Enhanced responsiveness of rat cardiac myocytes to muscarinic cholinergic stimulation during chemically-induced hypoxia

Michał Gajewski¹, Hanna Laskowska-Bożek², Joseph A. Moutiris³, Sławomir Maśliński¹,³ and Jan Ryzewski²

¹Department of Biochemistry, Institute of Rheumatology, Warsaw, Poland; ²Department of Pathophysiology, Institute of Rheumatology, Warsaw, Poland and ³Department of Pathophysiology, Medical Academy, Warsaw, Poland

Abstract. In contrast to adrenaline, exogenously administrated cholinergic agonist, carbachol have very little effect on the contractility of rat cardiac myocytes, unless its contractile has been increased by adrenergic agonist. This interaction between the muscarinic and adrenergic pathways has been suggested to be the major means by which muscarinic agonist alters adrenergic function. When the cardiac myocytes were incubated in the medium contained the mitochondrial respiratory inhibitor potassium cyanide (chemically-induced hypoxia) the spontaneous contractility was ceased. The contractility partly recovered when the cells were exposed to adrenergic stimulation. We showed that during chemical hypoxia, in which cellular ATP is decreased (37% of control), the responsiveness of myocytes to muscarinic cholinergic stimulation significantly increase. Contraction of myocytes, stimulated by adrenaline was totally inhibited by 10⁻⁴ M of carbachol in control cells and 5x10⁻⁶ M of carbachol in cells with chemically-induced hypoxia. This increase in physiological response to muscarinic stimulation was associated with an increase of muscarinic receptors (630%). The results support the hypothesis that in ischaemic/hypoxic myocardium the role of cholinergic system may be more important than previously assumed.

Key words: myocytes, hypoxia, muscarinic cholinergic receptors, ATP
INTRODUCTION

In recent years the dynamic nature of the interaction of hormone and neurotransmitter receptors with the cell membrane has become increasingly apparent (Haigh et al. 1988). The role of parasympathetic neurotransmitter, acetylcholine (Ach) as an important regulator of myocardial contractility and heart rate has been established by both physiological and histological studies (Loffelholz and Pappano 1985). Ach produces a negative inotropic effect as well as a negative chronotropic effect in the intact animal, isolated heart, and muscle preparations (Blumenthal et al. 1968). The cardiac effect of Ach is mediated by its binding to muscarinic cholinergic receptors (M2 receptor) which are coupled to Na⁺ and K⁺ channels that modulate myocardial contractility. These muscarinic cholinergic receptor (MChR)-mediated responses are thought to occur after initial interaction of the receptor with one of the family of G proteins (Maxwell et al. 1991).

The heart is dually innervated by sympathetic (adrenergic) and parasympathetic (cholinergic) nerves. Stimulation of muscarinic cholinergic receptors is well known to exert a "physiological" or "functional" antagonism of β-adrenoceptor mediated effects (Christiansen et al. 1987). Moreover, acetylcholine released by the vagal nerves inhibits and norepinephrine (NE) liberated by the sympathetic nerves facilitates the function of the myocardium (Muscholl 1980). It seems that in the heart complex adrenergic-cholinergic interactions occur both prejunctionally, at the level of the autonomic nerve terminals, and postjunctionally. The inhibitory effect of Ach on cardiac tissue are therefore achieved by a direct influence of the cholinergic neurotransmitter on the cardiac muscle cells, and in part by an indirect influence on sympathetic neurotransmission (Vanhoutte and Levy 1980).

It is generally recognized that myocardial ischaemia increases β-adrenergic receptor density at the cell surface (Strasser et al. 1990). Moreover, myocardial ischaemia induces the presynaptic release of large quantities of endogenous catecholamines, mainly norepinephrine, which activate β- and α-adrenergic receptors (Schomig et al. 1984). It was shown that endogenous catecholamines in these concentrations are sufficiently high to induce receptor desensitization of β-adrenergic receptors in normal heart (Strasser et al. 1990). In acute myocardial ischaemia, however, agonist-promoted internalization receptors to the inside and functional uncoupling of β-adrenergic receptors is abolished. Moreover, during myocardial ischaemia, β-adrenergic receptors from intracellular pool of receptors are redistributed from the intracellular vesicles to the sarcolemma (Maisel et al. 1985).

