtained from 54 rats. Significance of difference was determined with an analysis of variance followed by Dunnett's multiple comparisons test with a significance level of $P<0.05$.

Results

Effects of LPC on $[Ca^{2+}]_i$ and cell-shape

Exposure of myocytes to LPC at a concentration of 15 μM produced an abrupt increase in $[Ca^{2+}]_i$ from 72 ± 5 nM to 3042 ± 431 nM ($n=6$), and the percentage of rod-shaped cells was reduced from 69 ± 2 to 5 ± 5%. These changes were completely developed within 2 min. Figure 1 shows the time-course of the increase in $[Ca^{2+}]_i$ and the morphological change induced by LPC in the absence of β-adrenoceptor antagonists. From the foregoing results, we decided that the effects of β-adrenoceptor antagonists should be determined at 5 min after addition of LPC.

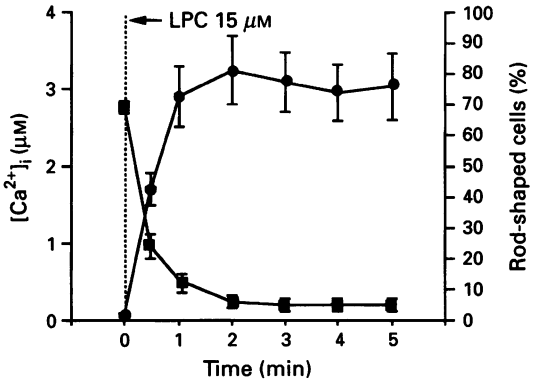

Figure 1 The time course of changes in $[Ca^{2+}]_i$ (●) and the percentage of rod-shaped cells (■) to total cells after addition of 15 μM LPC in isolated cardiomyocytes (mean ± s.e.mean, $n=6$).

Table 1 Pharmacological features, log octanol/water coefficients and IC_{50} of β-adreoceptor antagonists

Drugs	*βAA potency ratio*[a] *((+)-propranolol=1)*	*MSA*[a]	*log Octanol/water coefficients*[a] *(at 37°C, pH 7.4)*	IC_{50}[b] *(μM)*
(±)-Propranolol	1	+	20.2	
(−)-Propranolol	2	+	20.2	1.28
(+)-Propranolol	0.01−0.02	+	20.2	10.5
(−)-Penbutolol	4	+	179.0	0.64
(+)-Penbutolol	?	+	179.0	0.74
Acebutolol	0.3	+	0.02	>200
Pindolol	6	+	0.82	>200
Timolol	6	−	1.16	>20
Atenolol	1	−	0.68	>20
Practolol	0.3	−	0.15	>20

[a]All values were cited from Cruickshank & Prichard (1987). [b]The concentration required to inhibit the LPC-induced $[Ca^{2+}]_i$ increase by 50%, calculated from the results of Figure 3. MSA: membrane stabilizing activity indicated by local anaesthetic action. βAA: β-adrenoceptor antagonistic action.

The effect of β-adrenoceptor antagonists on the LPC-induced $[Ca^{2+}]_i$ increase

