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PROSTAGLANDIN E_2-INDUCED ARACHIDONIC ACID RELEASE AND CATECHOLAMINE SECRETION FROM CULTURED BOVINE ADRENAL CHROMAFFIN CELLS

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Abstract—We recently reported that prostaglandin E_2 (PGE_2) and arachidonic acid (AA) each induced a gradual secretion of catecholamines from cultured bovine adrenal chromaffin cells in the presence of ouabain by stimulation of phosphoinositide metabolism. In the present study, we examined the relationship between phospholipase A_2 and C activation and catecholamine secretion by PGE_2 in chromaffin cells. The phospholipase A_2 inhibitors *p*-bromophenacyl bromide and mepacrine did not affect the basal and ouabain-induced release, but dose-dependently blocked PGE_2-evoked phosphoinositide metabolism and the consequent catecholamine release at an IC_{50} value of 3 μM. PGE_2 induced rapid hydrolysis of [^{3}H]AA from prelabeled phospholipid pools: the release of [^{3}H]AA could be detected at as early as 15 sec and reached a plateau after 1 min. While the phospholipase C inhibitor neomycin did not inhibit PGE_2-induced AA release, phospholipase A_2 inhibitors dose-dependently inhibited it at IC_{50} values comparable to those for catecholamine release. Pretreatment of intact cells with the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate, but not with pertussis toxin, prevented AA release by PGE_2. These results demonstrate that PGE_2 activates phospholipase A_2 as well as phospholipase C in a pertussis toxin-insensitive manner and suggest that the released arachidonic acid may be involved in PGE_2-induced catecholamine release from chromaffin cells.

Bovine adrenal chromaffin cells secrete catecholamines on stimulation by cholinergic agonists or by depolarization of the plasma membrane [1, 2]. Although a transient increase in the intracellular free Ca^{2+} concentration appears to be the primary trigger for initiating release from the cells, exactly how Ca^{2+} acts to bring about exocytosis is still unknown. Recently, there has been considerable interest in exocytotic responses to the stimulation of non-nicotinic cell surface receptors of such molecules as histamine [3, 4], angiotensin II [5], bradykinin [6], GABA [7] and prostaglandins (PGs‡) [8, 9], all of which have in common the ability to stimulate phosphoinositide metabolism through their respective receptors. Previous studies in this laboratory [10–13] demonstrated that PGE_2 evoked a gradual secretion of catecholamines in the presence of ouabain by stimulation of phosphoinositide metabolism and that the activation of protein kinase C resulting from the stimulation of phosphoinositide metabolism, rather than Ca^{2+} mobilization, was mainly involved in PGE_2-induced catecholamine release. In many cases, agonist-stimulated phosphoinositide metabolism is accompanied by phospholipase A_2 activation with consequent liberation of free arachidonic acid (AA) from cellular phospholipids [14]. We observed recently that the addition of AA to chromaffin cells induced a dose-dependent stimulation of phosphoinositide metabolism and catecholamine secretion in the presence of ouabain [15]. These effects were specific for AA and were not dependent on AA metabolism by known cyclooxygenase and lipoxygenase pathways. These findings suggest that AA could serve as a link between phospholipase A_2 activation and phospholipase C activation, but the relationship between the two pathways remains unclear. In the present study, we studied the effect of PGE_2 on AA release and its relationship to PGE_2-induced secretion of catecholamines from chromaffin cells. A preliminary report of a part of this work will appear in abstract form [16].

