Muscarinic receptor-mediated increase in cytoplasmic free Ca^{2+} in isolated bovine adrenal medullary cells

Effects of TMB-8 and phorbol ester TPA

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The change in cytoplasmic free calcium, $[Ca^{2+}]_i$ in isolated bovine adrenal medullary cells during stimulation by acetylcholine (ACh) in Ca^{2+}-free incubation medium was measured using the fluorescent Ca^{2+} indicator quin2. ACh (1–100 μM) caused an increase in $[Ca^{2+}]_i$ by mobilization of Ca^{2+} from the intracellular pool. Nicotine (10 μM) did not increase $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. Pretreatment of the cells with atropine (10 μM) completely inhibited ACh-induced increase in $[Ca^{2+}]_i$, whereas pretreatment with hexamethonium (100 μM) did not. The intracellular Ca^{2+} antagonist 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), inhibited ACh-induced increase in $[Ca^{2+}]_i$. The activator of protein kinase C 12-*O*-tetradecanoylphorbol-13-acetate (TPA), but not its 'inactive' analog 4α-phorbol-12,13-didecanoate (PDD), also inhibited ACh-induced increase in $[Ca^{2+}]_i$. These findings suggest that in bovine adrenal medullary cells, stimulation of muscarinic ACh receptor causes an increase in $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from the intracellular pool and that protein kinase C is involved in 'termination' or 'down regulation' of this response.

Muscarinic receptor *Cytoplasmic free Ca^{2+}* *TMP-8* *Phorbol ester* *Protein kinase C* *Adrenal medulla*

1. INTRODUCTION

ACh stimulates the release of catecholamine from adrenal medullary cells by a process dependent on extracellular Ca^{2+} [1]. Studies using $^{45}Ca^{2+}$ showed that stimulation of nicotinic ACh receptor results in increased uptake of $^{45}Ca^{2+}$ into the cells and subsequent increase in catecholamine release from the cells [2–5]. Bovine adrenal medullary cells have both nicotinic and muscarinic receptors on their surface. Stimulation of muscarinic ACh receptor does not cause uptake of $^{45}Ca^{2+}$ and release of catecholamine [3], but increases PI turnover [6–8], the cyclic GMP level [9,10] and $^{45}Ca^{2+}$ efflux from the cells [3,11]. However, the role of muscarinic receptor in bovine adrenal medullary cells is still unknown.

Recently, it has become possible to measure intracellular $[Ca^{2+}]_i$ in bovine adrenal medullary cells both in the resting condition and during stimulation by secretagogues [12–14].

In this study, we examined the roles of nicotinic and muscarinic receptors in mobilization of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} using the fluorescent dye quin2. While the preparation of this manuscript was in progress, Kao and Schneider reported that stimulation of the muscarinic receptor causes an increase in $[Ca^{2+}]_i$ which is independent of extracellular Ca^{2+} [15].

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Abbreviations: ACh, acetylcholine; TMB-8, 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PDD, 4α-phorbol-12,13-didecanoate; PI, phosphatidylinositol

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2. MATERIALS AND METHODS

Isolated cells were prepared in large quantity by sequential digestion of bovine adrenal medullary slices with collagenase as described [16]. Adrenal medullary cells at a density of 7×10^6 per ml were incubated with quin2 acetoxymethyl ester (10 μM) in Krebs-Ringer phosphate buffer containing Ca^{2+} at 37°C for 1 h. The intracellular quin2 concentration was calculated to be approx. 1 mM. The cells were then washed with Ca^{2+}-free Ringer solution and kept in the same solution for 30 min at 0°C before starting experiments. All experiments were done at 37°C and fluorescence was measured simultaneously with a spectrofluorophotometer (excitation 339 nm, emission 492 nm). Cells were pre-incubated at 37°C for 5 min with or without cholinergic antagonists, TMB-8, TPA or PDD before challenge with ACh or nicotine. When necessary, EGTA (adjusted to the required pH) was added 2 min before stimulation. Fluorescence measurements were interrupted for addition of a stimulator and/or gentle up and down mixing of the cell suspension by pasteur pipette. After each experiment, digitonin at 50 μg/ml was added to lyse the cells and $[Ca^{2+}]_i$ concentration was calculated from the intracellular trapped quin2 as described in [17].