Figure 2 shows the effects of pretreatment for 5 min with β-adrenoceptor antagonists, including (−)- and (+)-propranolol, (−)- and (+)-penbutolol, pindolol, timolol, practolol, acebutolol and atenolol, all at a concentration of 20 μM on the $[Ca^{2+}]_i$-increase induced by exposure to 15 μM LPC for 5 min. (−)-propranolol and (−)-penbutolol, both of which possess βAA and MSA, significantly inhibited the $[Ca^{2+}]_i$-increase. The (+)-isomers of those two agents ((+)-propranolol and (+)-penbutolol) which possess much weaker βAA but a similar degree of MSA to (−)-propranolol or (−)-penbutolol, also inhibited the $[Ca^{2+}]_i$-increase induced by LPC. However, a higher concentration (50 μM) was necessary for (+)-propranolol to achieve a significant effect. The inhibitory effects of (−)- and (+)-propranolol and (−)- and (+)-penbutolol on the $[Ca^{2+}]_i$-increase induced by LPC were concentration-dependent (Figure 4a). The concentrations required to inhibit the $[Ca^{2+}]_i$-increase by 50% (IC_{50}) are also shown in Table 1. Other β-adrenoceptor antagonists, pindolol and acebutolol (with βAA and MSA), and timolol, atenolol and practolol (with βAA but without MSA), did not inhibit the $[Ca^{2+}]_i$-increase induced by LPC (Figure 2). In additional experiments, we increased the concentrations of pindolol and acebutolol to 200 μM. Even in the presence of 200 μM pindolol or acebutolol, LPC increased the $[Ca^{2+}]_i$ from 66±2 nM to 1863±506 nM ($n=4$) or to 2750±597 nM ($n=5$), respectively. These values are not significantly different from the value (2950±602 nM) in the absence of drugs ($n=5$). Thus, no inhibitory effect was found in the experiment at a higher concentration of both pindolol and acebutolol. In addition, lignocaine (100 μM), a local anaesthetic, did not inhibit the $[Ca^{2+}]_i$-increase induced by LPC (Figure 2).

The effect of β-adrenoceptor antagonists on LPC-induced cell-shape change

Figure 3 shows the effects of different β-adrenoceptor antagonists (20 μM) and lignocaine (100 μM) on the cell-shape change induced by LPC. (−)-propranolol, (−)- and (+)-penbutolol inhibited the cell-shape change, resulting in a significant increase in the percentage of rod-shaped cells. The

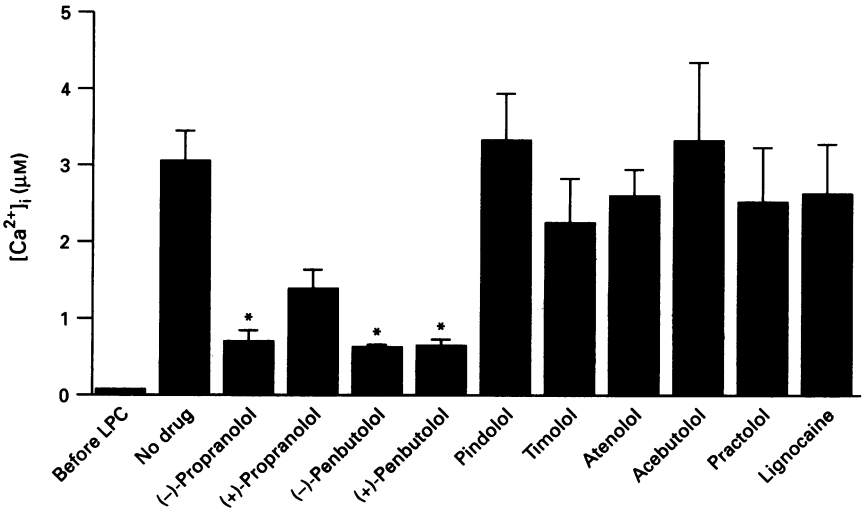

Figure 2 Effects of various β-adrenoceptor antagonists (20 μM) and lignocaine (100 μM) on the increase in $[Ca^{2+}]_i$ induced by LPC at 5 min in isolated cardiomyocytes (mean±s.e.mean, $n=5-30$, $^*P<0.05$ vs no drug).