Consequently, the balance of receptor internalization-externalization of myocytes is shifted toward an increase in functionally coupled receptors on the cell surface, and may contribute to the inadequate sensitivity of the infarcted heart to catecholamines. As a result, arrhythmias and the spreading of the infarcted zone in acute ischemia may be influenced by the sympathetic system (Strasser et al. 1990).

The effect of ischaemia or hypoxia on muscarinic cholinergic receptors on cardiac cells has been rarely studied. The parasympathetic activity has been found unchanged in ischaemic heart (Mukherjee et al. 1979) or decreased during hypoxia in dogs (Hammil et al. 1979, Vatner et al. 1988). On the other hand, according to Pantridge (1978), more than one-third of patients with acute myocardial infarction show evidence of sympathetic hyperactivity and almost half of the patients have parasympathetic hyperactivity. Moreover, Bergamashi (1978) noted that stimulation of the sympathetic nerves alone was seldom followed by ventricular arrhythmias, whereas concomitant stimulation of both sympathetic and parasympathetic cardiac nerves always produced cardiac arrhythmias during acute myocardial ischaemia.

The purpose of this study was to examine in detail the effect of hypoxia on the expression of muscarinic cholinergic receptors and the responsiveness of intact, cultured, neonatal rat cardiac myocytes to parasympathetic stimulation under hypoxic conditions.
METHODS

Chemicals

Atropine sulfate was obtained from Calbiochem (Los Angeles, CA), [3H]quinuclidinyl benzilate (QNB) (12Ci/mmol=444 GBq/mmol) and Nuclear Chicago Solubilizer (NCS) were purchased from Amersham International (Bucks, U.K.). Minimal essential medium (MEM) and phosphate-buffered saline (PBS) were obtained from Biomed, Poland; foetal calf serum (FCS) from Flow Laboratories, Rockville, USA: and ATP Monitoring Reagent from LKB-Wallac, Turku, Finland. All other reagents were purchased from Sigma Corp., St. Louis, USA.

Heart cell cultures

Cardiac myocytes were isolated from 1 to 3 days-old Wistar rats according to methods of Harrry and Farley (1963) and Halle and Wollenberg (1968) by 0.1% trypsin digestion of the heart tissue; details of the procedure were described earlier (Grąbczewska et al. 1983, Gajewski et al. 1988). The cell suspension was plated for up to 3 h (different plating period), and the unattached cells (myocytes enriched) were removed and replaced to culture dishes (Buja et al. 1985). Cells were resuspended in MEM enriched with l-glutamine (1mM) and 10% inactivated FCS (culture medium) and cultured at 37°C, 5% CO2 and 100% humidity for 72 h. The cells were then scraped from the surface of the dishes with a rubber policeman and counted.

Metabolic inhibition and recovery

Experiments on 3-day-old cultures were initiated by replacing the growth medium with medium supplemented with the potassium cyanide, a convenient inducer of hypoxia in biological system, due to its potent inhibition of cytochrome oxidase, the predominant O2-consuming enzyme, required for mitochondrial ATP production. Control cultures were maintained in growth medium with out metabolic inhibitor. In other experiments, the medium containing metabolic inhibitor was replaced with fresh medium without metabolic inhibitor, and the cultures were maintained for another 24 h. Experiments with control and treated cultures were performed concurrently. The doses of metabolic inhibitor were chosen on the basis of studies of Buja et al. (1985).

Measurements of changes in beating rate

Measurements of changes in physiological response were performed according to Haigh et al. (1988). Cells grown on Falcon dishes were placed in a chamber and continuously perfused via inlet and outlet ports. The chamber was placed on inverted microscope, at 37°C. Beating was determined by monitoring the movement of the border of a single cardiac cell with a video motion detector.

