Functional shift from muscarinic to nicotinic cholinergic receptors involved in inositol trisphosphate and cyclic GMP accumulation during the primary culture of adrenal chromaffin cells

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Specificities of cholinergic receptors for the accumulation of inositol trisphosphates (InsP_3) and cyclic GMP and mobilization of intracellular Ca^{2+} in relation to culture periods were investigated in primary cultures of bovine adrenal chromaffin cells. At 0.5 day in culture, muscarine, a specific agonist for muscarinic receptors, caused a greater effect on intracellular Ca^{2+} mobilization and the accumulation of $\text{Ins}(1,3,4)\text{P}_3$ than did the nicotinic-specific agonist nicotine. On the contrary, at 5 days, nicotine produced a greater effect on the accumulation of $\text{Ins}(1,3,4)\text{P}_3$ and intracellular calcium mobilization than did muscarine. Furthermore, at 0.5 day, the muscarinic antagonist atropine strongly inhibited the increase in InsP_3 accumulation that was induced by the nonspecific agonist carbachol, whereas at 5 days the inhibitory effect of atropine was greatly lowered. On the other hand, the nicotinic receptor antagonists hexamethonium and *d*-tubocurarine showed a much higher inhibitory potency at 5 days compared with 0.5 day in culture. Cholinergic receptor subtypes involved in cyclic GMP accumulation showed functional shifts similar to those in InsP_3 formation. Binding experiments with a muscarinic ligand excluded the possibility that the reduction in muscarinic effects on InsP_3 and cyclic GMP formation and intracellular Ca^{2+} mobilization were due to disappearance of the muscarinic receptor itself. These data show that cholinergic receptors linked to the accumulation of InsP_3 and cyclic GMP and Ca^{2+} mobilization functionally shift from muscarinic to nicotinic during primary culture of adrenal chromaffin cells.

INTRODUCTION

In various types of cells it seems established that mobilization of Ca^{2+} from its intracellular stores following receptor stimulation is the consequence of inositol trisphosphate (InsP_3) generation (Berridge & Irvine, 1984).

Our previous results clearly showed that nicotinic, but not muscarinic, receptor stimulation mobilized Ca^{2+} from intracellular stores in cultured bovine adrenal chromaffin cells (Sasakawa *et al.*, 1986). An intriguing problem is, therefore, what mechanisms are involved in the nicotinic receptor-mediated intracellular Ca^{2+} mobilization. There are two possibilities; one is that stimulation of the nicotinic receptors leads to generation of InsP_3 , which in turn mobilizes intracellular Ca^{2+} . The other is that nicotinic receptors are not coupled to InsP_3 formation. This implies that the mechanisms involved in the nicotinic receptor-mediated intracellular Ca^{2+} mobilization might be as yet unidentified. We have tested which possibility is the case.

Contrary to our results, in isolated bovine adrenal chromaffin cells, it had been reported that muscarinic cholinergic receptor stimulation induces mobilization of intracellular Ca^{2+} (Kao & Schneider, 1985; Check & Burgoyne, 1985; Misbahuddin *et al.*, 1985). A potentially important difference in experimental conditions is the duration of the primary culture of chromaffin cells. We used cells that had been cultured for 5 days, whereas the authors cited above performed experiments shortly after isolation of the chromaffin cells.

We have found that at early periods (< 2 days) of primary culture, muscarinic but not nicotinic stimulation leads to InsP_3 and cyclic GMP formation and Ca^{2+} mobilization, whereas at late periods (> 4 days) of culture, nicotinic but not muscarinic receptors became preferentially coupled to formation of InsP_3 and cyclic GMP as well as Ca^{2+} mobilization.

MATERIALS AND METHODS

Primary culture of bovine adrenal chromaffin cells

Chromaffin cells were isolated from fresh bovine adrenal medulla by retrograde perfusion with 0.025% collagenase as described previously (Sasakawa *et al.*, 1984). The cells were cultured in Minimum Essential Medium (Gibco) supplemented with 10% (v/v) foetal calf serum (Hyclone; Logan, UT, U.S.A.), 100 units of penicillin G/ml, 100 μg of streptomycin sulphate/ml, 40 μg of gentamicin sulphate/ml and 25 units of mycostatin/ml. The medium also contained 10 μM -fluorodeoxyuridine, 10 μM -cytosine arabinoside and 5 μM -uridine to prevent the proliferation of non-neuronal cells. The cells were cultured at 37 °C in an atmosphere of air/ CO_2 (19:1).

