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SUPPRESSION BY PHOSPHOLIPASE A_2 INHIBITORS OF SECRETION OF CATECHOLAMINES FROM ISOLATED ADRENAL MEDULLARY CELLS BY SUPPRESSION OF CELLULAR CALCIUM UPTAKE

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Abstract—The involvement of phospholipase A_2 in the secretion of catecholamines and cellular uptake of $^{45}Ca^{2+}$ was investigated in isolated bovine adrenal medullary cells. In these cells, stimulation of cholinergic receptors by carbamylcholine causes the activation of receptor-linked Ca-channels and influx of Ca^{2+} is known to trigger the secretory process. Phospholipase A_2 inhibitors, such as quinacrine, chloroquine, quinine and *p*-bromophenacyl bromide, all inhibited the secretion of catecholamines evoked by carbamylcholine in a dose-dependent manner. These phospholipase A_2 inhibitors also inhibited the cellular uptake of $^{45}Ca^{2+}$ evoked by carbamylcholine with similar dose–response curves to those for inhibition of catecholamine secretion. The inhibition by phospholipase A_2 inhibitors was found to be distinct from inhibition by *d*-tubocurarine which competitively blocks acetylcholine receptors, and from inhibition by diltiazem which acts as a Ca-antagonist at Ca-channels. Phospholipase A_2 inhibitors seem to suppress the secretion of catecholamines by interfering with the linkage between acetylcholine receptors and Ca-channels by the membrane effects including the inhibition of endogenous phospholipase A_2 activity of the adrenal medullary cells.

Stimulation of the acetylcholine receptor of adrenal medullary cells causes a rapid and prominent uptake of Ca^{2+} by the cells which is the prerequisite for catecholamine secretion: stimulus-secretion coupling [1]. In these cells, acetylcholine has been shown to alter the metabolism of membrane phospholipids during stimulus-secretion coupling, and to increase the ^{32}P incorporation into membrane phospholipids [2, 3] and the release of prostaglandin from the cells [4]. However, the causal relation between the metabolism of membrane phospholipid and the secretion of catecholamines has not been fully understood. It is also very important to know how neurotransmitter–receptor interaction initiates the cellular uptake of Ca^{2+} which leads to the secretion of catecholamines.

Recently, it has been shown that activation of cellular phospholipase A_2 [EC 3.1.1.4] is a critical step in the initiation of Ca^{2+}-dependent cell functions [5–7]. Receptor-mediated activation of phospholipase A_2 has been shown to be involved in the acceleration of the deacylation–reacylation cycle of membrane phospholipids leading to the alteration of phospholipid turnover and compositions [8, 9] and the release of arachidonic acid and subsequent formation of prostaglandins [4, 10, 11]. Conversely, drugs with phospholipase A_2 inhibiting properties, e.g. quinacrine, propranolol and local anesthetics, have been shown to inhibit agonist-induced cellular responses [12, 13].

In this paper, in an attempt to clarify the involvement of phospholipase A_2 in the secretion of adrenal catecholamines, we investigate the effect of quinacrine, chloroquine, quinine and *p*-bromophenacyl bromide on carbamylcholine-evoked secretion of catecholamines and cellular uptake of Ca^{2+} in isolated bovine adrenal medullary cells, since these compounds have been reported to be phospholipase A_2 inhibitors.

MATERIALS AND METHODS

Cell preparation. Fresh bovine adrenal glands from a local slaughterhouse were used throughout. Adrenal medullary cells were isolated by stepwise collagenase digestion of adrenal medullary slices as reported previously [14]. Isolated cells were suspended in Krebs-Ringer phosphate (KRP)† buffer (NaCl 154 mM, KCl 5.6 mM, $CaCl_2$ 2.2 mM, $MgCl_2$ 1.1 mM, glucose 10 mM and NaH_2PO_4 0.85 mM–Na_2HPO_4 2.15 mM, pH 7.4) containing 0.5% BSA and used in the experiments on catecholamine secretion and $^{45}Ca^{2+}$ uptake.

Catecholamine secretion. Secretion of catecholamines was started by the addition of a cell suspension (10^6 cells in 0.5 ml of KRP) to 1.5 ml of preheated KRP (37°, 5 min) which contained carbamylcholine as stimulant or other test compounds. Incubation was carried out for 1 min and terminated by transferring the incubation tube to an ice-cold bath and 5 min later cells were sedimented by centrifugation at 600 *g* for 5 min. Catecholamines secreted into the medium were estimated by the ethylenediamine condensation method after con-

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† Abbreviations: KRP, Krebs–Ringer phosphate; BSA, bovine serum albumin; IC_{50}, half-maximal inhibitory concn.

densation by aluminium hydroxide adsorption [15].

