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INVOLVEMENT OF PHOSPHOINOSITIDE METABOLISM IN GABA-INDUCED CATECHOLAMINE RELEASE FROM CULTURED BOVINE ADRENAL CHROMAFFIN CELLS

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Abstract—The effects of GABA on catecholamine release and phosphoinositide metabolism were studied in cultured bovine adrenal chromaffin cells. GABA and muscimol, a specific agonist for the $GABA_A$ receptor, each evoked a gradual secretion of catecholamines from the cells in the presence of ouabain, an inhibitor of Na^+,K^+-ATPase. This release was inhibited by bicuculline, a specific antagonist for the $GABA_A$ receptor, or by picrotoxin, a blocker of GABA-gated Cl^- channels, and was potentiated by diazepam or pentobarbital. GABA or muscimol induced a concentration-dependent formation of inositol phosphates. This accumulation of inositol phosphates was also inhibited by bicuculline, picrotoxin or removal of extracellular Ca^{2+}, and also potentiated by diazepam and pentobarbital. Nicardipine suppressed GABA-induced catecholamine release in the presence of ouabain and accumulation of inositol phosphates, while verapamil, diltiazem, and ω-conotoxin failed to inhibit these responses to GABA. The phosphoinositide-specific phospholipase C inhibitor neomycin also inhibited both GABA-induced accumulation of inositol phosphates and stimulation of catecholamine release in the presence of ouabain. These results taken together indicate that GABA evoked catecholamine release from the chromaffin cells in the presence of ouabain by stimulation of phosphoinositide metabolism in a Ca^{2+}-sensitive manner via activation of $GABA_A$ receptor-coupled Cl^- channels.

Adrenal chromaffin cells have as their main functions the synthesis, storage and secretion of the catecholamines epinephrine and norepinephrine. In most species thus far investigated, the secretory response is elicited via nicotinic cholinergic receptors, and a transient increase in the cytosolic Ca^{2+} appears to be the primary trigger for initiating catecholamine release from the cells [1, 2]. Recently there has been considerable interest in secretory responses to the stimulation of non-nicotinic cell surface receptors of such molecules as histamine, angiotensin II, bradykinin and prostaglandins (PGs) [3–10], and these stimulus-secretion mechanisms may involve phosphoinositide metabolism, the products of which mediate many cellular responses including stimulus-secretion coupling [11, 12]. We recently reported that a small increase in catecholamine release evoked by PGE_2 or the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate was manifested by ouabain, an inhibitor of Na^+,K^+-ATPase [9, 10]. While cardiac glycosides such as ouabain have long been known to increase the strength of contraction of cardiac muscle, exposure to ouabain enhances both spontaneous and evoked release of secretory product by causing accumulation of intracellular Na^+ followed by an increase in intracellular Ca^{2+} in secretory tissues including the adrenal medulla [13].

In the mammalian central nervous system γ-aminobutyric acid (GABA) is a neurotransmitter that causes an increase in the postsynaptic membrane conductance of Cl^- ions [14, 15]. In bovine adrenal medulla, the presence of GABA, GABA-synthesizing and metabolizing enzymes, and high-affinity binding sites for muscimol, a potent $GABA_A$ receptor agonist, and for flunitrazepam, a benzodiazepine receptor ligand, has been demonstrated [16]. Furthermore, it was reported that $GABA_A$ receptors are coupled to Cl^- channels in isolated bovine chromaffin cells [17], as well as in the central nervous system [15], and that GABA-activated Cl^- current is potentiated by benzodiazepines. $GABA_A$ receptors appear not only to modulate the secretion of catecholamines mediated by acetylcholine in a manner reminiscent of brain GABAergic receptors, but also to evoke the secretion of catecholamines [16, 18–20]. While GABA was recently reported to elevate the cytosolic Ca^{2+} concentration by inducing a $GABA_A$-receptor-linked Cl^- current [21], the mechanisms of GABA-induced secretion of catecholamines are not well understood. The purpose of the present paper is to characterize the signal-transduction mechanism(s) operative in GABA-induced catecholamine release from bovine adrenal chromaffin cells. We report here that GABA stimulated phosphoinositide metabolism via activation of $GABA_A$ receptor-coupled Cl^- channels and drastically induced catecholamine release from the cells in the presence of ouabain.

