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Calcium Movement in Carbachol-stimulated Cell-line

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ABSTRACT

It has been well known that the intracellular calcium concentration ($[Ca^{2+}]_i$) in living cell is very sensitive to live or to survive, but the transmembrane system of calcium ion, especially mechanism of calcium ion movement in unexcitable state has been little elucidated. Though many proposed theories for calcium ion transport have been reported, it is still unclear that how could the sustained maintenance in cytosolic calcium level be done in cell. Since one of possible mechanisms of calcium transport may be related to the acetylcholine receptor-linked calcium channel, author performed experiment to elucidate this mechanism of calcium influx related to cholinergic receptor in m1 muscarinic receptor-transfected RBL-2H3 cell-line.

1) The effects of carbachol both on calcium ion influx and on the secretion of hexosaminidase were respectively observed in the manner of time-related or concentration-dependent pattern in this model.

2) The effects of several metal cations on calcium transport were shown in carbachol-induced cell-line.

3) Atropine was administered to examine the relationship between cholinergic receptor and calcium ion influx in this model.

4) PMA (Phorbol 12-myristate 13-acetate) or PTx (Pertussis toxin) was respectively administered to examine the secondary mediator which involved pathway of calcium ion movement in carbachol-induced cell-line.

The results of this experiments were as follows;

1) Carbachol significantly stimulated both the calcium influx and the secretion of hexosaminidase in the manner of the concentration-dependent pattern.

2) Atropine potently blocked the effects of carbachol in concentration-response manner.

3) Administered metal cations inhibited the calcium influx in carbachol-stimulated this model to the concentration-related pattern.

4) PMA did not inhibit carbachol-induced secretion of hexosaminidase, but blocked the calcium influx in this cell-line.

5) The suppression of carbachol-induced hexosaminidase secretion was shown in PTx-treated cell-line.

Key Words: Carbachol (carbamylcholine: CBC), Ca^{2+} influx, Calcium channel, Metal cations, Hexosaminidase, m1 Muscarinic receptor-transfected RBL-2H3 cell, Atropine, PMA, PTx

INTRODUCTION

Generally, in excitable cells, the phenomenon that the mechanism of increased calcium ion influx and followed elevation of cytosolic calcium level being appeared is primarily undergoing via voltage-dependent calcium channel co-committed with sodium ion influx, but the

mechanism of calcium ion movement in unexcitable cell is still unclear. It has been already reported that the unidendified signalling generated from cell inside for the calcium ion influx and rapid changes of the cytosolic Ca^{2+} level being reponsible for the opening of Ca^{2+} channel on plasma membrane (Putney, 1990). Many papers were published on calcium ion movement; that be released with histamines in anaphylaxis by alkaline metal ions (Foreman and Mongar, 1972), that induced to depleting of intracellular calcium store activated calcium current in mast cells (Hoth and Penner, 1992), and that be done via receptor-operated calcium channel shown in rat hepatocytes (Kass *et al.*, 1990). After the Ca^{2+} influx stimulated by cholinergic agonist, carbachol, the intracellular Ca^{2+} stores was increased in acetylcholine receptor-stimulated cell (Felder *et al.*, 1991 & 1992). The Ca^{2+} influx drives cholinergic agonist-stimulated intacellular calcium level to increase calcium ion stores for mobilization and for release from storage organelles. (Shuttleworth, 1994).

Since the mechanism of increasing intracellular calcium level through this route is still undefined in unexcitable living state, the study for the pathway involved to the intracellular signalling via this receptor is one of the interesting current subjects to be investigated in communication between outside part and inside part through membrane for cell. As one of proposed mechanisms of calcium influx may be ralated the acetylcholine-receptor stimulation, author intended to show the effect of cholinergic agent on calcium transport in m1 muscarinic receptor-transfected RBL-2H3 cell-line.