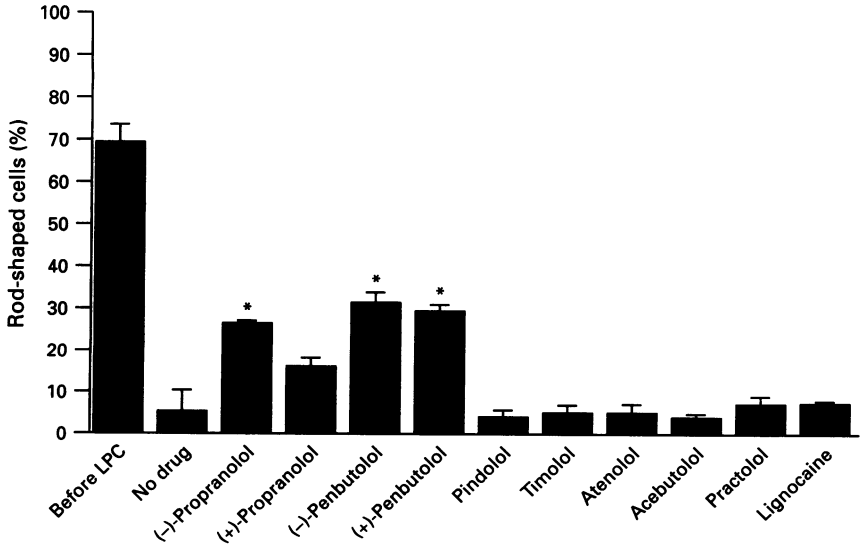

Figure 3 Effects of various β-adrenoceptor antagonists (20 μM) and lignocaine (100 μM) on the morphological change induced by LPC in isolated cardiomyocytes (mean±s.e.mean, $n=5-20$, $^*P<0.05$ vs no drug).

inhibitory effects on the cell-shape change of these drugs were concentration-dependent (Figure 4b). (+)-propranolol at a concentration of 20 μM did not inhibit the cell-shape change induced by LPC, but at 50 μM it did inhibit significantly ($30 \pm 2\%$, $n=6$, $P<0.05$). The IC_{50} for the cell-shape change of these drugs could not be calculated, because even the highest concentration we used did not completely inhibit the cell-shape change induced by LPC. Timolol, pindolol, acebutolol, atenolol, practolol and lignocaine did not inhibit the cell-shape change induced by LPC.

The effect of β-adrenoceptor antagonists on LPC-induced CPK release

Table 2 shows the effect of β-adrenoceptor antagonists on CPK release induced by LPC. The percentage of released CPK to total CPK was $9 \pm 1\%$ in the cells after 5 min incubation without LPC. Significant release of CPK ($45 \pm 2\%$) occurred 5 min after the addition of LPC, and both (−)-propranolol (20 μM) and (+)-propranolol (50 μM) significantly inhibited the CPK release induced by LPC, but acebutolol (20 μM) and timolol (20 μM) did not.

Discussion

LPC produced Ca^{2+}-overload (as shown by an increase in $[Ca^{2+}]_i$ and concomitant cell-rounding) in the cardiomyocytes. The aim in the present study was to clarify the effect of β-adrenoceptor antagonists on the Ca^{2+}-overload induced by LPC, and to determine the protective mechanism of the drug. Our results show that effects of β-adrenoceptor antagonists on the Ca^{2+}-overload induced by LPC are not homogeneous. (−)-propranolol and (−)-penbutolol, both of which possess βAA, significantly inhibited the Ca^{2+}-overload induced by LPC, while the (+)-isomer of those two agents, possessing weaker βAA (Cruickshank & Prichard, 1987), also inhibited. On the other hand, pindolol and timolol which possess more potent βAA than (±)-propranolol (Cruickshank & Prichard, 1987), did not inhibit the Ca^{2+}-overload induced by LPC. These data suggest that βAA is not the primary determinant of the inhibitory effect of β-adrenoceptor antagonists on the Ca^{2+}-overload induced by LPC.

Table 2 The effect of β-adrenoceptor antagonists on CPK release induced by LPC for 5 min in isolated cardiomyocytes

Treatment	n	*CPK release (%)*
No drug−LPC	6	9±1
No drug+LPC (15 μM)	20	45±2†
(−)-Propranolol (20 μM)+LPC	9	31±3*†
(+)-Propranolol (50 μM)+LPC	10	32±2*†
Acebutolol (20 μM)+LPC	5	48±3*†
Timolol (20 μM)+LPC	5	46±7*†

All data are shown as mean±s.e. mean. *Significantly different from no drug+LPC group: †significantly different from no drug−LPC group.

