Pergamon

Biochemical Pharmacology, Vol. 48, No. 10, pp. 1982–1985, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)00359-9

Short Communication

MUSCARINIC RECEPTOR-MEDIATED CALCIUM EFFLUX FROM CULTURED BOVINE ADRENAL CHROMAFFIN CELLS

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(*Received* 7 *February* 1994; *accepted* 8 *August* 1994)

Abstract—The effect of stimulation of the muscarinic receptor on Ca^{2+} mobilization in cultured bovine adrenal chromaffin cells was examined. Acetylcholine (ACh) increased the uptake of $^{45}Ca^{2+}$ and $[Ca^{2+}]_i$ whose levels decreased with time after reaching peaks. It also enhanced the efflux of $^{45}Ca^{2+}$ from the cells. Its effect was inhibited by the specific muscarinic receptor antagonist atropine (Atr), but not by the nicotinic receptor antagonist hexamethonium (C_6). The increase in muscarine (Mus)-stimulated $^{45}Ca^{2+}$ efflux was reduced concentration-dependently by deprivation of extracellular Na^+. These results suggest that muscarinic stimulation of the ACh receptor stimulates Na^+/Ca^{2+} exchange in cultured bovine adrenal chromaffin cells.

Key words: muscarine, calcium efflux; Na^+/Ca^{2+} exchange; chromaffin cell

Acetylcholine (ACh†), a neurotransmitter in the peripheral nervous system, stimulates the secretion of catecholamine from adrenal chromaffin cells by a process dependent on extracellular Ca^{2+} [1]. There are both nicotinic and muscarinic receptors on the surface of bovine adrenal chromaffin cells. Stimulation of the nicotinic ACh receptor results in increased uptake of Ca^{2+} into the cells and a subsequent increase in catecholamine secretion from the cells [2–4], whereas stimulation of the muscarinic ACh receptor does not cause uptake of extracellular Ca^{2+} and secretion of catecholamine [5], but increases phosphatidylinositol turnover [6–8] as well as intracellular levels of free Ca^{2+} ($[Ca^{2+}]_i$) [9] and cyclic GMP [10, 11]. We have reported that muscarinic stimulation causes efflux of Ca^{2+} from isolated bovine adrenal chromaffin cells [5]. A previous report suggested that phosphatidylinositol turnover was linked to the regulation mechanism of Ca^{2+} mobilization and cyclic GMP synthesis by muscarinic stimulation [12]. However, little is known about the mechanism of decrease of elevated $[Ca^{2+}]_i$ in adrenal chromaffin cells after physiological stimulation.

In this study, we examined the mechanism of calcium efflux after stimulation of cultured bovine adrenal chromaffin cells with muscarine. We found that muscarine-stimulated efflux of Ca^{2+} from these cells in culture depended on extracellular Na^+ concentration and suggest that it is mediated by stimulation of Na^+/Ca^{2+} exchange.

Materials and Methods

Bovine adrenal chromaffin cells were dispersed enzymatically as described previously [13]. Briefly, the medulla was sliced with a hand slicer, and the slices digested in medium containing 0.1% collagenase, 0.01% soybean trypsin inhibitor, and 0.5% bovine serum albumin in balanced salt solution (BSS: 135 mM NaCl, 5.6 mM KCl, 1.2 mM $MgSO_4$, 2.2 mM $CaCl_2$, 10 mM glucose, and 20 mM HEPES/NaOH, pH 7.4). Cells were plated in 35-mm culture dishes at a density of 2×10^6 cells/dish to measure $^{45}Ca^{2+}$ uptake and efflux or on 22 × 22 mm cover glasses in 35-mm culture dishes at a density of 1×10^6 cells/dish to measure intracellular calcium, and were maintained for 3 days as monolayer cultures in Eagle's basal medium supplemented with 5% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), gentamycin (40 μg/mL), fungizone (2.5 μg/mL) and 10 μM cytosine arabinoside.