Measurement of cytosolic $[\text{Ca}^{2+}]$

For 0.5 day culture, the cells were seeded in 75 cm^2 flasks (Corning) at a density of 9×10^6 cells/ml. For 5 days in culture, the cells were seeded in 35 mm dishes (3×10^6 cells/dish). Most cells were attached to the

Abbreviations used: InsP_3 , inositol trisphosphate; $\text{Ins}(1,3,4)\text{P}_3$, inositol 1,3,4-trisphosphate; $\text{Ins}(1,4,5)\text{P}_3$, inositol 1,4,5-trisphosphate; QNB, quinuclidinyl benzilate; $[\text{Ca}^{2+}]$, concentration of free calcium.

plastic dishes at 5 days. Cultured adrenal chromaffin cells were scraped off, if necessary, collected by centrifugation (200 g, 6 min), and suspended in a Locke's solution, consisting of 154 mM-NaCl, 5.6 mM-KCl, 2 mM-CaCl₂, 1 mM-MgCl₂, 3.6 mM-NaHCO₃, 5.6 mM-glucose, 10 mM-Hepes and 0.1% bovine serum albumin (pH 7.4). The cells were incubated at a density of 3×10^6 cells/ml with 2 μ M-fura-2 acetoxymethyl ester (Williams *et al.*, 1985) for 45 min at 37 °C. After the incubation, cells were washed and resuspended in the solution (2×10^6 cells/ml) without fura-2 acetoxymethyl ester and incubated for another 30 min. Immediately before use, 0.6 ml of cell suspension was centrifuged (5000 g, 10 s) and the cells were washed with and resuspended in Ca²⁺-free Locke's solution (2×10^6 cells/ml). The cell suspension was transferred to a quartz cuvette (37 °C) in a fluorescence spectrophotometer (JASCO, model CAF-100). The cells were stirred continuously and gently. Fluorescence intensity was measured at an excitation wavelength of 340 nm and an emission wavelength of 500 nm. Test agents were added with a microsyringe directly into the cuvette without interrupting continuous recording. The concentration of calcium was calibrated by the method of Grynkiewicz *et al.* (1985).

[³H]InsP₃ measurement in cultured chromaffin cells

The cells were labelled with *myo*-[2-³H]inositol (10 μ Ci/ml; Amersham) for 12 h, collected in a plastic tube and washed three times with Locke's solution. Since most cells did not attach to the plastic flasks or dishes at 0.5 day, we performed experiments by suspending cells (9×10^6 cells/ml) in 1 ml of Locke's solution supplemented with bovine serum albumin (1 mg/ml) and 10 mM-LiCl. The cells were preincubated at 37 °C for 20 min. Muscarine or nicotine was added to the incubation mixture and the cells were incubated for another 2 min. At the end of the incubation, the tubes were centrifuged at 3000 g for 30 s, followed by aspiration of the supernatant. To the cell pellet was added 1.5 ml of 10% (w/v) trichloroacetic acid. The cells were homogenized by sonication (30 s) and the homogenate was transferred to a 1.5 ml plastic tube followed by centrifugation at 10000 g for 60 s. The supernatant was transferred to a 10 ml glass tube and stored at -20 °C until separation of the InsP₃. No changes were detected during storage for at least 2 weeks.

For 5 days in culture, the cells were seeded in 35 mm dishes (3×10^6 cells/dish, 1 ml). Most cells were attached to the dishes by this time. Cells were labelled with [³H]-inositol (10 μ Ci/dish) for 12 h. Subsequent procedures were identical with those for the 0.5 day experiment, except that incubations were done in dishes where the cells were still attached.

The frozen supernatant was thawed at room temperature and trichloroacetic acid was extracted four times with 4 ml of water-saturated diethyl ether. Inositol phosphates were separated by anion-exchange chromatography according to the method of Batty *et al.* (1985) who modified the method of Berridge *et al.* (1982). The fraction of InsP₃ which was eluted with 0.8 M-ammonium formate/0.1 M-formic acid from the anion exchange column was collected and its radioactivity was measured by scintillation counting. The fraction of InsP₄ which was eluted with 1.0 M-ammonium formate/0.1 M-formic acid showed no significant increase 3 min after the stimulation

by 300 μ M-carbachol; we did not routinely analyse the InsP₄ fraction.