MATERIALS AND METHODS

Materials. PGs were generous gifts from Ono Pharmaceuticals (Osaka, Japan). [^{3}H]AA (2812 GBq/mmol) and *myo*-[2-^{3}H]inositol (740 GBq/mmol) were purchased from Du Pont-New England Nuclear (Boston, MA, U.S.A.) and Amersham (U.K.) respectively, and *p*-bromophenacyl bromide (pBPB) and mepacrine, from Nacalai Tesque (Kyoto, Japan). Pertussis toxin was from Seikagaku Kogyo (Tokyo, Japan); 12-*O*-tetradecanoylphorbol 13-acetate (TPA) from

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‡ Abbreviations: PG, prostaglandin; AA, arachidonic acid; pBPB, *p*-bromophenacyl bromide; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; 4α-PDD, 4α-phorbol 12,13-didecanoate; IP, inositol phosphate; HBS, HEPES-buffered saline solution; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; G-protein, GTP-binding protein.

Funakoshi Pharmaceuticals (Tokyo); 4α-phorbol 12,13-didecanoate (4α-PDD) from Boehringer Mannheim; and neomycin sulfate from Wako Pure Chemicals (Osaka). All other chemicals were of reagent grade.

Cell culture and catecholamine release. Chromaffin cells were prepared from bovine adrenal medulla by collagenase digestion and purification on Percoll gradients as described previously [10]. The purity of chromaffin cells in our preparation was more than 90%. Cells were cultured for 3–4 days prior to use in Dulbecco's modified Eagle's medium [or when measuring inositol phosphates (IPs), in Ham's F-10 medium] supplemented with 10% heat-inactivated fetal calf serum, cytosine arabinoside (2.8 μg/mL), streptomycin (100 μg/mL), penicillin (100 U/mL), and nystatin (250 U/mL). In experiments on catecholamine release, cells cultured in 24-well plates (2×10^5 cells/well) were washed with HEPES-buffered saline solution (HBS) containing 125 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 15 mM $NaHCO_3$, 11 mM glucose and 15 mM HEPES (pH 7.4), and reactions were started by the addition of test agents unless otherwise indicated. Secretion was determined by measurement of the percentage of total cellular catecholamines (norepinephrine and epinephrine) released into the medium [17].

Measurement of [^{3}H]IP formation. Chromaffin cells cultured in 35-mm dishes (3×10^6 cells/dish) precoated with poly-L-lysine were labeled with [^{3}H]-inositol (111 kBq/dish) for 45–60 hr. The cells were then washed three times with HBS and preincubated with HBS containing 10 mM LiCl for 10 min at 37°. Reactions were started by the addition of test agents unless otherwise indicated. After incubation for 2 min at 37°, the medium was quickly aspirated; and 5% trichloroacetic acid was then added to each dish. Separation of [^{3}H]IPs was carried out by Bio-Rad AG1 × 8 chromatography essentially as described previously [18]. Inositol monophosphate (IP_1), inositol bisphosphate (IP_2) and inositol trisphosphate (IP_3) were eluted serially with 5 mM disodium tetraborate–180 mM sodium formate, 0.1 M formic acid–0.4 M ammonium formate and 0.1 M formic acid–1.0 M ammonium formate. Radioactivity in the eluates was determined by scintillation counting using Clearsol (Nacalai Tesque).

Release of [^{3}H]AA. Suspended chromaffin cells at a density of 10^6 cells/mL were labeled with 7.4 kBq/mL of [^{3}H]AA for 30 min at 37° in HBS. The cells were then washed twice with HBS and incubated in fresh HBS for 30 min at the same cell density, and again washed twice with HBS. The cells thus labeled with [^{3}H]AA (10^6 cells/mL) were incubated for the indicated times with test agents. After the incubation, the cells were quickly chilled and centrifuged at 500 *g* for 30 sec, and the medium was analysed for the radioactivity. More than 90% of [^{3}H]AA released in the medium remained intact as analysed by TLC.