3. RESULTS AND DISCUSSION

Fig.1 shows the changes in fluorescence of quin2-loaded cells after exposure to different concentrations of ACh in the presence of extracellular Ca^{2+} at 2.2 mM. $[Ca^{2+}]_i$ was increased dose-dependently by ACh and was maximal with 100 μM ACh. The concentrations of $[Ca^{2+}]_i$ both in the resting condition and after stimulation by ACh were similar to those in [12,14].

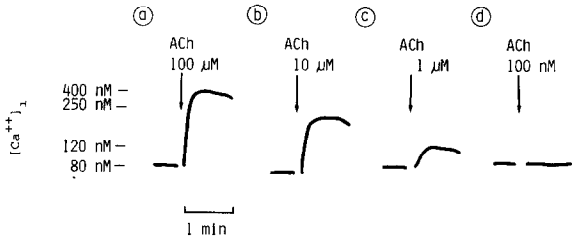

Fig.1. Effect of various concentrations of ACh (a–d) on the $[Ca^{2+}]_i$ level in quin2-loaded isolated bovine adrenal medullary cells in the presence of extracellular Ca^{2+} at 2.2 mM. The arrow indicates the time of addition of ACh to the cuvette containing 2 ml of cell suspension (7×10^6 cells/ml) and then mixing by gentle up and down movement of the cell suspension. Cell fluorescence was recorded before and after the addition of ACh.

Fig.2 shows the increases in $[Ca^{2+}]_i$ in adrenal medullary cells incubated without Ca^{2+} with different concentrations of ACh. When the cells were incubated in the absence of extracellular Ca^{2+}, the resting $[Ca^{2+}]_i$ decreased. Subsequent stimulation with ACh resulted in slight increase in $[Ca^{2+}]_i$. Nicotine (10 μM) also caused slight increase in $[Ca^{2+}]_i$. This increase in $[Ca^{2+}]_i$ in the presence of nicotine may be due to nicotinic receptor-mediated uptake of Ca^{2+} by the cells, because the incubation medium contained a micromolar concentration of Ca^{2+}. To clarify this point, we stimulated quin2-loaded adrenal medullary cells after removal of extracellular Ca^{2+} by addition of 2 mM EGTA (fig.3). In this medium, nicotine did not change the $[Ca^{2+}]_i$ level, whereas ACh caused increase in $[Ca^{2+}]_i$, possibly by release of Ca^{2+} from the in-

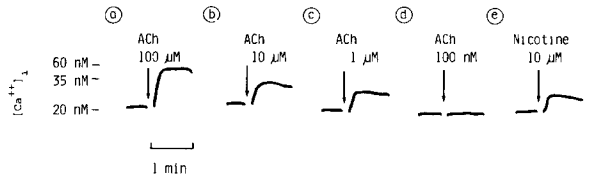

Fig.2. Changes in $[Ca^{2+}]_i$ caused by ACh (a–d) and nicotine (e) in medium without added Ca^{2+} (containing a micromolar concentration of contaminating Ca^{2+}). Cells were pre-incubated at 37°C for 5 min and then ACh or nicotine was added at the time indicated by an arrow.

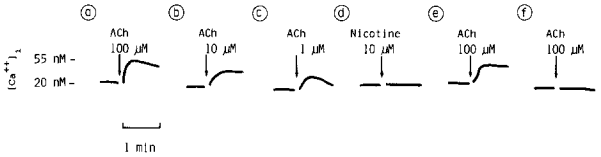

Fig.3. Effect of cholinergic agonist on the $[Ca^{2+}]_i$ level in Ca^{2+} free medium in the absence or presence of antagonist. Cells were pre-incubated for 5 min in the absence (a–d) or presence of an antagonist, such as hexamethonium 100 μM (e) or atropine 10 μM (f). EGTA was added at 2 mM to all cell suspensions 2 min before the addition of ACh or nicotine.

tracellular pool. Atropine (10 μM), a muscarinic antagonist, inhibited ACh-induced increase in $[Ca^{2+}]_i$ whereas hexamethonium (100 μM), a nicotinic antagonist, did not affect ACh-induced increase in $[Ca^{2+}]_i$. These results indicate that the ACh-induced increase in $[Ca^{2+}]_i$ is caused mainly by uptake of Ca^{2+} into the cells by nicotinic receptor stimulation and partly by release of Ca^{2+} from the intracellular Ca^{2+} pool by muscarinic receptor stimulation. The increase in $[Ca^{2+}]_i$ mediated by muscarinic receptor does not seem sufficient for initiation of catecholamine secretion [15].