$^{45}Ca^{2+}$ uptake. For measurement of cellular uptake of Ca^{2+}, 1.5 ml of KRP containing 1.5 μCi of $^{45}CaCl_2$, 3×10^{-4} M carbamylcholine and 0.5% BSA was preheated at 37° for 5 min, and then cells (4×10^6 cells in 0.5 ml of KRP) were added to the medium and incubated for 1 min. The reaction was terminated by the addition of hexamethonium (final concn 10^{-3} M) and transferring the tubes into an ice-cold bath. Cells were sedimented by centrifugation and washed 4 times with 5 ml of ice-cold Ca^{2+}-free KRP containing 0.5% BSA. Cells were finally solubilized in 10% Triton X-100 and $^{45}Ca^{2+}$ radioactivity was measured by a liquid scintillation counter with an efficiency of 78%.

Materials. Carbamylcholine and chloroquine were from Sigma. Quinacrine, quinine and hexamethonium were from Nakarai Chemical Co. Ltd, Japan. *p*-Bromophenacyl bromide was from Aldrich. Diltiazem was from Tanabe Seiyaku Co. Ltd, Japan. $^{45}CaCl_2$ (0.8 Ci/mmole) was purchased from Amersham International Ltd. All the chemicals were dissolved in water except *p*-bromophenacyl bromide which was insoluble in water and dissolved in dimethylsulfoxide. The final concn of the vehicle in the reaction mixture was always less than 0.5% and this concn of dimethylsulfoxide did not affect the secretion of catecholamines and the uptake of $^{45}Ca^{2+}$ by itself.

RESULTS

The spontaneous secretion of catecholamines during the 1-min incubation period was less than 1% of the total catecholamines in the cells. Stimulation with carbamylcholine caused a rapid secretion of catecholamines which was transient and levelled off within 1 min. The half-maximal concn of carbamylcholine for secretion of catecholamines was 3.3×10^{-5} M and the maximal concn was 3×10^{-4} M. Under the maximal conditions, 6.6 ± 0.5% of catecholamines in the cells were secreted into the medium. The phospholipase A_2 inhibitors, quinacrine, chloroquine, quinine and *p*-bromophenacyl bromide, all inhibited the secretion of catecholamines evoked by carbamylcholine in dose-dependent manners (Fig. 1). The inhibitory effects of quinacrine, chloroquine and quinine were reversible

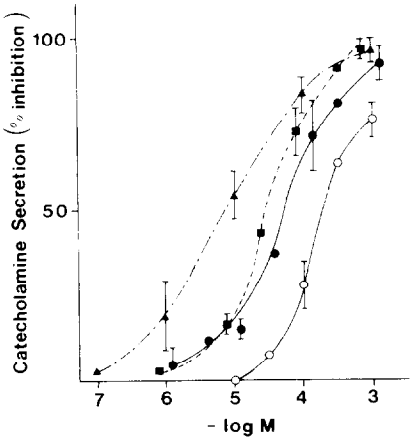

Fig. 1. Effect of phospholipase A_2 inhibitors on carbamylcholine-induced secretion of catecholamines from isolated adrenal medullary cells. Cells (10^6 cells/tube) were incubated in 2 ml of KRP buffer (NaCl 154 mM, KCl 5.6 mM, $CaCl_2$ 2.2 mM, $MgCl_2$ 1.1 mM, glucose 10 mM, and NaH_2PO_4 0.85 mM–Na_2HPO_4 2.15 mM, pH 7.4) containing 0.5% BSA and stimulated for 1 min by carbamylcholine (3×10^{-4} M) with or without phospholipase A_2 inhibitors. Catecholamines secreted into the medium during 1 min of stimulation were estimated. Data show the dose–response curves for suppression by various phospholipase A_2 inhibitors of carbamylcholine-induced catecholamine secretion. Abscissa represents concns of phospholipase A_2 inhibitors. (▲----▲) Quinacrine, (●——●) chloroquine, (■-----■) quinine, (○——○) *p*-bromophenacyl bromide. The data are means ± S.D. from four to six separate experiments.