The aim of this study in this model was to determine whether or not this cell-line would be responded to cholinergic receptor-related agents which were supposed to affect the muscarinic receptor coupled with calcium channel. In search for the relationship of calcium transport with the acetylcholine receptor, carbachol was selected as stimulant for cholinergic receptor in unexcitable this model. Therefore, the anthor designed the experiment to examine the effects of carbachol both on Ca^{2+} influx and on secretion of hexosaminidase in m1 muscarinic receptor-transfected RBL-2H3 cell. Atropine or several metal cations were treated to elucidate the mechanism of Ca^{2+} influx through cholinergic receptor in this cell-line. And, to observe the secondary mediator involved calcium movement via calcium channel-linked cholinergic receptor, PMA or PTx was seperately treated to carbachol-induced this cell-line.

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources; radiolabeled compounds from DuPont/NEN, Boston; carbachol from Aldrich, Milwaukee; atropine from Fisher Scientific, New York; Several metal cations salts from Sigma, St Louis; PMA (Phorbol 12-myristate 13-acetate) from LC Services Corporation, Woburn; PTx (Pertussis toxin) from List Biologicals, Campbell.

Cell culture

Muscarinic m1 receptor-transfected RBL-2H3 cells were used in this experiment (Jones *et al.*, 1991). The cells were maintained in culture and plated in 24-well plates in growth media as described previously (Yamada *et al.*, 1992; Maeyama *et al.*, 1986). Cultures in the multiwell plates contained radiolabled compound as required (Hide & Beaven, 1991). Experiments were performed in a glucose-saline, Pipes-buffered medium that contained 110 mM NaCl, 5 mM KCL, 5.6 mM glucose, 0.4 mM $MgCl_2$, 0.1% BSA, 25 mM Pipes-NaOH (pH 7.2), and 1 mM Ca^{2+} (Ca^{2+}-containing medium) or 0.1 mM EGTA instead of Ca^{2+}(Ca^{2+}-free medium).

Uptake of $^{45}Ca^{2+}$

Cultures in 24-well plates were incubated overnight at 37℃ in growth medium. The cultures were washed and replaced with 0.2 ml of the Ca^{2+}-containing buffer. After a 10 min incubation (37℃), the buffer was replaced with containing $^{45}Ca^{2+}$ (5 μCi/ml), the different concentration of administered agents, several metal ions or antagonist for examinig the inhibitory responses. At the indicated times, the reaction was stopped by washing the cultures with ice-cold Ca^{2+}-free buffer that contained 100 μM La^{3+}

and cells were lysed with 0.5 ml deionized water for the assay of intracellular $^{45}Ca^{2+}$. The amount of $^{45}Ca^{2+}$ uptaken per culture was calculated from the specific activity of $^{45}Ca^{2+}$ in the medium. In experiments, values were expressed as a percentage of maximal uptake (% of control) in the absence of inhibitor (control).

Measurement of hexosaminidase (HA)

Aliquots (10 μl) of medium and cell lysate (in 1 ml 0.1% Triton X-100) were incubated with 10 μl of 1mM para-nitrophenyl-N-acetyl-beta-D-glucosaminide in 0.1 M sodium citrate buffer (pH4.5) at 37°C for 1 h. At the end of the incubation, 250 μl of 0.1 M Na_2CO_3 /0.1 M $NaHCO_3$ buffer was added. Absorbance was read at 400 nm. Values (mean±SE) were expressed as the actual release (% of total HA) after correction for spontaneous release (2 to 3%) or percentages of maximal responses.

Data analysis

At least more than three cultures were used for each data point in one experiment. The response to stimulant in the presence of inhibitor was expressed as a percentage of the response to the stimulant in the absence of inhibitor (% of control). Experiments were repeated several times and mean values (±SE) for more than 3 experiments were shown. Values for all responses in the absence (control) or presence of inhibitor were presented in the Figures.

RESULTS

Effect of Carbachol on $^{45}Ca^{2+}$ uptake

Stimulation of m1 muscarinic receptor-transfected RBL-2H3 cells with carbachol caused the progressive increases of accumulation of $^{45}Ca^{2+}$ in the cell to the incubation time-dependent manner. The extent of calcium uptake was maximal increased at the 5 min incubation (Fig. 1A).