The effect of the intracellular Ca^{2+} antagonist TMB-8 [18] on muscarinic receptor-mediated increase in $[Ca^{2+}]_i$ was examined. As shown in fig.4, TMB-8 (100 μM) inhibited ACh-induced increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, indicating that TMB-8 inhibits Ca^{2+} release from the intracellular pool mediated by muscarinic receptor stimulation. This result seems consistent with the report that TMB-8 inhibited carbamylcholine-stimulated $^{45}Ca^{2+}$ efflux from $^{45}Ca^{2+}$-preloaded cultured cells [19].

Next we examined whether protein kinase C regulates Ca^{2+} mobilization using phorbol ester to activate protein kinase C [20]. Results showed that the muscarinic receptor-mediated increase in $[Ca^{2+}]_i$ was inhibited by TPA, but not by PDD, a phorbol ester analog that does not activate protein kinase C [20]. TPA was inhibitory at concentrations of above 1 nM and caused complete inhibition at a concentration of 100 nM (fig.4). These findings suggest that the phorbol ester TPA inhibited muscarinic receptor-mediated Ca^{2+} release from the intracellular pool by activating protein kinase C.

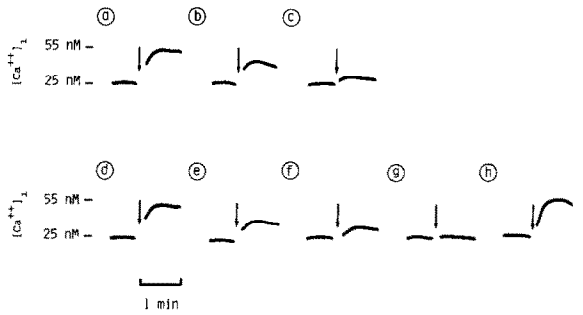

Fig.4. Effect of TMB-8, TPA and PDD on ACh-induced change in $[Ca^{2+}]_i$ level. Cell suspensions were pre-incubated for 5 min in Ca^{2+}-free medium in the absence (a,d) or presence of 1 μM TMB-8 (b), 100 μM TMB-8 (c), 1 nM TPA (e), 10 nM TPA (f), 100 nM TPA (g) or 100 nM PDD (h). EGTA was added at 2 mM to cell suspensions 2 min before addition of ACh (100 μM) at the time indicated by an arrow.

The Ca^{2+}-ionophore A23187 also stimulated the release of Ca^{2+} from the intracellular Ca^{2+} pool, but its effect was not influenced by pre-treatment of the cells with TPA (100 nM) (not shown).

In bovine adrenal medullary cells, stimulation of the muscarinic receptor caused increase in not only PI turnover [7,8], but also the cyclic GMP level [9,10] and $^{45}Ca^{2+}$ efflux from the cells [3,11]. The possibility that cyclic GMP is involved in muscarinic receptor-mediated release of Ca^{2+} from internal stores can be excluded by the fact that increase in the cyclic GMP level by ACh is dependent on extracellular Ca^{2+} [10]. Moreover, the inhibitory effect of TPA on muscarinic receptor-mediated increase in $[Ca^{2+}]_i$ could not be overcome by addition of 1 mM dibutyryl cyclic GMP (not shown). The increase in turnover of PI does not require the presence of extracellular Ca^{2+} [21]. It is known that increase in PI turnover leads to formation of inositol phosphate and diacylglycerol [22–24]. Inositol 1,4,5-triphosphate may be involved in mobilization of Ca^{2+} from its intracellular pool [25–27], and diacylglycerol, like TPA, stimulates protein kinase C [20,23,24]. It is of particular interest that diacylglycerol, which is formed concomitantly with inositol phosphate, inhibits Ca^{2+} mobilization by activation of protein kinase C.

Recently, a similar inhibitory action of phorbol ester on receptor-mediated intracellular Ca^{2+} movement has been observed in other tissues, such as isolated hepatocytes [28] and neutrophils [29].

To summarize, these results demonstrate by direct measurement that in isolated bovine adrenal medullary cells, increase in $[Ca^{2+}]_i$ released from the intracellular pool is mediated by muscarinic stimulation. The phorbol ester TPA inhibited this increase in $[Ca^{2+}]_i$, possibly by activating protein kinase C, suggesting that protein kinase C may have a negative influence on muscarinic receptor-mediated Ca^{2+} release from the intracellular pool.

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