MATERIALS AND METHODS

Materials. *myo*-[2-^{3}H]Inositol (20 Ci/mmol) was purchased from Amersham (Japan). Muscimol, bicuculline, verapamil, diltiazem, nifedipine and nicardipine were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). GABA, pentobarbital, phenobarbital, nicotine and ouabain were from Nacalai Tesque (Kyoto, Japan). Picrotoxin, diphenylamine-2-carboxylic acid (DPC), neomycin, diazepam and nitrazepam came from Wako Pure

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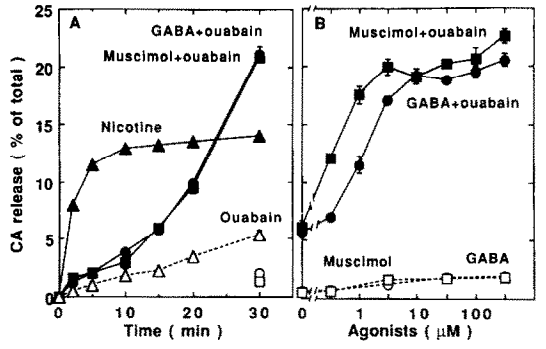

Fig. 1. Time course and dose dependency of catecholamine release from bovine adrenal chromaffin cells induced by GABA or muscimol. (A) Cells (2×10^5 cells/well) were incubated for the indicated times at 37° with 20 μM nicotine (▲), 10 μM GABA (○), 10 μM muscimol (□), 100 μM ouabain (△), 10 μM GABA and 100 μM ouabain (●), or 10 μM muscimol and 100 μM ouabain (■). (B) Cells were incubated for 30 min at 37° with the indicated concentrations of GABA (○, ●), or muscimol (□, ■) in the presence (●, ■) or absence (○, □) of 100 μM ouabain. The percentage of catecholamines released into the medium was determined as described in Materials and Methods. Values shown are means ± SE of triplicate experiments. Most SE values were small and bars indicating SE were included within the symbols.

Chemical Industries (Osaka, Japan). All other chemicals were of reagent grade.

Chromaffin cell preparation. Chromaffin cells were prepared from bovine adrenal medulla by collagenase digestion and purification on Percoll gradients as described previously [22]. Chromaffin cells were purified further by differential plating [23]. Cells were cultured in Dulbecco's modified Eagle's medium [or when inositol phosphates (IPs) were measured, 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 units/mL), and nystatin (250 units/mL). The cultures were maintained at 37° in 5% CO_2/95% air for 3–4 days prior to use.

Catecholamine release. Chromaffin cells cultured in 24-well culture plates (2×10^5 cells/well) were used in the experiments for catecholamine release. The cells 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. Cellular catecholamines (norepinephrine plus epinephrine, 2–3 μg/10^5 cell) and catecholamines released into the medium were extracted with 0.4 M perchloric acid and analysed on a Waters HPLC model 510 equipped with an electrochemical detector (Eicom ECD-100). Secretion was determined by calculating the percentage of total cellular catecholamines released into the medium [24].

Measurement of [^{3}H]IP *formation*. Chromaffin cells cultured in 6-well culture plates (3×10^6 cells/well) precoated with poly-L-lysine were labeled with [^{3}H]inositol (6 μCi/well) 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. For reaction termination, the medium was quickly aspirated and 5% (w/v) trichloroacetic acid solution was added to each well. Separation of [^{3}H]inositol monophosphate (IP_1), [^{3}H]inositol bisphosphate (IP_2) and [^{3}H]inositol trisphosphate (IP_3) was carried out by Bio-Rad AG1-x8 chromatography essentially as described by Berridge *et al.* [25]. Radioactivity in the eluates was determined by a Packard Tri-Carb 2200 CA liquid scintillation analyser. Total [^{3}H]IPs represent the sum of [^{3}H]IP_1, [^{3}H]IP_2 and [^{3}H]IP_3.