Incubation-time was not seemed to be important in limiltation of 5 min, author chosed two incubation time, 2 min and 3 min for observing

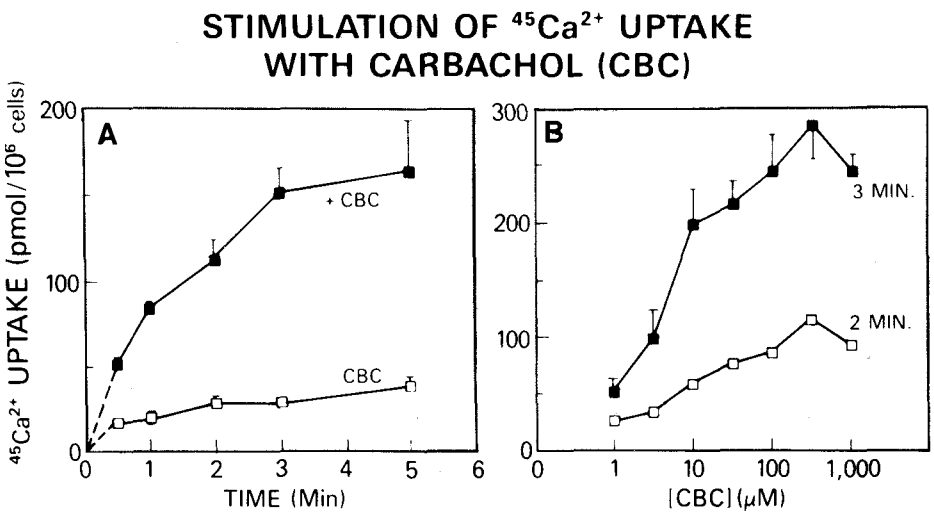

Fig. 1. Stimulation of $^{45}Ca^{2+}$ up'take with carbachol.

A) Time-dependent curve Cells were incubated with $^{45}Ca^{2+}$ (in 1 mM Ca^{2+}) for the time points in the absence (open symbols) and 1 mM carbachol (closed symbol).

B) Concentration-dependent curve to incubation time Cells were incubated with carbachol indicated concentration both at 2 min (opensymbols) and at 3 min(closed symbols). All Values were mean±SE from more than seperated experiment(3 cultures per experiment).

concentration-dependent effect of carbachol. Increase of concentration of administered carbachol (from 1 μM to 1 mM) stimulated the $^{45}Ca^{2+}$ uptake, but the maximal increase of uptake was shown with 500 μM (Fig. 1B).

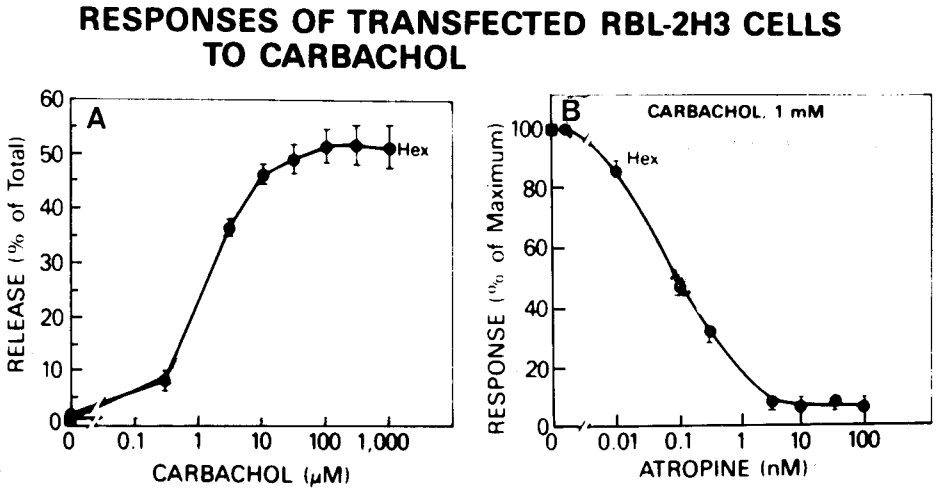

Fig. 2. Inhibition of atropine on secretion of hexosaminidase with carbachol (1mM)
A) Dose-response effect of carbachol on hexosaminidase secretion Cells were inculated with the indicated concentration of carbachol
B) Effct of atropine on carbachol-stimulated cell-lines The indicated concentrations of atropine were pretreated for 15 min before stimulating with carbachol. All values were mean±SE.