For the measurement of $^{45}Ca^{2+}$ uptake, the incubation medium contained 3 μCi of $^{45}Ca^{2+}$. After incubation, the dishes were immediately chilled on ice and washed three times with ice-cold Ca^{2+}-free BSS. The $^{45}Ca^{2+}$ taken up into the cells was extracted with 1% Triton X-100 and counted in a liquid scintillation counter. The intracellular Ca^{2+} ($[Ca^{2+}]_i$) level in single chromaffin cells was measured using the fluorescent Ca^{2+} indicator fura-2. Cells cultured on cover glasses were incubated at 37° for 30 min in 1 mL of BSS containing 2 μM fura-2/acetoxy methyl ester. Then the cells on the cover glasses were transferred to a small incubation bath (approx. 0.5 mL) on the platform of a microscope. The temperature was maintained at 37° and the incubation bath was perfused with BSS at a rate of 0.8 mL/min during the experimental period. Fluorescence was measured in single chromaffin cells on the cover glass using a fluorescence spectromicroscope (excitation, 340/380 nm, emission, 510 nm). The intracellular Ca^{2+} level was calculated using the equation described previously [14].

For measurement of $^{45}Ca^{2+}$ efflux, cells were washed and incubated as described above in BSS containing $^{45}Ca^{2+}$ (3 μCi/mL) for 1 hr at 37°. After incubation, the cells in each well were washed 19 times with 1-mL volumes of BSS at intervals of 30 sec to remove unincorporated $^{45}Ca^{2+}$. The cells were then incubated 11 times with 1 mL volumes of

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† Abbreviations: IP_3, inositol 1,4,5-trisphosphate; BSS, balanced salt solution; ACh, acetylcholine; C_6, hexamethonium.

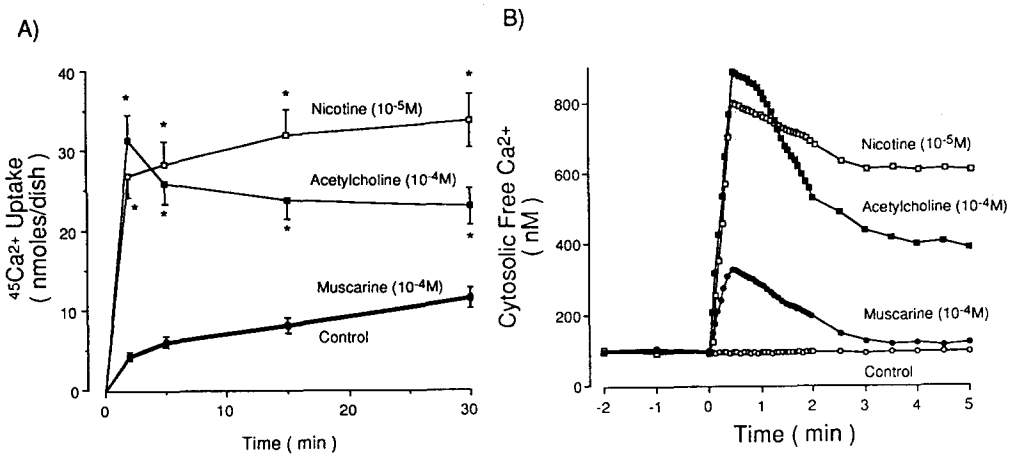

Fig. 1. (A) Effects of ACh, nicotine and muscarine on $^{45}Ca^{2+}$ uptake by cultured bovine adrenal chromaffin cells. Cells were incubated with ACh (10^{-4} M), nicotine (10^{-5} M) or muscarine (10^{-4} M) in BSS containing $^{45}Ca^{2+}$ as described in Materials and Methods. Data are means ± SE for four to six separate experiments. *, Significantly greater than control value ($P < 0.01$). (B) Effects of ACh, nicotine and muscarine on $[Ca^{2+}]_i$ in cultured bovine adrenal chromaffin cells. Cells were preloaded with 2 μM fura-2/acetoxy methyl ester as described in Materials and Methods, and then incubated with or without ACh (10^{-4} M), nicotine (10^{-5} M) or muscarine (10^{-4} M). Data are means for three to seven separate experiments. The maximal standard error was ±9.7%. All peaks of fura-2 fluorescence with test compounds were significantly greater than the control level ($P < 0.01$). Following the level of $[Ca^{2+}]_i$ with muscarine was not significantly greater than the control level.