H.p.l.c. analysis of InsP₃ isomers

The fractions of InsP₃ were obtained as described in the above, except that [³H]inositol used for labelling the cells was increased to 50 μ Ci/dish. InsP₃ fractions were applied to hydroxyapatite columns (2 ml of wet bed volume) and washed with 5 ml of distilled water. InsP₃ was eluted with 4 ml of 10% (v/v) triethylamine and the fractions were extracted twice with 1.5 ml of water-saturated ethyl acetate to remove triethylamine. The aqueous phase was dried by Speed Vac concentrator (Savant, Hicksville, NY, U.S.A.), and the dried residue was reconstituted with 0.2 ml of elution buffer for h.p.l.c. as described below. Isomers of InsP₃ were separated by h.p.l.c. by a modification of the method of Irvine *et al.* (1985). The authentic standard for [³H]Ins(1,4,5)P₃ (3.6 Ci/mmol; New England Nuclear) was processed in parallel with the cell-derived samples. [³H]Ins(1,3,4)P₃ was prepared as described in the literature (Irvine *et al.*, 1985). ATP (30 μ g/ml) was mixed together with the samples as an internal marker and monitored by u.v. absorbance at 259 nm. The samples were subjected to h.p.l.c. with an anion exchange column (Whatman Partisil 10 SAX) and an isocratic running buffer consisting of 0.65 M-ammonium formate (pH 3.7 with phosphoric acid) at a flow rate of 1.8 ml/min. The fractions were collected at 20 s intervals. Radioactivity in the fractions was determined by scintillation counting. Both cell-derived samples and the authentic Ins(1,4,5)P₃ and Ins(1,3,4)P₃ yielded no peaks before 5 min of the run time.

[³H]QNB binding assay

The number and apparent dissociation constant of muscarinic receptors in cultured adrenal chromaffin cells were estimated by use of [³H]quinuclidinyl benzilate (QNB) (Yamamura & Snyder, 1974), a specific ligand for muscarinic receptors. In brief, cells cultured for 0.5 or 5 days were collected and washed twice with 0.32 M-sucrose. They were resuspended in 0.32 M-sucrose at a final cell concn. of 1.5×10^7 cells/ml and were homogenized with a Dounce homogenizer. The homogenate was centrifuged at 600 g for 5 min. The resultant supernatant was centrifuged at 100000 g for 60 min, and the final pellet was resuspended at a protein concn. of 1–1.4 mg/ml. The tissue extract (50 μ l) and [³H]QNB (25 μ l) were added to 2 ml of 50 mM-sodium/potassium phosphate buffer (pH 7.3). The mixture was incubated with or without 0.1 μ M-atropine at 25 °C for 60 min. The reaction was terminated by filtering the incubation mixture through a glass fibre filter (Whatman GF/B), followed by three washes with the ice-cold phosphate buffer. The filter was immersed in a liquid scintillator and was counted for radioactivity. The measurement of protein was done by the method of Lowry *et al.* (1951).

Measurement of cyclic GMP accumulation

The chromaffin cells were cultured for 5 days in a 24-multiwell plate at a density of 0.25×10^6 cells/cm². Culture conditions for 0.5 day were similar to those for InsP₃ experiments, except that the cell density was 2×10^6 cells/ml. The composition of Locke's solution was identical with that used for the InsP₃ experiments.

3-Isobutylmethylxanthine was added to inhibit cyclic GMP phosphodiesterase. After preincubation for 10 min, cholinergic agonists were added to the incubation mixture, which was further incubated for 5 min. After the 5 min incubation with agonists, the incubation mixture was aspirated and 150 μ l of 0.1 M-HCl was added. For the cells cultured for 0.5 day, the reaction was terminated by centrifugation at 3000 g for 30 s, followed by aspiration of the supernatant and addition of 150 μ l of 0.1 M-HCl. The cells were sonicated and were centrifuged at 1000 g for 10 min. A portion of the resultant supernatant was subjected to a radioimmunoassay with specific antibodies according to the method of Honma *et al.* (1977) using commercially available kits (Yamasa Shoyu Co., Choshi, Japan). Cellular protein was measured by the method of Lowry *et al.* (1951).