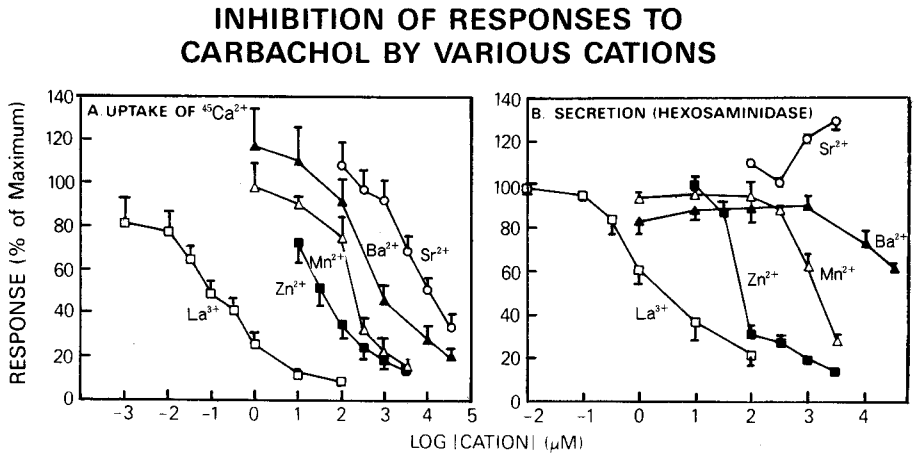

Fig. 3. Effects of several metal cations on $^{45}Ca^{2+}$ uptake (A) and hexosaminidase secretion (B) with carbachol-stimulated cell-lines Cell were incubated with $^{45}Ca^{2+}2$ (in 1 mM Ca^{2+}), and the indicated concentrations of metal ions and 1mM carbachol for 5 min for the mesurement of intracellular $^{45}Ca^{2+}$ or 15 min for the mesurement of secretion of hexosaminidase. All values were expressed as a percentage of response in the absence of metal ions and were the mean±SE from five seperate experiments with each three cultures per data points.

Inhibition of atropine on carbachol-induced cells

Cells were incubated with the indicated concentration of carbachol for release of hexosaminidase for 5 min. Carbachol treatment showed the increases of secretion of hexosaminidase correlated with $^{45}Ca^{2+}$ uptake concentration-dependent manners (Fig. 2A). When cells were preincubated for 15 min with atropine to the indicated concentrations before treatment with carbachol(1 mM), atropine significantly blocked this stimulating effects of carbachol (1 mM) in the concentration-dependent manners (Fig. 2B).

Effect of several metal cations on carbachol-stimulated cells

Cultures which were stimulated with 1 mM carbachol for 5 min for measurement of intracellular $^{45}Ca^{2+}$, or for 15 min for measurement of the secretion of hexosaminidase were incubated with $^{45}Ca^{2+}$ (in 1mM Ca^{2+}) and with the indicated concentrations of metal ions.

1) Uptake of $^{45}Ca^{2+}$ influx: Several metal cations (La^{3+}, Zn^{2+}, Mn^{2+}, Ba^{2+}, and Sr^{2+}) were treated to the carbachol-induced cell, respectively. All of administered cations significantly blocked the $^{45}Ca^{2+}$ uptake induced by carbachol in dose-dependent manners, the potency of inhibition was as follows: $La^{3+} > Zn^{2+} > Mn^{2+} > Ba^{2+} > Sr^{2+}$ (Fig. 3A).