ATP measurement

Intracellular ATP level was determined according to the firefly luciferase luminometric method (Noronha-Dutra and Steen 1982). Briefly, the cells were incubated at 37°C, 100% humidity and 5% CO2. An aliquot of Triton X-100 was added (final concentration - 2%) to disintegrate the cell membrane, inactivate ATP-ases and release the intracellular ATP. 20 μl samples were taken at zero time and a different intervals thereafter and transferred to the cuvettes containing 800 μl of Tris-EDTA buffer. After 60 second incubation at room temperature 200 μl of the luciferin-luciferase enzyme (LKB 1243-200 ATP Monitoring Reagent) was added and the ATP level measured.

Muscarinic cholinergic receptors binding assay

Binding assay was performed using [3H]quinuclidinyl benzilate (QNB) (specific activity 12 Ci/mmol). A cell suspension (3x10^5 cells in 0.2 ml MEM solution) was equilibrated in an atmosphere of 5% carbon dioxide, 100% humidity at 37°C for
30 min. It was then treated for 10 min with 20 µl of 10^{-4}M atropine sulfate (nonspecific binding) or phosphate buffered saline (PBS) (total binding) followed by 20 µl [^{3}H]QNB (0.8 µM; 2 µCi). The reaction was continued for 10 min: enough for saturation of binding sites (Grąbczewska et al. 1983). The reaction was stopped by addition of 2 ml of cold MEM solution and immediate centrifugation (2500 x g, 1 min). The radioactivity of pellets was measured after solubilization with 1 ml of Nuclear Chicago Solubilizer (18 h, 37°C). The specific binding was obtained as the difference of the radioactivity of the total and nonspecific binding of [^{3}H]QNB to 3x10^{5} myocytes and was regarded as the expression of muscarinic cholinergic receptors.

Biochemical assays

Activity of superoxide dismutase (SOD) was measured by the method of Misra and Fridovich (1972), and activity of catalase (CAT) by the spectrophotometric method of Beers and Sizer (1952). Activity of glutathione peroxidase (GPx) was measured by the coupled enzyme assay of Paglia and Valentine (1967) using hydrogen peroxide as a substrate. Protein concentration was determined by the method of Lowry et al. (1951).

Statistical analysis

Data from experimental groups and controls were compared with a two-tailed Student’s test. For two group comparisons, differences were considered significant when \( P<0.05 \). Data were expressed as the mean ±SE.

RESULTS

Characterization of muscarinic cholinergic receptors binding

An experiment in which [^{3}H]quinuclidinyl benzilate (QNB) binding was measured after 20 minutes of
Muscarinic responsiveness of hypoxic myocytes

with the concavity upwards means that the binding of [3H]QNB does not occur at a single class of independent binding sites. In binding studies performed with myocytes treated with potassium cyanide (KCN) specific binding reached equilibrium within 20 minutes. Thus, time to equilibrium was similar for preparation from control and treated myocytes.

Sensitivity of beating to cholinergic agonist in rat cardiac myocytes cultured in medium supplemented with adrenaline

The myocytes isolated from newborn rat hearts were small, round, and typical of neonatal heart cells. Transferred to the plastic cluster dishes, they quickly attached to the bottom of the wells and formed monolayer. Light microscopy of a 48-h culture revealed a network of adjoining cells which had lost their independence and were beating at a rate of 150±20 beats/min (SE, n=20, data are the mean of 2 determinations of 10 separate cultures).

Figure 2 shows the initial results of the experiments in which cultured myocytes received only cholinergic stimulation for 1 h. The degree of modulation of myocyte contractility and the intracellular ATP level were estimated, under the influence of cholinergic stimulation. It was shown, that after 1 h of cholinergic stimulation, intracellular ATP levels remained unchanged, unrelated to
the concentration of the cholinergic agonist. The present experiments indicate that the exposure of myocytes for 1 h to different concentration of the cholinergic agonist, carbachol (10^{-8} M-10^{-4} M), does not cause significant changes of their spontaneous contractility. A reduction of about 30% was observed only with very high concentration of carbachol; such high concentration may have cytotoxic effects.