RESULTS

Effect of phospholipase A_2 inhibitors on PGE_2-induced catecholamine release

Recently, we demonstrated that PGE_2 and AA each evoked a gradual secretion of catecholamines from bovine adrenal chromaffin cells in the presence of ouabain with a similar time course [11, 15]. To assess the participation of AA in the PGE_2-induced catecholamine release, we examined the effect of pBPB and mepacrine, phospholipase A_2 inhibitors, on the release. As shown in Fig. 1, neither pBPB nor mepacrine had any effect on the basal or

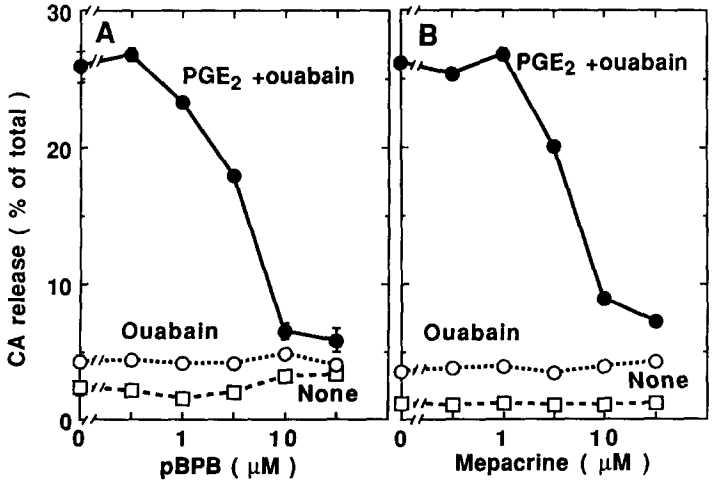

Fig. 1. Dose dependency of pBPB and mepacrine for inhibition of PGE_2-induced catecholamine release. After cells had been preincubated for 10 min at 37° with the indicated concentrations of pBPB (A) or mepacrine (B), they were further incubated for 30 min at 37° without (□) or with (○) 100 μM ouabain, with 1 μM PGE_2 and 100 μM ouabain (●). The percentage of catecholamines released into the medium was determined as described in Materials and Methods. Values shown are the means ± SE of triplicate experiments.

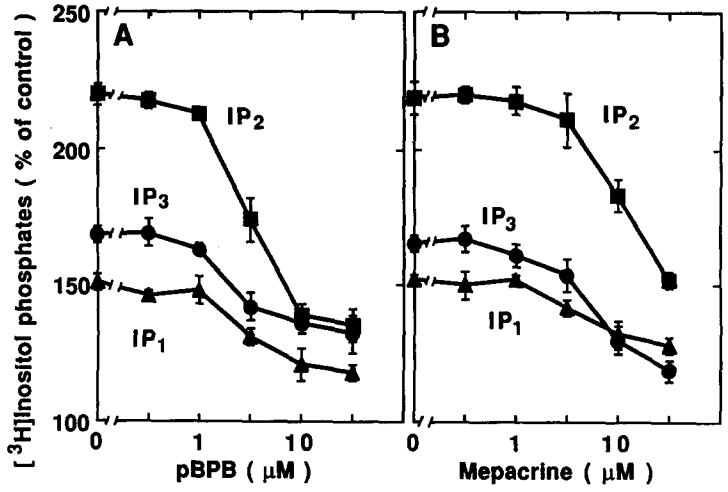

Fig. 2. Dose dependency of pBPB and mepacrine for inhibition of PGE_2-induced [^{3}H]IP formation in chromaffin cells. After [^{3}H]inositol-labeled cells had been preincubated for 10 min at 37° with the indicated concentrations of pBPB (A) or mepacrine (B), the cells were further incubated for 2 min at 37° with 1 μM PGE_2. [^{3}H]IPs formed were measured as described in Materials and Methods. The values (means ± SE, N = 3) of IP_1 (▲), IP_2 (■) and IP_3 (●) are expressed as percentages of control.