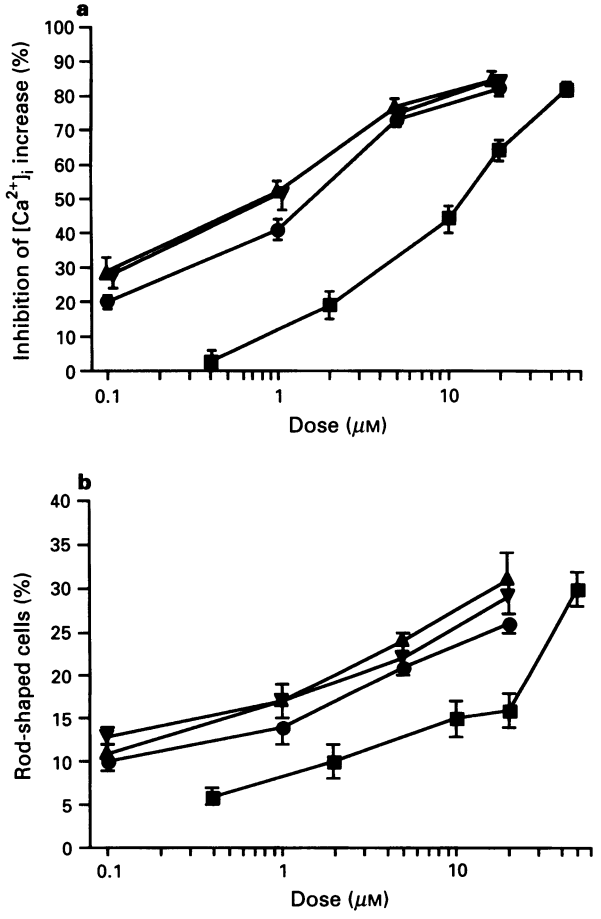

Figure 4 Concentration-dependent inhibition by (−)- and (+)-propranolol and (−)- and (+)-penbutolol on LPC-induced $[Ca^{2+}]_i$ increase (a) and the cell-shape change (b) in isolated cardiomyocytes (mean±s.e.mean, $n=5-7$): (●) (−)-propranolol; (■) (+)-propranolol; (▲) (−)-penbutolol; (▼) (+)-penbutolol.

The property which (−)- and (+)-propranolol, (−)- and (+)-penbutolol possess in common is MSA (Table 1). It is, therefore, possible that MSA may play a role in the protection against the Ca^{2+}-overload induced by LPC. However, acebutolol and pindolol, both having MSA, were not effective at a concentration of 20 μM. It is reported that the concentrations required to elicit MSA for pindolol and acebutolol should be as high as 300 μM and 134 μM, respectively (Giudicelli *et al.*, 1969; Sada & Ban, 1980). On the other hand, those for (−)- and (+)-propranolol were about 10 μM (Dohadiwalla *et al.*, 1969), and (−)- and (+)-penbutolol were about 0.4 μM (Kodama *et al.*, 1978). The concentration (20 μM) of the β-adrenoceptor antagonists would be not sufficient for pindolol and acebutolol to elicit MSA. For this reason, pindolol and acebutolol at higher concentration (200 μM) were also tested in the present study. However, even at 200 μM pindolol and acebutolol did not inhibit the Ca^{2+}-overload induced by LPC. Further, lignocaine which possesses local anaesthetic action failed to inhibit the Ca^{2+}-overload induced by LPC. These findings suggest that MSA alone cannot explain the protective effect of β-adrenoceptor antagonists on the Ca^{2+}-overload induced by LPC.

In the present study, LPC increased CPK release, which has been considered to reflect membrane damage (Murphy *et al.*, 1985; Fujioka *et al.*, 1991). Both (−)- and (+)-propranolol, being effective in preventing the Ca^{2+}-overload induced by LPC, also reduced the CPK release. On the other hand, acebutolol and timolol which were not effective, did not inhibit the CPK release. It is suggested, therefore, that preserving membrane integrity probably contributes to the inhibitory effect of (−)- and (+)-propranolol on the Ca^{2+}-overload induced by LPC.