RESULTS

We first examined the cholinergic receptor subtypes that are involved in Ca^{2+} release from intracellular stores during primary culture of chromaffin cells (Fig. 1). At early periods of culture (0.5 or 1 day), muscarine, a muscarinic receptor agonist, caused a greater effect than nicotine, a nicotinic agonist. This is consistent with the results of others (Kao & Schneider, 1985; Check & Burgoyne, 1985; Misbahuddin *et al.*, 1985) who have used chromaffin cells shortly after isolation. However, at later periods (2–5 days), nicotine exceeded muscarine in releasing Ca^{2+} from intracellular stores, in agreement with our previous findings (Sasakawa *et al.*, 1986).

Since the most important candidate for second messengers that mediate intracellular Ca^{2+} mobilization is $\text{Ins}(1,4,5)\text{P}_3$ (Berridge & Irvine, 1984; Irvine *et al.*, 1986; Stoehr *et al.*, 1986), we next examined the cholinergic receptor specificity involved in the formation of InsP_3 . Although carbachol caused a statistically significant increased accumulation of InsP_3 even in the absence of extracellular Ca^{2+} , the measurable increment was too small to be assessed reliably in the absence of extracellular Ca^{2+} (Table 1). Therefore, we used a condition of presence of extracellular Ca^{2+} to determine InsP_3 accumulation.

We examined the cholinergic receptor specificity for InsP_3 accumulation at 0.5 and 5 days in culture. Fig. 2 shows an elution profile of InsP_3 isomers separated by h.p.l.c. with isocratic running buffer, which is slightly different from the method of Irvine *et al.* (1985) who used a gradient buffer. This may explain the slight differences in retention time between $\text{Ins}(1,3,4)\text{P}_3$ and ATP. At 0.5 day, muscarine (Fig. 2b) had a greater effect than nicotine (Fig. 2c) on InsP_3 formation. The major isomer detected 2 min after stimulation by agonists was $\text{Ins}(1,3,4)\text{P}_3$. At 5 days, nicotine became more effective than muscarine and the isomer observed was still $\text{Ins}(1,3,4)\text{P}_3$ (Figs. 2e and 2f). Fig. 3 shows the time course of accumulation of two InsP_3 isomers. At 0.5 day, muscarine greatly enhanced the accumulation of the $\text{Ins}(1,3,4)\text{P}_3$ in a time-dependent manner, whereas nicotine caused a lesser effect (Figs. 3b and 3c). On the contrary, at 5 days nicotine produced a greater effect on the accumulation of $\text{Ins}(1,3,4)\text{P}_3$ than did muscarine (Figs. 3e and 3f). $\text{Ins}(1,4,5)\text{P}_3$ was not clearly detected at 5, 15 or 120 s except that at 5 days $\text{Ins}(1,4,5)\text{P}_3$ was increased slightly by nicotine at 5 s (Fig. 3f). It is likely that the earliest time (5 s) in our method was not rapid

Fig. 1. Changes in muscarine- and nicotine-induced Ca^{2+} release from intracellular stores during the primary culture of adrenal chromaffin cells

Fluorescence intensity of fura-2-loaded cells was measured in Ca^{2+} -deprived medium as described in the Materials and methods section. Effects of muscarine (100 μM) (○) and nicotine (100 μM) (●) were examined during the time course of the primary culture. Peak values of net increase in the fluorescence intensity by agonists (excitation at 340 nm and emission at 500 nm) observed at 10–15 s after stimulation were plotted as an augmentation of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). The basal level of $[\text{Ca}^{2+}]_i$ was about 100 nM throughout the culture periods. Data presented are one of two experiments, which showed a similar result. Magnitudes of experimental errors are less than 10% in each case.

Table 1. Effect of calcium deprivation from the incubation medium on InsP_3 accumulation in cultured adrenal chromaffin cells

Cultured (5 days) cells prelabelled with [^3H]inositol (10 $\mu\text{Ci}/\text{dish}$) were incubated with 300 μM -carbachol for 2 min in Locke's solution at 37 °C containing 10 mM-LiCl and 0.1% bovine serum albumin with or without Ca^{2+} . [^3H] InsP_3 was separated by the method of Batty *et al.* (1985). * $P < 0.05$ versus each control ($n = 4$).