RESULTS

Synergistic effect of GABA and ouabain on catecholamine release

GABA and muscimol, a specific agonist for the $GABA_A$ receptor, each evoked catecholamine release from cultured bovine adrenal chromaffin cells, but they gave only a small release (1–2%) over the basal level of 1% by 30 min (Fig. 1). As shown in Fig. 1A, the secretory response elicited by exposure of the cells to ouabain alone gradually increased over a 30-min incubation period, and ouabain markedly enhanced GABA- and muscimol-induced catecholamine release with quite similar time courses. In contrast, 20 μM nicotine evoked a rapid and transient release of 11% of the total within 5 min, which then leveled off. The differences in the rate and magnitude of the secretory responses to GABA plus ouabain and to nicotine suggest that these two stimuli are acting by different mechanisms. Figure 1B shows the dose dependency of GABA and muscimol for catecholamine release in the presence or absence of ouabain. In the presence of ouabain, GABA and muscimol dose-dependently potentiated catecholamine release with a half-maximal concentration of 2 and 0.5 μM, respectively.

It is thought that GABA produces its action through the opening of Cl^- channels coupled to its receptor. In order to determine whether GABA-induced release was mediated by the activation of $GABA_A$ receptor-coupled Cl^- channels, we next examined the effects of bicuculline, a specific antagonist for $GABA_A$ receptors [26], picrotoxin, a blocker of GABA-gated Cl^- channels [27], and DPC, another Cl^- channel blocker [28], on GABA- and muscimol-induced catecholamine release in the presence of ouabain. As shown in Fig. 2, all three of these blockers completely inhibited both GABA- and muscimol-induced release with a half-maximal concentration of 10, 10 and 100 μM, respectively. On the other hand, these agents had no effect on the basal release or on the ouabain-induced release (data not shown). We further examined the effect of diazepam and nitrazepam, anxiolytic benzodiazepines that increase the current flow through $GABA_A$ receptor-coupled Cl^- channels [29], and that of pentobarbital and phenobarbital, barbiturates that prolong the burst duration of GABA receptor-coupled Cl^- channel. As shown in Table 1, these agents potentiated the GABA- and muscimol-induced release in the presence of ouabain. Again these agents showed no effect on the basal or

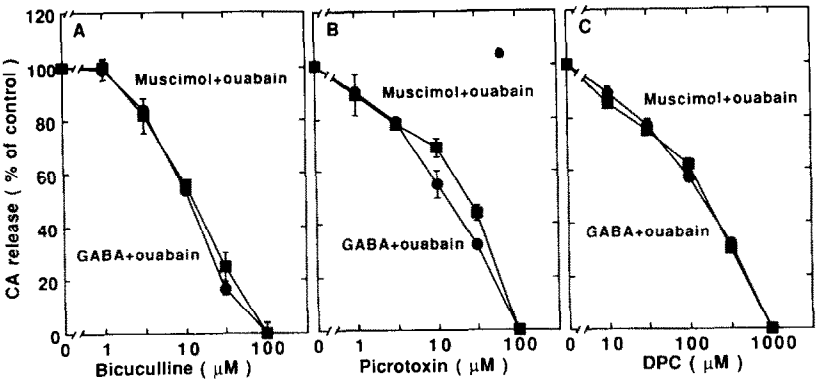

Fig. 2. Dose dependency of inhibition by bicuculline, picrotoxin, and DPC of GABA- or muscimol-induced catecholamine release. After cells were preincubated for 10 min at 37° with the indicated concentrations of bicuculline (A), picrotoxin (B), or DPC (C), the cells were further incubated for 30 min at 37° with 10 μM GABA and 100 μM ouabain (●), or 10 μM muscimol and 100 μM ouabain (■). Catecholamine release was determined as described in Materials and Methods. The data were corrected by subtraction of ouabain alone-induced release (4.82 ± 0.56%) from secretagogue plus ouabain-evoked release, and are expressed as a percentage of the release elicited by seretagogue plus ouabain in the absence of inhibitor. Values shown are means ± SE of triplicate experiments. In the absence of inhibitor, the percentage of the release elicited by GABA plus ouabain and muscimol plus ouabain was 21.21 ± 0.71 and 20.25 ± 1.01%, respectively.