2) Secretion of hexosaminidase: The treatments of La^{3+}, Zn^{2+}, Mn^{2+},and Ba^{2+} inhibited the secretion of hexosaminidase respectively, but only the treatment of Sr^{2+} was shown stimulation effect of the secretion of hexosaminidase, but the shape of inhibition curve were definetely different with those of uptake of calcium influx (Fig. 3B). The potency of inhibition was as follows: $La^{3+} > Zn^{2+} > Mn^{2+} > Ba^{2+}$. Both inhibitions of the $^{45}Ca^{2+}$uptake and of the hexosaminidase secretion were shown the concentration-dependent manner.

Effects of PMA (20 nM) or PTx(0.2 μg/ml) on carbachol-induced cell

PMA or PTx was pretreated for 3 h before stimulating with the indicated concentrations of carbachol to examine one of possible mechanisms of $^{45}Ca^{2+}$ influx in carbachol-stimulated

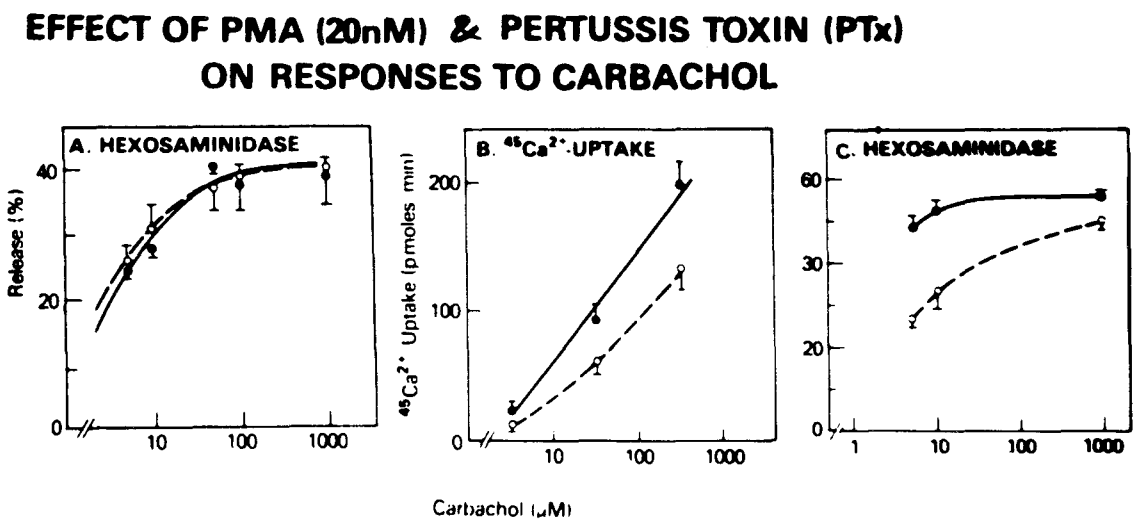

Fig. 4. Effects of PMA (20 nM) both on hexosaminidase secretion (A) and on $^{45}Ca^{2}$ influx (B), and effect of PTx (0.2 μg/ml) on hexosaminidase secretion (C) in carbachol-stimulated cell-lines Cells were exposed inhibitors (PMA or PTx; open circles) or control (closed circle) for 3 h before stimulated with the indicated concentrations of carbachol. Values were mean±SE from more than 3 seperated experiments (3 cultures per experiment).

cells. PMA did not show the inhibition of secretion of hexoseaminidase (Fig. 4A), but blocked $^{45}Ca^{2+}$ influx (Fig. 4B). PTx inhibited the secretion of hexosaminidase in carbachol-induced cells (Fig. 4C). The inhibitory effects of two agents were shown in dose-response manners.