Treatment	InsP_3 (d.p.m.)
With extracellular calcium	
Control	1130 $\pm$ 110
300 μM -carbachol	3000 $\pm$ 310*
Without extracellular calcium	
Control	1140 $\pm$ 70
300 μM -carbachol	1480 $\pm$ 80*

enough to detect the increases in $\text{Ins}(1,4,5)\text{P}_3$ in the bovine adrenal chromaffin cells.

We noted that radioactivities of the InsP_3 fraction from both unstimulated and stimulated cells at 5 days were always higher than those at 0.5 day in culture, although the reason for this is unknown. This implies that a straightforward comparison of ^3H counts between 0.5 day and 5 days is meaningless.

We next examined the dose-response relations of specific agonists and antagonists for InsP_3 accumulation,

Fig. 2. Separation of isomers of InsP_3 by h.p.l.c. in adrenal chromaffin cells cultured for 0.5 day (a,b,c) and 5 days (d,e,f)

Cells prelabelled with [^3H]inositol were stimulated by muscarine ($100\ \mu\text{M}$) (b,e) or nicotine ($100\ \mu\text{M}$) (c,f) for 2 min. Isomers of [^3H]Ins P_3 extracted from cells were separated by h.p.l.c. with an anion exchange column basically according to the method of Irvine *et al.* (1985) except that we use an isocratic running buffer (0.65 M-ammonium formate, pH 3.7 with phosphoric acid). Results from one experiment, which is typical of two that gave similar results, are shown. Arrows show the peaks of ATP, authentic [^3H]Ins(1,4,5) P_3 (New England Nuclear) and [^3H]Ins(1,3,4) P_3 . (a) and (d) are data on unstimulated cells.

which mostly reflected the changes in the Ins(1,3,4) P_3 isomer. At 0.5 day in culture, muscarine effectively increased Ins P_3 accumulation, whereas nicotine had a lesser effect than did muscarine (Fig. 4a). On the contrary, at 5 days in culture, nicotine showed a greater effect than muscarine on Ins P_3 accumulation (Fig. 4b). Furthermore, at 0.5 day, the muscarinic antagonist atropine strongly inhibited the increase in Ins P_3 that was induced by the nonspecific agonist carbachol (Fig. 5a), whereas at 5 days in culture the inhibitory effect of atropine was greatly lowered (Fig. 5b). On the other hand, the nicotinic receptor antagonist hexamethonium showed a much higher inhibitory potency at 5 days compared with 0.5 day in culture. The inhibitory effects of another nicotinic antagonist, *d*-tubocurarine, showed changes similar to those of hexamethonium. We obtained qualitatively similar results when the cells were cultured in a suspension state (results not shown), indicating that changes in receptor subtypes are not due to secondary changes such as cell attachment to plastic dishes during the primary culture.

The reduction in muscarinic component cannot be explained by alterations in its binding sites; binding experiments with a muscarinic ligand ([^3H]QNB) showed that the affinities and the numbers of binding sites were not changed during the time course of a culture [at 0.5 day, the dissociation constant (K_d) was $145 \pm 15\ \text{pM}$ and the maximal number of binding sites (B_{max}) was $140 \pm 10\ \text{fmol/mg}$ of protein ($n = 3$); at 5 days, K_d was $175 \pm 15\ \text{pM}$ and B_{max} was $145 \pm 15\ \text{fmol/mg}$ of protein ($n = 3$) ($P > 0.05$)].

At 0.5 day in culture, muscarine significantly increased cyclic GMP accumulation at $1\ \mu\text{M}$, whereas nicotine failed to do so even at a concentration of $100\ \mu\text{M}$. On the contrary, at 5 days, nicotine significantly increased cyclic GMP accumulation at a concentration as low as $10\ \mu\text{M}$, whereas muscarine failed to do so at $10\ \mu\text{M}$. It is noteworthy that the unstimulated as well as $300\ \mu\text{M}$ -acetylcholine (a nonspecific agonist)-stimulated accumulation of cyclic GMP are constant during the culture periods, indicating that no general deterioration of cellular functions occurred during the primary culture.