BSS for 30 sec periods to determine basal efflux levels. They were then incubated 11 times with 1-mL volumes of reaction mixture with or without test agents for 30 sec periods to determine agonist-stimulated efflux levels. After agonist stimulation, the cells were solubilized in 1 mL of 1% Triton X-100 to determine their residual $^{45}Ca^{2+}$. Samples were counted in 10 mL of liquid scintillation fluid for 2 min periods. The total radioactivity of $^{45}Ca^{2+}$ in each well was determined as the sum of the radioactivity in each fraction and the residual radioactivity, and this value was used to calculate the fractional release of Ca^{2+} in each period.

$^{45}CaCl_2$ was obtained from the Amersham Corp. (Tokyo, Japan). Ach, nicotine, muscarine, hexamethonium and atropine were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Other chemicals used were commercial products of reagent grade.

Results and Discussion

Figure 1A shows the effects of ACh, nicotine and muscarine on $^{45}Ca^{2+}$ uptake into adrenal chromaffin cells. ACh (10^{-4} M) and nicotine (10^{-5} M) increased $^{45}Ca^{2+}$ uptake into the cells by approx. 540 and 440%, respectively, in 2 min. Muscarine did not increase $^{45}Ca^{2+}$ uptake into the cells. After 30 min, the effect of ACh on $^{45}Ca^{2+}$ uptake decreased, but that of nicotine did not. Figure 1B shows the effects of ACh, nicotine and muscarine on the $[Ca^{2+}]_i$ in adrenal chromaffin cells. ACh (10^{-4} M) and nicotine (10^{-5} M) increased $[Ca^{2+}]_i$ to approx. 910 and 780 nM whereas muscarine (10^{-4} M) increased it to only about 340 nM. This muscarine-induced $[Ca^{2+}]_i$ may be released from intracellular Ca^{2+} pools because muscarine has no effect on $^{45}Ca^{2+}$ uptake into the cells. The ACh-induced $[Ca^{2+}]_i$ gradually decreased after reaching a peak. Bovine adrenal chromaffin cells are known to have both nicotinic and muscarinic receptors on their surface. Therefore, the decreases in $^{45}Ca^{2+}$ uptake and $[Ca^{2+}]_i$ induced by ACh may be mediated by the muscarinic ACh receptor on the cells. On the level of muscarine-induced $[Ca^{2+}]_i$ (Fig. 1B),

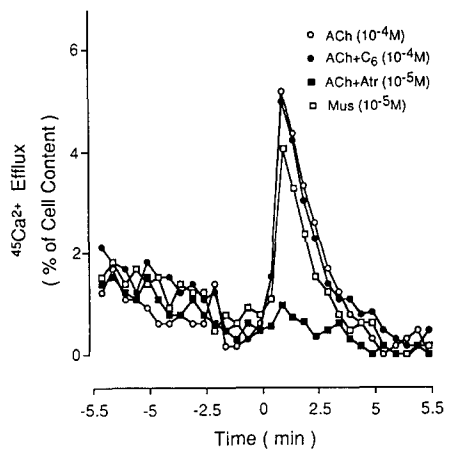

Fig. 2. Effects of ACh receptor antagonists on ACh-induced $^{45}Ca^{2+}$ efflux from cultured bovine adrenal chromaffin cells. Cells were preloaded with $^{45}Ca^{2+}$ as described in Materials and Methods. The nicotinic receptor antagonist hexamethonium (C_6; 10^{-4} M) or muscarinic receptor antagonist atropine (Atr; 10^{-6} M) was added 5 min before ACh (10^{-4} M), and the cells were then incubated for 5.5 min. Date are means ± SE for three to four separate experiments. The maximal standard error was ±10.6%.

the following level was slightly higher than control level (no significance). It remains to be explained whether this following level is dependent on the release of Ca^{2+} from intracellular pools or an increase in Ca^{2+} uptake.