DISCUSSION

It is a widespread and one of the most important phenomena that the increasing influx of calcium ion would be followed after stimulation of the signalling pathway (Taylor & Marshall, 1992). Especially, the electrophysiological basis of unexcitable membrane is so poorly understood that it is hard to find a specific name for response, even though the stimulating signal is the one of important indicator for regulation of the calcium transport in this type of condition. Second messenger-activated calcium influx and chloride conductance being activated by external agonist and by internal messegers were reported (Matthews *et al.*, 1989a,b), and currently, the dubbed ROCs (receptor-operated calcium channels) and the putative current pathways renamed SMOCs (second messenger-operated calcium channels) which was questioned a certain kind of involving second messengers have not been proven(Penner *et al.*, 1988).

The mechanism of calcium ion influx in electrically excitable cells has been detailly characterized in present: it was reported that the intracellular Ca^{2+} concentration and regulation of ions transports were related (Van Scott and Paradiso, 1992), and the voltage-operated calcium current could be easily measured, even though the receptor-mediated calcium influx in nonexitable cells be difficult (Neher, 1992).

As author wanted to observe whether the unexcitable RBL-2H3 cell-line transfected with the gene for m1 muscarinic receptor could respond to affect or not the treatments of carbachol and the related agents which induce the changes of the increment of calcium ion uptake and stimulation of hexosaminidase release, so author designed the experiment to perform the calcium transport in unexcitable cell transfected with the muscarinic receptor. For this experiment, RBL-2H3 cell transfected with m1 muscarinic receptor-this model could be artificially stimulated with carbachol-was selected to elucidate one of mechanism of calcium influx via calcium channel linked-muscarinic receptors.

There were reported that this RBL-2H3 cell-line was used to define the mechanism of exocytosis (Beaven *et al.*, 1884; Mohr & Fewtrell, 1987; Hide & Beaven, 1991), that the increase of calcium influx after receptor-activation in this model (Meldolesi, 1991) was shown, and that carbachol induced the secretion in transfected cell with muscarinic receptor (Jones *et al.*, 1991). Therefore, the aim of study in this paper was focused on behaviors of this muscarinic receptor transfected cell-lines treated with carbachol, on changes in calcium influx treated with several metal cation in carbachol-induced culture cell-line, and on relationship of calcium movement and second messengers in this model.

Muscarinic acetylcholine receptors play important roles in numerously physiological functions including higher cognitive process in CNS and fine muscle operation in periphery (Wess, 1993), and these muscarinic acetylcholine receptors are associated with activation of receptor-operated calcium influx in normal state such as in human airway smooth muscle (Murry *et al.*, 1993), in platelet (Rink, 1990), and in lymphocytes (Liburdy, 1992). The secretion mechanism from neutrophils (Lew, 1990) and in tumor cell (Felder *et al.*, 1993) have been reported, and the calcium ion influx was reduced tyrosine kinase inhibitors (Yule *et al.*, 1994).

The studies for the ions transport modulated with muscarinic receptor agents have been increased in recent years; the chloride conductance (Bajnath *et al.*, 1992), Na^+-H^+ exchange (Wu & Tseng, 1993), many other anions secretion and transport (Chandan *et al.*, 1991a,b.c), and Na^+ and Cl^-fluxes (Traynor *et al.*, 1991). Carbachol (Carbamylcholine; CBC), a stable synthetic analogue of acetylcholine, significantly inhibited C-type calcium current [Ica(L)] which had been augmented by beta-adrenergic stimulation (Han *et al.*, 1994). Because carbachol is one of most potent agonists on acetylcholine re-

ceptor (Re *et al.*, 1993), Ca^{2+} influx drives agonist-activated $[Ca^{2+}]_i$, and Ca^{2+} entry is inbibited by La^{3+} (Martin and Shuttlewarth, 1994). The 0.1 mM of carbachol elevated the cytosolic calcium concentration and regulated the Na^+ and Cl^-transport (increared cytosolic calcium propably inhibited Na^+ absorption) (Al-Bazzaz, 1994), and carbachol-induced muscle contraction was reduced by papaverine which is believed to relax smouth muscle by reducing transmembrane calcium transport and cyclic nucleotide phosphodiesterase activity (Diederichs, 1991).