DISCUSSION

Of the two possibilities stated in the Introduction, the first one appears to be the case; the stimulation of nicotinic receptors leads to the formation of Ins P_3 in bovine adrenal chromaffin cells cultured for 5 days. Furthermore, it is clearly shown that cholinergic receptor subtypes involved in the accumulation of Ins P_3 and intracellular Ca^{2+} mobilization shift from muscarinic to nicotinic during primary culture of the adrenal chromaffin cells. Therefore, the previous and apparently inconsistent results (Forsburg *et al.*, 1986; Kao & Schneider, 1985; Check & Burgoyne, 1985; Misbahuddin *et al.*, 1985; Sasakawa *et al.*, 1986) can be explained by the differences in the period of the primary culture of chromaffin cells. Moreover, our findings are supported by recent evidence (TerBush & Holz, 1986) that nicotinic stimulation can translocate protein kinase C, implying that the nicotinic mechanism is involved in phospholipase

Fig. 3. Time course of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$ accumulation stimulated by muscarine (*b,e*) or nicotine (*c,f*) in adrenal chromaffin cells cultured for 0.5 day (*a,b,c*) and 5 days (*d,e,f*)

Cells prelabelled with $[\text{H}]\text{inositol}$ were stimulated by muscarine ($100\ \mu\text{M}$) (*b,e*) or nicotine ($100\ \mu\text{M}$) (*c,f*) for various time periods. Other experimental conditions were identical to those in Fig. 2. (*a*) and (*d*) are data on unstimulated cells. ●, $[\text{H}]\text{Ins}(1,3,4)\text{P}_3$; ■, $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$.

Fig. 4. Effect of muscarinic and nicotinic agonists on InsP_3 accumulation in adrenal chromaffin cells cultured for 0.5 day (*a*) and 5 days (*b*)

$[\text{H}]\text{inositol}$ ($10\ \mu\text{Ci}/\text{dish}$)-prelabelled cells were stimulated by specific agonists. Incubation with agonists lasted 2 min. $[\text{H}]\text{InsP}_3$ was separated by a method described in the legend to Fig. 2. $[\text{H}]\text{InsP}_3$ accumulation is expressed as a percentage, where 0% represents the basal level and 100% the maximal level attained by $300\ \mu\text{M}$ -acetylcholine. Points and bars are means and S.E.M. of three independent experiments, each of which was done in quadruplicate. At 0.5 day in culture (*a*), the basal and the maximal levels of InsP_3 were 500 ± 70 and 1100 ± 60 d.p.m., respectively. At 5 days in culture (*b*), the basal and the maximal levels were 1200 ± 400 and 2900 ± 1080 d.p.m., respectively. Symbols: ■, no agonist (basal level); ○, muscarine; ●, nicotine.

Fig. 5. Effect of muscarinic and nicotinic antagonists on carbachol-induced InsP_3 accumulation in adrenal chromaffin cells cultured for 0.5 day (*a*) and 5 days (*b*)

$[\text{H}]\text{inositol}$ ($10\ \mu\text{Ci}/\text{dish}$)-prelabelled cells were stimulated by $300\ \mu\text{M}$ -carbachol, a nonspecific cholinergic agonist, in the presence of various concentrations of specific antagonists, which were added 3 min before the addition of carbachol. Incubation with carbachol lasted 2 min. $[\text{H}]\text{InsP}_3$ was separated by a method described in the legend to Fig. 2. $[\text{H}]\text{InsP}_3$ accumulation is expressed as a percentage, where 0% represents the basal level and 100% a maximal level attained by $300\ \mu\text{M}$ -carbachol. Points and bars are means and S.E.M. of three independent experiments, each of which was done in quadruplicate. At 0.5 day in culture (*a*), the basal and the maximal levels of InsP_3 were 500 ± 60 and 1400 ± 300 d.p.m., respectively. At 5 days in culture (*b*), the basal and the maximal levels were 1600 ± 400 and 3700 ± 1100 d.p.m., respectively. Symbols: ■, no antagonist (maximal level); ○, atropine; ●, hexamethonium; ▲, *d*-tubocurarine.