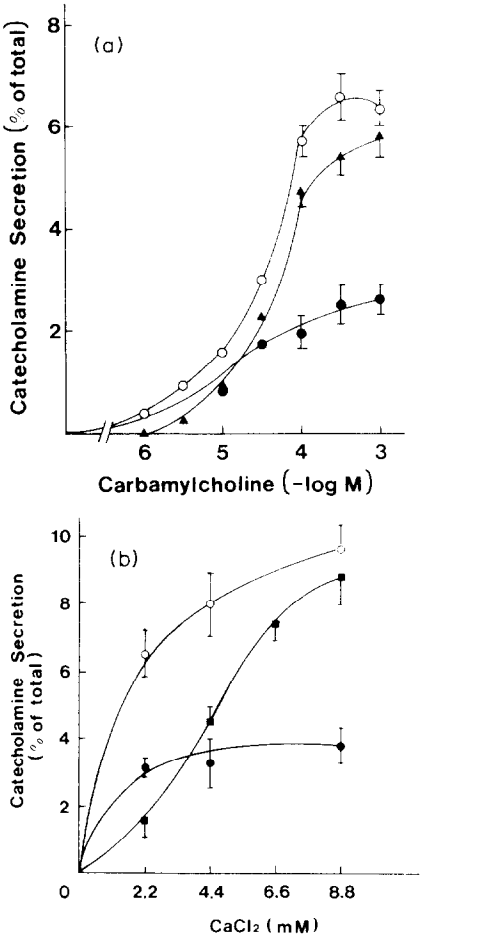

Fig. 2. Inhibition of carbamylcholine-induced catecholamine secretion by phospholipase A_2 inhibitors under various concns of carbamylcholine and Ca^{2+}. (a) Cells (10^6 cells/tube) were stimulated by various concns of carbamylcholine for 1 min and the inhibitory effects of *d*-tubocurarine (7×10^{-7} M) and quinacrine (10^{-5} M) were measured. Control (○), *d*-tubocurarine (▲), quinacrine (●). The data are means ± S.D. from three to seven separate experiments. (b) Cells (10^6 cells/tube) were stimulated by carbamylcholine (3×10^{-4} M) for 1 min under various concns of Ca^{2+} and the effect of diltiazem (3×10^{-6} M) and quinacrine (10^{-5} M) were examined. Control (○), diltiazem (■), quinacrine (●). The data are means ± S.D. from three to four separate experiments. Catecholamines secreted into the medium were expressed as % of total catecholamines in the cells.

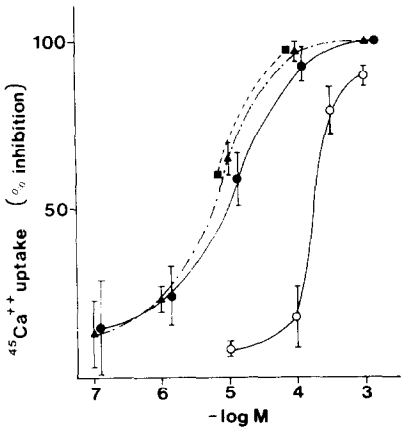

Fig. 3. Effect of phospholipase A_2 inhibitors on carbamylcholine-induced $^{45}Ca^{2+}$ uptake by isolated adrenal medullary cells. Cells (4×10^6 cells/tube) were incubated with $^{45}CaCl_2$ (1.5 μCi, 3.3×10^6 cpm) in 2 ml of KRP buffer (pH 7.4) containing 0.5% BSA and stimulated for 1 min by carbamylcholine (3×10^{-4} M) with or without phospholipase A_2 inhibitors. The cells were washed 4 times with Ca^{2+}-free KRP buffer containing 0.5% BSA and $^{45}CaCl_2$ in the cells was extracted and counted by a liquid scintillation counter. Data show the dose–response curves for suppression by various phospholipase A_2 inhibitors of carbamylcholine-induced $^{45}Ca^{2+}$ uptake. $^{45}Ca^{2+}$ uptake in control experiments was 4460 ± 1710 cpm/4×10^6 cells. Abscissa represent concns of phospholipase A_2 inhibitors. (▲——▲) Quinacrine, (●——●) chloroquine, (■-----■) quinine, (○——○) *p*-bromophenacyl bromide. The data are means ± S.D. from four separate experiments.

while that of *p*-bromophenacyl bromide was irreversible (data not shown). These compounds were not cytotoxic at the concns used.