ouabain-induced release. On the other hand, these agents dose-dependently inhibited PGE_2-induced catecholamine release in the presence of ouabain with a half-maximal inhibitory concentration of 3 μM. In previous experiments, we demonstrated that both PGE_2- and AA-induced catecholamine release in the presence of ouabain is mediated by stimulation of phosphoinositide metabolism [10, 14]. Therefore, we next examined the effect of pBPB and mepacrine on PGE_2-induced [^{3}H]IP formation. As reported previously [10], 1 μM PGE_2 induced a rapid formation of IPs in chromaffin cells, and [^{3}H]IP_1, [^{3}H]IP_2 and [^{3}H]IP_3 levels (mean ± SE, N = 3) at 2 min were 151 ± 3, 220 ± 4 and 168 ± 3% of the control, respectively. As shown in Fig. 2A, pBPB at a 10 μM concentration reduced PGE_2-induced IP formation by 65% for IP_1, 70% for IP_2 and 53% for IP_3. The inhibitory effect of pBPB on PGE_2-enhanced [^{3}H]IP formation (Fig. 2A) was parallel to that on PGE_2-evoked catecholamine release (Fig. 1A). Mepacrine also inhibited PGE_2-induced [^{3}H]-IP formation similarly, while the concentration that caused half-maximal inhibition of [^{3}H]IP formation was slightly higher than that causing half-maximal inhibition of catecholamine release (compare Fig. 1B with Fig. 2B).

Release of [^{3}H]*AA by* PGE_2 *from bovine chromaffin cells*

To further explore the possibility of participation of AA in PGE_2-induced catecholamine release, we examined whether PGE_2 could release [^{3}H]AA from prelabeled chromaffin cells. As shown in Fig. 3A, 1 μM PGE_2 induced a rapid release of [^{3}H]AA into the culture medium, and [^{3}H]AA could be detected at as early as 15 sec. The [^{3}H]AA level reached a plateau after 1 min, being 700% of the control. Figure 3B shows the dose dependency of PGs for [^{3}H]AA release at 3 min. PGE_1 and PGE_2 were the most effective in causing [^{3}H]AA release, followed by $PGF_{2\alpha}$ and PGD_2. Dose–response curves of PGs for [^{3}H]AA release were quite similar to those for IP_3 formation and catecholamine release reported previously [11, 19].

In order to determine whether PGE_2-induced [^{3}H]AA release occurred by direct activation of phospholipase A_2 or required prior action of phospholipase C, we examined the effect of pBPB and mepacrine on the release. As shown in Fig. 4, both pBPB and mepacrine completely inhibited [^{3}H]AA release from prelabeled chromaffin cells with a half-maximal inhibitory concentration of 2 μM. When comparing the dose–inhibition curves of pBPB for the effects shown in Figs 1, 2 and 4, a correlation can be seen among PGE_2-induced catecholamine release in the presence of ouabain, [^{3}H]IP formation and [^{3}H]AA release. On the other hand, neomycin, a phosphatidylinositol-specific phospholipase C inhibitor, which blocked PGE_2-stimulated IP formation at 1 mM [12], had no effect on PGE_2-induced [^{3}H]AA release at 1 mM (Fig. 5). These results indicate that phospholipid hydrolysis with the release of AA by direct activation of phospholipase A_2, a process that can be inhibited by pBPB and mepacrine, may be involved in PGE_2-induced catecholamine release from chromaffin cells in the presence of ouabain. Calcium is known to play an important role in the activation of phospholipase A_2 as well as phospholipase C. In a Ca^{2+}-free medium, PGE_2 failed to stimulate [^{3}H]-AA release.

To study further the signal transduction coupled to PGE_2, we examined the effect of the phorbol esters TPA and 4α-PDD, and pertussis toxin on [^{3}H]AA release induced by PGE_2. Although a 10-min pretreatment of chromaffin cells with 1 μM TPA