The influence of the adrenergic stimulation on energy levels as well as on the spontaneous contractility of the myocytes, is shown in Fig. 3. Cultured myocytes, kept for a period of 1 h in 10^{-6} M adrenaline, did not show significant decrease in ATP levels, parallel to a small, insignificant increase in spontaneous contractility (Fig. 3A). The lack in reduction of ATP levels, and of increased degree of contractility, was also observed during exposure of myocytes to lower concentrations of adrenaline (i.e. 10^{-8} M - 10^{-7} M) (data not shown).

Higher concentration of adrenaline (10^{-5} M) had a significant influence on myocytes (Fig. 3B). The frequency of contractions was significantly increased (about 300 beats/min), upon exposure to the adrenergic agonist. The energy levels of the same myocytes were also reduced about 10 min after exposure to adrenaline. The reduction in ATP levels and the increased frequency of contractions of the myocytes, reached peak values in a concentration of 10^{-4} M adrenaline (Fig. 3C). The addition of propranolol (10^{-4} M), to the myocytes exposed to adrenaline, caused inhibited contractility, compared with that in the absence of propranolol (data not shown).

The results of concomitant stimulation of both adrenergic and cholinergic systems, are shown on

![Graph showing contractility (%)](image)

**TABLE I**

<table>
<thead>
<tr>
<th>ATP level (nmol/mg protein) in myocytes treated with adrenaline in the absence (A) and presence of carbachol (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
</tr>
<tr>
<td>After 1 h stimulation with adrenaline</td>
</tr>
<tr>
<td>10^{-4} M</td>
</tr>
<tr>
<td>10^{-5} M</td>
</tr>
<tr>
<td>10^{-6} M</td>
</tr>
<tr>
<td>Control</td>
</tr>
</tbody>
</table>

All values represent means ±SE, n=10; a, b - significant difference (P<0.001) between a and b
Table I. The myocytes submitted exclusively to adrenergic stimulation for 1 h, showed a significant decrease in ATP levels, which was dose-dependent (Table I A).

Significant changes in ATP levels, were observed, with concentration of $10^{-6}$M adrenaline, and concentration of $10^{-5}$M caused a 50% reduction in ATP level of the myocytes. The highest concentration of adrenaline used, $10^{-4}$M decreased by 75% the initial energy levels.

The results of the experiments in which myocytes were incubated, for 1 h, concomitantly with adrenaline and carbachol, are shown on Table IB. The reduction in ATP levels, caused by adrenaline in concentration of $10^{-4}$M, was not changed in any way, by carbachol, in concentrations of $10^{-7}$M to $10^{-4}$M.

The 50% reduction in ATP level of the myocytes exposed to adrenaline $10^{-5}$M (Table I A), was inhibited by the concomitant cholinergic stimulation by carbachol $10^{-5}$M to $10^{-4}$M.

The effect of carbachol ($10^{-7}$M - $10^{-4}$M) on the contractility of the myocytes, following adrenergic stimulation ($10^{-5}$M), are shown in Fig. 4. The increase in contractility induced by $10^{-5}$M adrenaline, was inhibited to 80% by carbachol ($10^{-6}$M). Concentrations of $10^{-5}$M of carbachol, caused a strong inhibition in contractility and almost complete inhibition was observed with concentration of $10^{-4}$M (Fig. 4).

In our experiments, we have tried to create the same conditions as in vivo, in which the heart, receiving concomitantly adrenergic and cholinergic stimulation, keeps permanent levels of energy and of contractility. Thus, the next part of the experiments has been carried out in cultured myocytes stimulated with $10^{-5}$M adrenaline, and exposed to different concentration of carbachol.