In this experiment, carbachol-treated cell-line showed stimulating effect of calcium ion influx which was blocked atropine, it means that this calcium movement would be through calcium channel which linked to muscarinic receptor. And the inhibitory effects of several metal cations on carbachol-stimulated cell-lines were shown in this paper, this means that one of toxicological mechanisms of metal ions would be attributed to blocking action on the calcium channel coupled with muscarinic receptor. It has been reported that the effects of several metal ions on Ca^{2+} influx (divalent cations-indued toxicity) in RBL-2H3 cell was one of metal-induced toxicity (Templeton and Chaitu, 1990).

In this cell-line, the treatment with low concentration of PMA, inhibitor of phosphokinase C and calcium transport (Ui and Katada, 1990), showed little effect on stimulation of secretion of hexosaminidase in carbachol-stimulated cell, but exhibited the inhibition of increasing influx of calcium ion. Since PMA did not block the carbachol-induced hexosaminidase secretion in this model that supposed to be not directly induced to muscarinic receptor but indirectly intracellular effect, it can be assumed that there is a certain pathway of calcium ion transport involved to intracellular messengers operated with linked-cholinergic receptor which was blocked by atropine. As it was significantly blocked the secretion of hexosaminidase in carbachol-stimulated cell by PTx which was direct inhibition of transport of calcium ion through calcium channel, the results would be confirmed that carbachol-induced responses appeared to be partially dependent on a G-protein as indicated by PTx which can prevent certain type of receptor through phospholipase C activation in this cell-line.

These results also show that carbachol-induced stimulations were revealed in part phospholipase C involved toxin-sensitive and toxin-insensitive calcium channel in other part. It was also apparent that another Ca^{2+}-dependent mechanism existed for the activation of phospholipase C in this cell-line, accordingly activation of phospholipase C through certain types of receptors can be prevented by treatment of the cells with PTx, which is known to inactivate the certain types of G-proteins (Beaven *et al.*, 1987; Sagi-Eisenberg *et al.*, 1985) other receptors appear to be operated via toxin-insensitive G-protein.

From this study, the result in this model can be concluded that Ca^{2+} influx reveals the existance of at least two possible pathway for calcium ion transport, one of them might be through G-protein-dependent muscarinic receptor and the other through G-protein-independent muscarinic receptor, and this calcium channel is easily blocked by metal cations.

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=국문초록=

Calcium수송기전에 미치는 Carbachol의 영향

삼육대학교 약학과

이 종 화

Calcium수송에 대한 기전을 추구하기위하여, carbachol을 사용하여 m1 muscarinic receptor-transfected RBL-2H3 cell-line에서 다음과 같은 실험결과를 얻었기에 이에 보고한다.

1) Carbachol의 투여로 이들 cell-line에서 Ca^{2+} influx가 농도에 따라 증가하였고, hexosaminidase 분비양도 의의있게 증가하였다.

2) Atropine 투여로 Carbachol의 상승작용이 의의있게 억제되었다.

3) 수종의 금속양이온을 투여하여 carbachol의 Ca^{2+}수송에 대한 영향을 관찰한 바, 이들 금속이온들은 Ca^{2+}의 influx를 의의있게 억제하였다.

4) PMA(20 nM) 투여로 carbachol의 hexosaminidase의 분비는 억제되지 못했지만 Ca^{2+} influx는 억제되었다.

5) PTx (0.2 μg/ml) 투여로 carbachol의 hexosaminidase 분비가 의의있게 억제되었다.

위의 결과로 미루어 보아, 이 세포의 muscarinic receptor가 calcium channel을 통한 calcium수송에 매우 중요한 영향을 나타내는데, 이들 calcium ion channel은 적어도 두 종류가 존재하며, 하나는 G-protein-dependent calcium channel에 의하며, 다른 하나는 G-protein-independent calcium channel에 대한 작용에 의한 것으로 생각된다. 또한 이 calcium channel들은 2가 또는 3가의 다른 금속 ion들에 의하여 calcium수송이 억제된다.