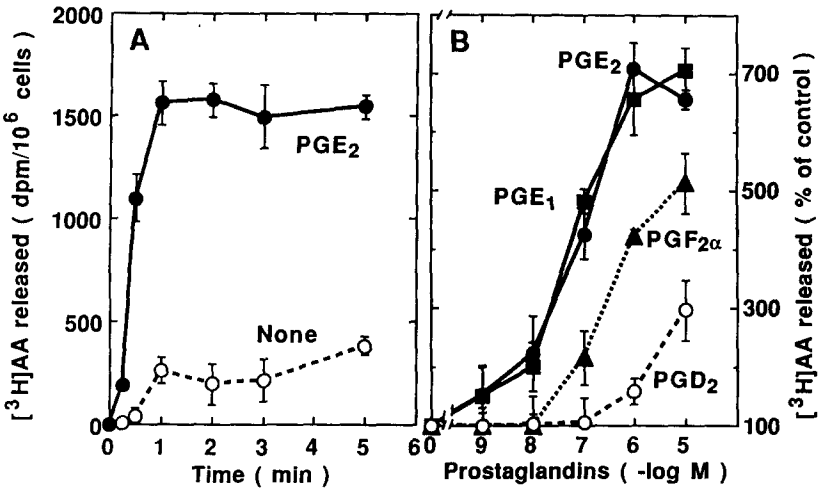

Fig. 3. Time course and dose dependency of the effect of PGs on [^{3}H]AA release from cultured bovine adrenal chromaffin cells. (A) Chromaffin cells were incubated at 37° for the indicated time without (○) or with (●) 1 μM PGE_2. (B) Chromaffin cells were incubated for 3 min at 37° with the indicated concentrations of PGE_1 (■), PGE_2 (●), $PGF_{2\alpha}$ (▲) or PGD_2 (○). [^{3}H]AA release was determined as described in Materials and Methods. Values shown are the means ± SE of triplicate experiments. The values of B are expressed as percentages of control.

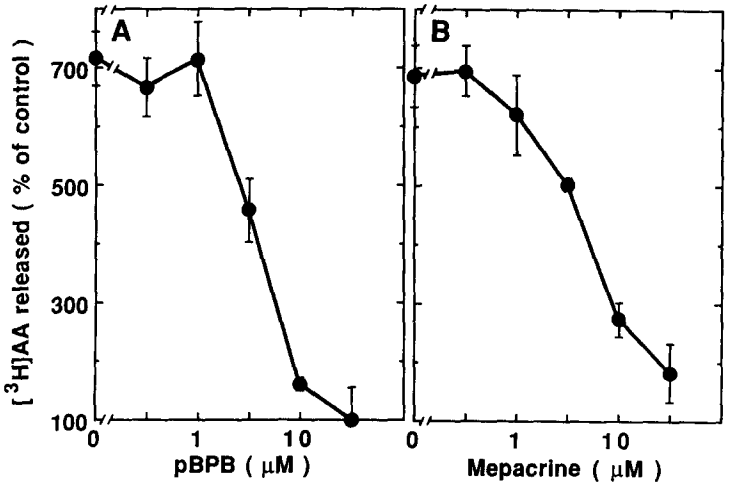

Fig. 4. Dose dependency of pBPB and mepacrine for inhibition of PGE_2-induced AA release. Chromaffin cells labeled with [^{3}H]AA were incubated for 3 min with 1 μM PGE_2 in the presence of various concentrations of pBPB (A) or mepacrine (B). [^{3}H]AA release was determined as described in Materials and Methods. Values shown are the means ± SE of triplicate experiments.

stimulated 2-fold the basal release of [^{3}H]AA, TPA completely inhibited PGE_2-induced [^{3}H]AA release (Fig. 5). 4α-PDD, an inactive phorbol ester analogue, did not affect either the basal or PGE_2-induced release. [^{3}H]AA release was not prevented by 6 hr pretreatment of the cells with pertussis toxin (100 ng/mL). Under these conditions, pertussis toxin-sensitive G-proteins were almost completely inactivated by ADP-ribosylation [17, 19]. These results demonstrate that PGE_2 may activate phospholipase A_2 by receptor occupation via interaction with a pertussis toxin-insensitive G-protein.