**ATP and antioxidant enzymes activity depletion and recovery after chemically-induced hypoxia**

After incubation of cultured myocytes with $7.5 \times 10^{-4}$M KCN for 4 h, there was a 63±2% decrease of ATP level (Fig. 5A). The ATP level diminished very rapidly within 15 min, decrease of the

![ATP level in cultured myocytes incubated with potassium cyanide (KCN) ("chemically-induced hypoxia"): solid line control; n=19; dashed line $7.5 \times 10^{-5}$M KCN, n=12; dashed dotted line $7.5 \times 10^{-4}$M KCN, n=16. Error bars represent ±S.E; B, Changes (in % of control) of the activities antioxidant enzymes: solid line, superoxide dismutase (SOD); dashed line, catalase (CAT), dashed dotted line, glutathione peroxidase (GPx). Values are means ±S.E: n is 4-6 determinants for each point.](image-url)
TABLE I

Expression of muscarinic cholinergic receptors on rat neonatal cardiac myocytes after incubation with KCN

<table>
<thead>
<tr>
<th>Time of incubation with KCN(h)</th>
<th>Binding of $[^3]$HJQNB (cpm/3x10$^5$ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0.25 (n=10)</td>
<td>1845±192</td>
</tr>
<tr>
<td>0.5 (n=10)</td>
<td>1477±171</td>
</tr>
<tr>
<td>1.0 (n=10)</td>
<td>1877±176</td>
</tr>
<tr>
<td>2.0 (n=10)</td>
<td>1399±182</td>
</tr>
<tr>
<td>3.0 (n=10)</td>
<td>1384±167</td>
</tr>
<tr>
<td>4.0 (n=12)</td>
<td>1221±159</td>
</tr>
</tbody>
</table>

Number of experiments in parentheses. All values are means ±SE; $^*$P<0.001 vs. control; $^{**}$P<0.001 vs. control; a, b - significant difference (P<0.0001) between a and b

activities antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) was also observed (Fig. 5B). Treatment of cardiac myocytes with 7.5x10$^{-5}$M was associated with a moderate reduction in ATP content, and a moderate decrease of the activities of antioxidant enzymes (data not shown).

In another series of experiments, medium containing KCN was removed, and the cells with chemically-induced hypoxia were maintained in the medium without metabolic inhibitor for an additional 24 h (Table III).

After the recovery period, the cultures exhibited no differences in ATP content, compared to control level. Moreover, the partial recovery in activities of antioxidant enzymes was also observed (data not shown). These data indicate that changes in antioxidant activities and ATP level are temporary and that the cells retain the ability for the full recovery when KCN is removed.

Changes in muscarinic cholinergic receptor expression during chemically-induced hypoxia

The degree of the expression of the cholinergic muscarinic receptors on the membrane of the cultured myocytes, that were in a reversible form of injury, was estimated by radioactivity ligand binding studies using $[^3]$HJQNB.

It was evidenced (Table II) that the muscarinic receptor expression on the surface of the myocytes,

TABLE III

Reversibility of changes in expression of muscarinic receptors and ATP level in myocytes incubated with KCN

<table>
<thead>
<tr>
<th>Groups</th>
<th>ATP (mmol/mg protein)</th>
<th>Binding of $[^3]$HJQNB (cpm/3x10$^5$ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - 4 h without KCN</td>
<td>23.0±1.5 (10)</td>
<td>1345±195</td>
</tr>
<tr>
<td>4 h with 7.5x10$^{-5}$M KCN</td>
<td>17.3±2** (8)</td>
<td>3380±340**</td>
</tr>
<tr>
<td>4 h with 7.5x10$^{-4}$ KCN</td>
<td>8.5±2.5* (10)</td>
<td>7300±322*</td>
</tr>
<tr>
<td>Control 4 h without KCN +24 h without KCN</td>
<td>24.0±3 (6)</td>
<td>1825±95</td>
</tr>
<tr>
<td>4h with 7.5x10$^{-5}$ KCN +24 h without KCN</td>
<td>22.0±3 (8)</td>
<td>1350±123</td>
</tr>
<tr>
<td>4h with 7.5x10$^{-4}$M KCN +24 h without KCN</td>
<td>25.5±3 (8)</td>
<td>1745±134</td>
</tr>
</tbody>
</table>

Number of experiments in parentheses. All values are means ±SE; $^*$P<0.001 vs. control; $^{**}$P<0.0001 vs. control
was significantly increased after 15 min exposure to KCN, not depending on its concentration (7.5x10^{-5}M - 7.5x10^{-4}M).