Among the β-adrenoceptor antagonists tested, (−)- and (+)-propranolol, (−)- and (+)-penbutolol, which were effective, possess higher lipophilicity than other β-adrenoceptor antagonists (Table 1). Both (−)- and (+)-penbutolol being the most lipophilic, were most effective. High lipophilicity of these drugs is related, at least partially, to the protective effects of β-adrenoceptor antagonists against the Ca^{2+}-overload induced by LPC. Because of the amphipathic property of LPC, LPC binds to the cytoplasmic membrane and associates with it

(Man *et al.*, 1990). β-adrenoceptor antagonists with a high lipophilicity may also associate with the membrane and could disturb the interaction between LPC and the membrane. The importance of high lipophilicity of β-adrenoceptor antagonists for protecting the membrane is supported by data from another experiment. It has been reported that lipophilic β-adrenoceptor antagonists protect cells from lipid peroxidation of sarcolemma mediated by oxygen radicals, resulting not from βAA but from their interaction with membrane lipids (Mak & Weglicki, 1988). Moreover it has been found that the increase in $[Ca^{2+}]_i$ induced by LPC can be inhibited by flunarizine (Ver Donck *et al.*, 1992), which possesses high lipophilicity (Michiels *et al.*, 1983). Our previous study (Hoque *et al.*, 1995) revealed that non-esterified fatty acids (NEFA) accumulate in the myocardium after addition of exogenous LPC in perfused rat hearts, and (+)-propranolol inhibited the accumulation of NEFA. This result indicates that LPC accelerates catabolism of membrane phospholipids, and (+)-propranolol inhibits it. From these data and the results in the present study, it is possible that high lipophilicity of (+)- and (−)-propranolol and (+)- and (−)-penbutolol inhibit the interaction between LPC and sarcolemma. In the present study, however, (−)-propranolol was more potent than (+)-propranolol in inhibiting the Ca^{2+}-overload and CPK release induced by LPC, although both isomers have an identical lipophilicity. Therefore, high lipophilicity alone cannot completely explain the protective effect of β-adrenoceptor antagonists on the LPC-induced Ca^{2+}-overload. Further studies are necessary to clarify the exact mechanism(s) of the protective effect of β-adrenoceptor antagonists against the LPC-induced Ca^{2+}-overload.

β-adrenoceptor antagonists provide various beneficial effects on ischaemic and post-ischaemic reperfusion injury. However, recent studies (Lu *et al.*, 1990; Takeo *et al.*, 1990; Liu X. *et al.*, 1991; Kramer *et al.*, 1991; Fujioka *et al.*, 1991) have revealed that the beneficial effects on the ischaemic injury cannot be explained by their βAA alone. Our previous study (Hoque *et al.*, 1993) also indicated that (+)-propranolol, but not timolol, attenuated post-ischaemic reperfusion injury in perfused rat working hearts, as well as (±)-propranolol did. Thus, the data in the present study are consistent with those in the study of effects of β-adrenoceptor antagonists on ischaemic injury. Because LPC accumulates in the myocardium during ischaemia and reperfusion (Corr *et al.*, 1982; Kinnaird *et al.*, 1988), the β-adrenoceptor antagonist which possesses an additional action to inhibit the Ca^{2+}-overload induced by LPC may be more effective in protecting the myocardium from ischaemic injury.

In summary, the action of β-adrenoceptor antagonists to preserve membrane integrity, which is not associated with βAA but with other actions that remain to be clarified, may be important for the inhibitory effect of β-adrenoceptor antagonists on the Ca^{2+}-overload induced by LPC. Our results indicate the possibility that the inhibitory effect on the Ca^{2+}-overload induced by LPC is an additional mechanism of the anti-ischaemic effects of β-adrenoceptor antagonists.

The authors wish to express their thanks to Mr T. Yokoyama for his technical assistance, to Mrs K. Fukataki for her secretarial work and to all the other members of this Department for their help in carrying out this study.

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(Received August 8, 1995
Revised January 9, 1996
Accepted February 21, 1996)