DISCUSSION

Although AA is well known as a precursor of PGE_2 and other eicosanoids, the present study demonstrates that PGE_2 induces a rapid hydrolysis of [^{3}H]AA from prelabeled chromaffin cells. The dose dependency and specificity of PGs for AA release (Fig. 3B) are quite similar to those for IP formation and diacylglycerol formation, and those for displacement of [^{3}H]PGE_2 binding to adrenal medullary membranes reported previously [10, 12, 19], indicating that these processes are

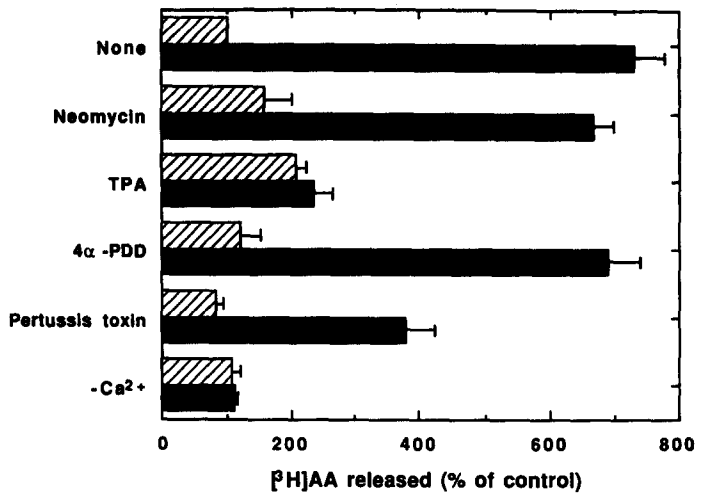

Fig. 5. Effect of various agents on [^{3}H]AA release induced by PGE_2. After cells had been pretreated for 10 min at 37° with 1 mM neomycin, 1 μM TPA or 1 μM 4α-PDD; or for 6 hr with 100 ng/mL pertussis toxin; or preincubated for 5 min in the Ca^{2+}-free solution containing 0.1 mM EGTA, they were further incubated for 3 min at 37° without (hatched column) or with (closed column) 1 μM PGE_2. Release of [^{3}H]AA was measured as described in Materials and Methods.

mediated through the PGE receptor. The receptor-mediated generation of AA occurs mainly by two mechanisms: one involving direct activation of phospholipase A_2 and the other phospholipase C followed by diglyceride lipase. The results obtained here and in previous experiments support the pathway that phospholipase A_2 activation is the more proximal event than phospholipase C activation for the following reasons: first, while the phospholipase C inhibitor neomycin did not inhibit it, the phospholipase A_2 inhibitors pBPB and mepacrine dose-dependently blocked the PGE_2-induced release of AA (Figs 4 and 5). Second, as compared with the rapid release of AA from chromaffin cells (Fig. 3A), PGE_2-induced diacylglycerol formation was fairly slow [12], and PGE_2-induced Ca^{2+} mobilization occurred after a lag time of 10–30 sec [13]. In several cell types, phospholipase A_2 and phospholipase C have been suggested to be regulated by distinct G-proteins, based on the difference in susceptibility to pertussis toxin and phorbol ester [20–24]. While TPA inhibited phosphoinositide metabolism, it had no effect on AA release or augmented the release [20–22]. However, TPA was also reported to inhibit AA release [23] and, furthermore, the effects of TPA on AA release in rabbit platelets were both stimulatory and inhibitory, depending on agonists [24]. On the other hand, pertussis toxin inhibited [^{3}H]AA release, but not phosphoinositide metabolism [23]. In our studies, pretreatment of chromaffin cells with TPA, but not with pertussis toxin, blocked PGE_2-enhanced phosphoinositide metabolism [18, 19] and PGE_2-induced [^{3}H]AA release (Fig. 5). These results suggest that these processes may occur via the same pathway in an inter-dependent manner. Recently, AA has been shown to stimulate phosphoinositide metabolism in several tissues and cells [25–27]. Assuming that phospholipase A_2 and phospholipase C are activated by the same receptor, the sites of action of TPA may be distal to the coupling of receptor to GTP-binding protein, as reported previously [18].