The higher concentrations of KCN (7.5x10^{-4}M) caused a reduction in the energy level of the same myocytes by about 63%. After a 4 h exposure to 7.5x10^{-4}M KCN, an increase of up to 630% of the cholinergic muscarinic receptors was observed (Table II).

In both cases, the increase was proved to be reversible. In optimal conditions, and 24 h later the energy levels of cells and the level of the muscarinic cholinergic receptors expression on their surface, were recovered (Table III).

**Increased sensitivity of beating to cholinergic agonist in cardiac myocytes during chemically-induced hypoxia**

Figure 6 shows the susceptibility of the myocytes undergoing chemically-induced hypoxia, to modulation of their contractility by cholinergic stimulation. The synchronous, spontaneous contraction of the myocytes cultured in optimal conditions, enhanced by adrenergic stimulation (adrenaline 10^{-5}M), was almost completely diminished by carbachol in concentration 10^{-4}M (Fig. 4).

Hypoxia (incubation with KCN) caused cessation, just after 2-3 min, in spontaneous contractility. When the myocytes, treated with KCN for 1 h, were transferred to optimal conditions, the spontaneous contractions partly reappeared (data not shown).

Myocytes, in chemically-induced hypoxia (inhibition of spontaneous contractions) after administration of adrenaline (10^{-5}M) restore their ability to contract rhythmically (100±24 contractions/min vs. 150±20 contractions/min in optimal conditions).

**Fig. 6. Effect of carbachol to inhibit adrenaline (10^{-5}M) stimulation of contractility of myocytes during "chemically-induced hypoxia".** Solid line 5 min of chemical hypoxia with adrenaline; dashed line 1 h of chemical hypoxia with adrenaline; dashed dotted line 4 h of chemical hypoxia with adrenaline. Each point represents the mean of 7-10 determinations in 5-6 separate cultures (±SE).

**Fig. 7. A, Effect of carbachol on ATP level in "hypoxic" myocytes treated with adrenaline (10^{-5}M).** After 1 h of "chemically-induced hypoxia" with concomitant adrenergic stimulation (adr) the ATP level was significantly decreased (*** P<0.0001 vs control). B, ATP level in "hypoxic" myocytes after 1-3 h of concomitant stimulation of both adrenaline (10^{-5}M) and carbachol; 10^{-6}M (solid bars); 4x10^{-6}M (hatched bars); 10^{-5}M (open bars). Data are means values ±SE: number of experiments in parantheses. Asterisk indicates P<0.005 vs adrenergic stimulated "hypoxic" controls (in the absence of carbachol).
Figure 6 indicates that the contractility of the myocytes kept for 5 min in contact with KCN and adrenaline, can be inhibited only with very high concentrations of carbachol i.e. $10^{-4}$ M.

The contractility of the myocytes, incubated for 1 h with $7.5 \times 10^{-4}$ M KCN and stimulated by adrenaline $10^{-5}$ M (Fig. 6) and exposed to carbachol, was completely inhibited by $2 \times 10^{-5}$ M of carbachol. It was in contrast to nonhypoxic myocytes where higher ($10^{-4}$ M) concentration of carbachol was needed to exert the same inhibitory antiadrenergic effect. Lower doses of carbachol ($5 \times 10^{-6}$ M) are sufficient to inhibit adrenergic stimulation when hypoxia lasts 4 h (Fig. 6).

The ATP level was reduced, in the myocytes incubated with $7.5 \times 10^{-4}$ M KCN. Adrenergic stimulation (adrenaline $10^{-6}$ M) induced a further reduction in ATP level. As previously the effect caused by the addition of carbachol was depending on how long the myocytes were exposed to the chemically-induced hypoxia (Fig. 7). The longer time of hypoxia, the lower concentrations of carbachol are effective as antiadrenergic agents.