Fig. 6. Effects of muscarinic and nicotinic agonists on cyclic GMP accumulation in adrenal chromaffin cells cultured for 0.5 day (*a*) and 5 days (*b*)

Cultured cells were incubated with agonists for 5 min at 37°C in Locke's solution, supplemented with $0.3\ \text{mM}$ -isobutylmethylxanthine and 0.1% bovine serum albumin. The content of cellular cyclic GMP was measured by radioimmunoassay. Symbols: ■, no agonist (basal level); ○, muscarine; ●, nicotine; ▲, acetylcholine. * $P < 0.05$ versus each basal level. Points and bars are means and S.E.M. of quadruplicate assays.

C activation. We propose that one has to define how long the chromaffin cells were cultured *in vitro* in order to interpret correctly any data on cholinergic receptor mechanisms in cultured chromaffin cells.

We observed that cholinergic receptors involved in cyclic GMP responses showed a functional shift similar to that observed in InsP_3 formation. This is consistent with the proposition by Snider *et al.* (1984) that cholinergic receptor-stimulated cyclic GMP formation is mediated through diacylglycerol generation, i.e. the other metabolite of polyphosphoinositides catalysed by phospholipase C.

The mechanisms by which functional shift of cholinergic receptors occur are unknown. It is unlikely that muscarinic receptors were conformationally changed into nicotinic ones in the plasma membrane, because it is evident that the primary structures of amino acid sequences of the two are quite different (Noda *et al.*, 1983; Kubo *et al.*, 1986). We also exclude the possibility of a loss of muscarinic receptors from the membranes, because we could not detect such changes by the [^3H]QNB binding assay. It is possible, therefore, that the coupling process somewhere between receptors and phospholipase C was changed.

Table 1 shows that carbachol-induced InsP_3 formation involves two mechanisms: one is extracellular calcium-dependent and the other is calcium-independent. Indeed, high concentrations of KCl increased InsP_3 accumulation in chromaffin cells, and this effect was inhibited by nifedipine (an inhibitor of voltage-sensitive calcium channels), suggesting that calcium influx induces InsP_3 accumulation in chromaffin cells (Sasakawa *et al.*, 1987). The ability to detect only $\text{Ins}(1,3,4)\text{P}_3$ species and the need for extracellular calcium may raise a possibility that the observations of $\text{Ins}(1,3,4)\text{P}_3$ alterations are a consequence rather than a cause of changes in $[\text{Ca}^{2+}]$. Our unpublished observations showed, however, that only high concentrations ($> 30 \mu\text{M}$) of nifedipine inhibited a carbachol-induced InsP_3 formation in chromaffin cells cultured for 5 days; much lower concentrations (about 30 nM) of nifedipine were sufficient to inhibit the carbachol-induced $^{45}\text{Ca}^{2+}$ uptake. It is unlikely, therefore, that carbachol (nicotinic)-induced InsP_3 formation mainly involves a voltage-sensitive calcium channel.

Another question is which type of cholinergic receptors are coupled to phospholipase C *in vivo*. If muscarinic receptors are coupled to the enzyme *in vivo*, there are two possibilities; one is that something important for maintaining the muscarinic function is missing in the medium for the primary culture. The other is that something unphysiological for the adrenal chromaffin cells affected the coupling process. Such substance(s) could be various antibiotics included in the culture media. If nicotinic receptors are coupled to the enzyme *in vivo*, the functional shift may reflect a 'repair process' of chromaffin cells damaged by the treatment with digestive enzymes such as collagenase. Our unpublished data, however, showed that collagenase treatment of chromaffin cells which had been cultured for 5 days did not affect the nicotinic coupling to phospholipase C. At present, we cannot determine which possibility is the case.

The reason that we could not detect a significant accumulation of InsP_4 under our experimental conditions may be due to a rapid degradation of InsP_4 to $\text{Ins}(1,3,4)\text{P}_3$. In fact, it has been shown that the metabolisms of

$\text{Ins}(1,4,5)\text{P}_3$ and InsP_4 are much faster than that of $\text{Ins}(1,3,4)\text{P}_3$ (Rossier *et al.*, 1987).

Our findings on functional shift of muscarinic to nicotinic receptors suggest that cultured bovine adrenal chromaffin cells are a useful model for investigations into plasticity of functions of cholinergic receptors.

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