Bovine adrenal medulla and chromaffin cells possess high affinity binding sites for PGE_2 and PGE_1, other PGs and PG metabolites having much lower affinities [28–30]. Recent studies including those from our laboratory [8–13, 19, 30] have shown that PGE_2 stimulates catecholamine release from cultured chromaffin cells, but the results of the release by PGs are apparently not consistent. We found that PGE_2-induced catecholamine release was markedly enhanced in the presence of ouabain and that the potency of PGs to stimulate catecholamine release in the presence of ouabain was well correlated with that to displace [^{3}H]PGE_2 from the membrane, demonstrating that PG-induced release is mediated through the PGE receptor. On the other hand, other groups [8, 9] showed that $PGF_{2\alpha}$ and PGD_2 enhanced the release more than did PGE_2 at low concentrations, but that PGE_2 was the most potent at a high concentration of 10 μM. Previously PGE_2 was also reported to inhibit the basal and nicotine-induced catecholamine release from chromaffin cells [30]. Because the stimulatory effect of these PGs on the release in the absence of ouabain was weak and because the effect of PGE_2 on the release might be a sum of stimulatory and inhibitory effects, these actions appeared not to be mediated by the PG receptor identified by ligand binding studies. We assume that the stimulatory effect of PGE_2 on the release is preferentially enhanced by ouabain, which makes it easy to characterize PGE_2-induced catecholamine release from chromaffin cells.

There is growing evidence for important roles of AA in cellular signaling, in which it acts both as a precursor in eicosanoid biosynthesis and as a signal

molecule in the regulation of various cell functions [31–33]. A number of reports argue for and against the participation of AA in stimulus–secretion coupling in chromaffin cells [34–38]. We also reported that exogenous AA itself stimulated phosphoinositide metabolism, and subsequent catecholamine release from the cells in the presence of ouabain [15]. Because the phospholipase C inhibitor neomycin inhibited both PGE_2- and AA-induced phosphoinositide metabolism and subsequent catecholamine release in the presence of ouabain [12, 15], the mechanisms of catecholamine release by PGE_2 and AA appear to be similar, if not identical, but are obviously different from that of the known nicotinic pathway or high K^+ that uses membrane depolarization to induce an uptake of extracellular Ca^{2+} [1, 2], and from that of Ca^{2+}-dependent exocytosis in permeabilized chromaffin cells. Ouabain also synergistically stimulates catecholamine release induced by muscarine, GABA and AA [7, 10, 15], possible through the activation of protein kinase C, as demonstrated with PGE_2 and TPA [12]. In the present study, a striking correlation was observed with regard to dose dependency of pBPB and mepacrine for the inhibition of the stimulatory effects of PGE_2 on [^{3}H]AA release, phosphoinositide metabolism and catecholamine release in the presence of ouabain (Figs 1, 2 and 4). The IC_{50} values for pBPB and mepacrine were as low as 3 μM, and are comparable to or lower than those given in previous reports [39–41]. Although these inhibitors reportedly have a number of non-specific pharmacological and biochemical effects such as inhibition of Ca^{2+} influx at higher concentrations [42, 43], pBPB did not block the AA-induced increase in intracellular Ca^{2+} concentration at 10 μM (S. Ito, unpublished observation). This result negates the possibility that pBPB inhibited AA actions by blocking Ca^{2+} influx.

In conclusion, we postulated that for secretion to take place, both phospholipase A_2 and phospholipase C may be activated by PGE receptor occupation and that the AA released as a consequence of phospholipase A_2 activation by PGE_2 can act at least in part as a second messenger to activate phospholipase C and thus stimulate phosphoinositide metabolism and the ensuing catecholamine release.

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