**DISCUSSION**

In the study of Mukherjee et al. (1979) experimental myocardial ischaemia did not result in a significant change in the number of $[^3H]$QNB binding sites. These data suggested that occlusion in dogs for 1 h of the proximal left anterior descending coronary artery significantly increased the number of $\beta$-adrenergic receptors of the ischaemic ventricle (LV), without changing the number of muscarinic cholinergic receptors in crude membrane isolated from the cardiac tissue. Preliminary experiments of Vatner et al. (1985) using a crude membrane preparation in dogs suggested that a decrease in muscarinic cholinergic receptors occured in LV failure. The current investigation (Vatner et al. 1988), using the enriched sarcolemmal preparation, confirms the previous findings of decreased muscarinic receptor density in heart failure, as measured by $[^3H]$QNB binding studies.

On the other hand, it was suggested that conclusions about the functionally status of the receptors in various crude or partially purified membrane fractions isolated from ischaemic hearts are difficult to relate to the properties of the receptors of the intact cells or tissues (Marsh and Sweeney 1989).

Ischaemia in vivo is a complex process that is difficult to characterize or control precisely. Accordingly, we tried to approach the problem of muscarinic cholinergic receptors in ischaemia by attempting to simplify and carefully define the experimental design and by studying the pharmacological functions of the muscarinic receptors in intact cells rather than in a membrane preparation.

It is commonly accepted that ischaemia is characterized by inhibition of mitochondrial respiration and by depletion of high energy phosphates (ATP) (Reimer and Jennings 1986). Thus, in our model of metabolic inhibition ("chemically-induced hypoxia") the myocytes were exposed to potassium cyanide (KCN), a convenient inducer of hypoxia in biological systems.

Buja et al. (1985) reported that 4 h incubation of intact cultured neonatal rat heart cells with $7.5 \times 10^{-4}$ M KCN and $4.5 \times 10^{-2}$ M 2-deoxyglucose (DOG: non-metabolized glucose derivative) led to a 43% increase in $\beta$-adrenergic receptors and a 41% decrease in ATP level, accompanied by minimal morphological changes in myocytes. It was also shown that the increased $\beta$-adrenergic receptor density in moderately injured myocytes was potentially reversible, if the injurious stimulus was removed and ATP level returned to normal.

It is commonly accepted that the ischaemic myocardium produces some amounts of high-energy phosphates by the anaerobic glycolysis-lactate pathway, whereby the end product of anaerobic glycolysis accumulates and tissue acidosis increases rapidly (Reimer and Jennings 1986). Thus, in our experiments we used only KCN (cytochrome oxidase inhibitor) in concentrations which were chosen on the basis of the study of Buja et al. (1985), without blocking anaerobic glycolysis. The cardiac myocytes after 4 h of this chemically-induced hy-
Muscarinic responsiveness of hypoxic myocytes

Hypoxia showed a 63%±3 decrease of ATP content, whereas the expression of the muscarinic cholinergic receptors was about 630% higher than that of the control cells, as measured by [3H]QNB binding studies, both effects being reversible.

In the study performed by Strasser and Marquetant (1990) the density of muscarinic cholinergic receptors in the plasma membrane of isolated perfused rat hearts was determined by saturation experiments using another muscarinic antagonist N-methylscopolamine. In these experiments the muscarinic receptor expression increased in the plasma membrane following an ischaemic insult. The time-course of this increase was quite similar to that of β-adrenergic receptors. Again, reperfusion led to a rapid return to control values.

The data described by Corr et al. [1982] indicate that it may not be a β-adrenergic receptor specific effect that leads to an increase of the receptors at the cell surface. More likely is a "non-specific" membrane linked alteration, which may include several receptor system independent of their activation. In these experiments, however, an increased responsiveness in the early phase of global ischemia could be demonstrated only for the β-adrenergic system. Similarly, despite the reversible increase of muscarinic receptor expression, the inhibitory pathway of the adenylyl cyclase systems becomes ineffective since the coupling protein, Gi, is rapidly impaired, and increased responsiveness to muscarinic stimulation in the early phase of ischemia was not confirmed (Strasser and Marquetant 1990).