To determine whether ACh-stimulated $^{45}Ca^{2+}$ efflux is mediated by activation of the muscarinic receptor, we

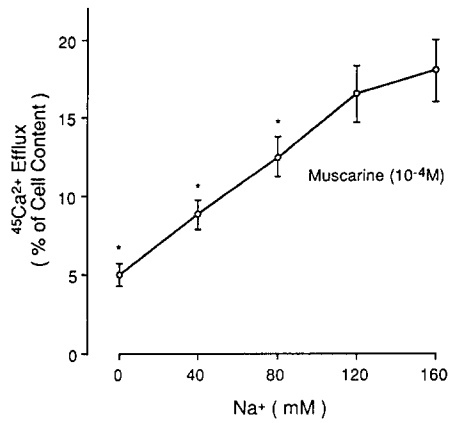

Fig. 3. Effect of extracellular Na^+ concentration on muscarine-induced $^{45}Ca^{2+}$ efflux from cultured bovine adrenal chromaffin cells. Cells were preloaded with $^{45}Ca^{2+}$ as described in Materials and Methods. The medium was changed to Na^+-deficient medium (with sucrose instead of Na^+), and the cells were then incubated for 5 min. $^{45}Ca^{2+}$ efflux 5 min after addition of muscarine was calculated. Data are means ± SE for three to four separate experiments.
*, Significantly less than with 160 mM Na^+($P < 0.01$).

examined the effects of the nicotinic receptor antagonist hexamethonium and the muscarinic receptor antagonist atropine on the efflux. As shown in Fig. 2, ACh-stimulated $^{45}Ca^{2+}$ efflux was inhibited by atropine, but not by hexamethonium. Muscarine (10^{-4} M) significantly stimulated $^{45}Ca^{2+}$ efflux from the cells. Under this experimental condition, ACh-stimulated $^{45}Ca^{2+}$ efflux was slightly greater than muscarinic stimulation. ACh may have a higher affinity with muscarinic receptor than muscarine because ACh is a physiological neurotransmitter to adrenal chromaffin cells. These findings indicate that stimulation of the muscarinic ACh receptor regulates Ca^{2+} efflux from adrenal chromaffin cells.

We also examined whether the increased $^{45}Ca^{2+}$ efflux induced by muscarinic receptor stimulation is dependent on extracellular Na^+ in experiments with various concentrations of extracellular Na^+. As shown in Fig. 3, muscarine-stimulated $^{45}Ca^{2+}$ efflux was dependent on extracellular Na^+ concentration. Therefore, the effect of muscarine in stimulating Ca^{2+} efflux across the plasma membrane may be mediated by a Na^+/Ca^{2+} exchange mechanism. However, the stimulatory action of muscarine on Ca^{2+} efflux was not completely abolished by the omission of extracellular Na^+. Therefore, other mechanisms such as Ca^{2+}-ATPase pumps may be involved in this muscarine-stimulated Ca^{2+} efflux from the cells.

In the present study we examined the mechanisms involved in stimulation of Ca^{2+} efflux from cultured bovine adrenal chromaffin cells by stimulation of the muscarinic acetylcholine receptor. ACh is known to stimulate the uptake of Ca^{2+} and to increase $[Ca^{2+}]_i$ in cultured bovine adrenal chromaffin cells (Fig. 1) [9, 15]. However, this increased $[Ca^{2+}]_i$ should be restored to a physiological level for response to a subsequent stimulus.

Diacylglycerol is produced concurrently with IP_3 on breakdown of PIP_2 by phospholipase C and is thought to activate protein kinase C by increasing the affinity of the enzyme for calcium [16]. ACh induces production of IP_3 through the muscarinic ACh receptor in adrenal chromaffin cells [9, 17, 18]. Acceleration of phosphatidylinositol turnover by muscarinic stimulation is reported to be linked to the regulation of Ca^{2+} mobilization [12]. In the present study, we observed enhanced $^{45}Ca^{2+}$ efflux from cultured bovine adrenal chromaffin cells during stimulation of the muscarinic receptor, and found that this muscarine-stimulated $^{45}Ca^{2+}$ efflux was dependent on extracellular Na^+ concentration (Fig. 3). The subtypes of muscarinic receptors are known to be coupled to potassium channels [19–21]. However, the relation between the activation of potassium channels and muscarine-stimulated $^{45}Ca^{2+}$ efflux in cultured bovine adrenal chromaffin cells is not clear.

We conclude from this study that stimulation of the muscarinic receptor increases Ca^{2+} efflux from cultured bovine adrenal chromaffin cells, and that this efflux may be explained by stimulation of Na^+/Ca^{2+} exchange mechanisms.

Acknowledgement—We thank Mr. Masayuki Shono for his expert technical assistance.

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