Secretion of adrenal catecholamines has been reported to be inhibited by *d*-tubocurarine and diltiazem, the former by direct competition at cholinergic receptor sites and the latter by direct antagonism of Ca^{2+}-channels [16, 17]. In order to clarify the mode by which phospholipase A_2 inhibitors suppressed carbamylcholine-evoked secretion of catecholamines, we examined whether the increase in concns of carbamylcholine or Ca^{2+} in the medium could overcome the inhibitory effects of these compounds. Inhibition of catecholamine secretion by quinacrine was not restored either by the increase in carbamylcholine or Ca^{2+} concns, although inhibition by *d*-tubocurarine was restored by the increase in carbamylcholine concn and that by diltiazem was overcome by the increase in Ca^{2+} concn (Fig. 2a and b). Similar results were obtained for the inhibitory effects of chloroquine and quinine. These observations indicate that the inhibitory effect of phospholipase A_2 inhibitors was not due either to the competition at cholinergic receptors or to the antagonism of Ca^{2+}-channels.

In stimulus-secretion coupling, cellular uptake of Ca^{2+} has been shown to be the critical step in triggerring catecholamine secretion. Accordingly, carbamylcholine, nicotine, high K^+ medium and Ca^{2+} ionophore A 23187 cause the secretion of catecholamines by promoting the cellular uptake of Ca^{2+} [1, 18, 19]. Therefore, it is very important to investigate whether phospholipase A_2 inhibitors alter the cellular uptake of Ca^{2+}. Carbamylcholine (3×10^{-4} M) induced a rapid uptake of $^{45}Ca^{2+}$ which showed a similar time course to that of catecholamine secretion. The phospholipase A_2 inhibitors, quinacrine, chloroquine, quinine and *p*-bromophenacyl bromide, all inhibited carbamylcholine-induced uptake of $^{45}Ca^{2+}$ in dose-dependent manners (Fig. 3). The IC_{50} of these compounds to the inhibition of cellular $^{45}Ca^{2+}$ uptake were close to that of catecholamine secretion.

DISCUSSION

Recently, evidence has been presented that acceleration of membrane phospholipid metabolism by phospholipase A_2 alters the physicochemical properties of the membranes and is involved in the manifestation of a variety of cell functions such as membrane fusion [20], increased ion permeability [21] and coupling of β-adrenergic receptor with adenyl cyclase [22]. The secretion of adrenal catecholamines has been shown to occur by exocytosis and fusion of chromaffin granules with plasma membranes is the most critical step in exocytosis [23, 24]. In this paper, we examined the role of phospholipase A_2 in the secretion of catecholamines from isolated adrenal medullary cells.

It has been well established that influx of Ca^{2+} into the cells is of the greatest importance in triggerring the secretion of adrenal catecholamines [1, 18, 19, 23]. In our present experiments, phospholipase A_2 inhibitors such as quinacrine, chloroquine, quinine and *p*-bromophenacyl bromide suppressed the secretion of catecholamines with simultaneous inhibition of cellular uptake of Ca^{2+}. These findings strongly indicate that inhibition of catecholamine secretion by these compounds has resulted from inhibition of cellular uptake of Ca^{2+}. We also demonstrated that those compounds had inhibited the secretion of catecholamine by a mechanism which is distinct from competition at receptor sites or direct antagonism of Ca^{2+}-channels. Therefore, in adrenal medullary cells, it will be postulated that receptor-mediated activation of phospholipase A_2 could alter the phospholipid turnover which, in turn, might lead to the increase in cellular Ca^{2+} availability. The signal generated from receptor stimulation seems to be transduced to putative Ca^{2+}-channels only when metabolism of membrane phospholipids is accelerated.

However, it is very important to distinguish whether these compounds have inhibited the uptake of Ca^{2+} via the inhibition of phospholipase A_2 or by a mechanism which is unrelated to phospholipase A_2 inhibition. Recently, quinacrine has been shown to modify phospholipid metabolism by an action unrelated to the phospholipase A_2 inhibition and it also has calmodulin antagonistic properties [25, 26]. Therefore, for the interpretation of the effects of so-called phospholipase A_2 inhibitors, caution should be exercised concerning how they actually modify the phospholipid metabolism of the cells, and such work is under progress in this laboratory.

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