On the contrary Haigh et al. (1988) documented the increased susceptibility of the slightly damaged myocytes to muscarinic stimulation. It was documented that under conditions in which cellular cholesterol is increased, the responsiveness of cultured chick atrial cells to muscarinic stimulation is significantly increased and the sensitivity of rate beating to the muscarinic agonist carbamylcholine (carbachol) increased 10-fold compared to control cells. This enhancement in physiological response was associated with an increase of total number of muscarinic receptors (2-fold), as measured by [3H]QNB binding studies.

The purpose of our study was to examine in detail the effects of chemically-induced hypoxia on the responsiveness of intact cultured cardiac myocytes to parasympathetic stimulation.

Although atrial myocardium receives the greatest amount of cholinergic innervation, there is considerable evidence that ventricular myocardium receives also a functionally significant parasympathetic innervation (Loffeholz and Pappano 1985).

However, there are regional variations in the heart's response to cholinergic stimulation. In contrast to atrial myocardium, exogenously administered acetylcholine has minimal or no effect on the contractility of mammalian ventricular myocardium (Chen and Wince 1988).

In these preliminary experiments we have shown that carbachol by itself caused no significant effect on the contractility and ATP level in myocytes isolated from whole hearts, unless its very high (10^{-5}M) concentrations were used. Similar results were obtained by Haigh et al. (1988) which showed that beating cells isolated from chick atria were totally inhibited at 10^{-5}M carbachol, whereas beating in cultures of ventricular cells decreased only by 10% at concentrations of carbachol as high as 10^{-4}M.

In vivo, heart is exposed to both sympathetic and parasympathetic activity. A simple model in which intact cells were exposed to adrenaline and carbachol may represent an approach which could also be relevant for the in vivo situation to elucidate the sympathetic/parasympathetic coregulation in the heart.

In the first series of our experiments, the ability of a maximally effective concentration of carbachol to reduce the contractile responsiveness of cultured myocytes exposed to adrenaline was examined. We showed that only relatively high concentrations of carbachol reduced the contractility of the myocytes isolated from whole hearts and exposed to adrenaline.

These results indicate the increased functional importance of the carbachol-mediated reduction in contractility of myocytes during simultaneous adrenergic stimulation under normoxic conditions.

It is possible that modulation of adrenergic receptor stimulation in the heart by cholinergic agonists may be the major means by which muscarinic
agonists alter heart function. This possibility was first suggested by Watanabe et al. (1981).

After addition of respiratory inhibitor, cultured myocytes ceased spontaneous contraction. When the myocytes in "chemically-induced hypoxia" were exposed to adrenaline a partial restoration of synchronous contraction was observed. The present data demonstrate that under conditions in which the "hypoxic" myocytes were exposed to adrenaline, the responsiveness of cardiac cells to muscarinic cholinergic stimulation was significantly increased. This increase in physiological response is associated with the potent increase in the expression of muscarinic cholinergic receptors. The relationship of these effects to changes in energy metabolism was also observed. The mechanism responsible for the increased responsiveness to muscarinic agonist observed during "chemically-induced hypoxia" remain to be established.

ACKNOWLEDGMENTS

The authors is greatly indebted to Piotr Świdziński M.Sc.Eng., from institute of Aviation, Warsaw, Poland for his excellent technical assistance. This study is a part of the doctor thesis submitted by the M. Gajewski. It was partly supported by the C.P.B.R. 11.6 by Central Cardiology Programme. Portions of this work were presented at the XXVI Meeting of the Polish Biochemical Society, Gdańsk, September 1990 (Gajewski et al.1991), and First International Congress of the Polish Neuroscience Society, Warsaw, September 1992 (Gajewski et al.1992).

REFERENCES


Muscarinic responsiveness of hypoxic myocytes


Paper presented at the 1st International Congress of the Polish Neuroscience Society; Session: Neurotransmitters and their receptors.