Table 1. Effect of benzodiazepines or barbiturates on GABA- or muscimol-induced catecholamine release

Addition	Catecholamine release (% of control)	
	GABA + ouabain	Muscimol + ouabain
None	100 ± 1.6	100 ± 2.1
Diazepam (1 μM)	130 ± 3.9	118 ± 9.2
Nitrazepam (1 μM)	165 ± 1.1	123 ± 1.1
Pentobarbital (10 μM)	182 ± 1.4	147 ± 7.0
Phenobarbital (10 μM)	197 ± 1.7	162 ± 8.5

Cells were incubated for 30 min at 37° with the indicated benzodiazepines (1 μM) or barbiturates (10 μM) in the presence of 1 μM GABA or 1 μM muscimol and 100 μM ouabain. Catecholamine release was determined as described in Materials and Methods. The percentage of the release elicited by GABA plus ouabain, muscimol plus ouabain and ouabain alone was 13.16 ± 0.55, 17.21 ± 0.76 and 4.61 ± 0.82%, respectively. The data are expressed as described in the legend for Fig. 2. Values shown are the mean ± SE of triplicate experiments.

ouabain-induced release (data not shown). These results indicate that GABA-induced catecholamine release in the presence of ouabain is mediated by the activation of $GABA_A$ receptor-coupled Cl^- channels.

GABA-stimulated phosphoinositide metabolism

In bovine chromaffin cells, activation of the nicotinic acetylcholine receptor stimulates the formation of IPs through voltage-sensitive Ca^{2+} channel activation following membrane depolarization [30]. It is likely, therefore, that GABA may activate the voltage-sensitive Ca^{2+} channel by membrane depolarization, and consequently induce accumulation of IPs. Thus, we examined the effect of GABA on IP formation, whose formation plays an important role in catecholamine release. For the evaluation of phosphoinositide metabolism, [^{3}H]inositol-labeled chromaffin cells were treated with GABA in the presence of 10 mM LiCl. Figure 3 shows the time course of [^{3}H]IP levels following the addition of 10 μM GABA. [^{3}H]IP_3 formation could be detected at as early as 30 sec, reached maximum at 1 min, and then gradually decreased. A rapid rise in [^{3}H]IP_2 formation was also observed, reaching a maximum value of 218% at 2 min. In contrast, [^{3}H]IP_1 accumulation was detectable at 30 sec, but in a much lesser amount, and increased continuously over a 3-min period. Figure 4 shows the effects of various concentrations of GABA and muscimol on [^{3}H]IP levels at 2 min. Formation of [^{3}H]IPs was dose-dependent over the concentrations from 100 nM to 100 μM. A fairly good correlation was observed between accumulation of [^{3}H]IP,

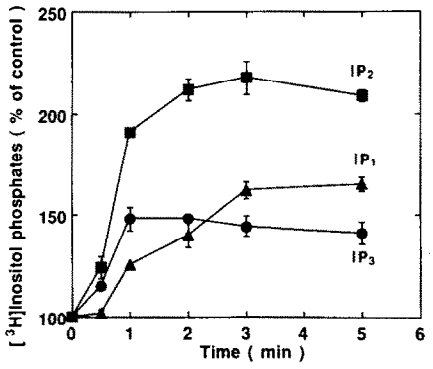

Fig. 3. Time course of the effect of GABA on [^{3}H]IP accumulation in chromaffin cells. Cells were labeled with [^{3}H]inositol in Ham's F-10 medium for 45–60 hr. After preincubation with 10 mM LiCl for 10 min, the cells were stimulated with 10 μM GABA. At the time points indicated, [^{3}H]IPs were extracted and analysed by AG1-x8 chromatography as described in Materials and Methods. The values (mean ± SE, N = 3) of IP_1 (▲), IP_2 (■) and IP_3 (●) are expressed as a percentage of the control at each time point. The dpm/dish in the control at zero time was 4825 ± 136 for IP_1, 4881 ± 177 for IP_2 and 2032 ± 58 for IP_3.

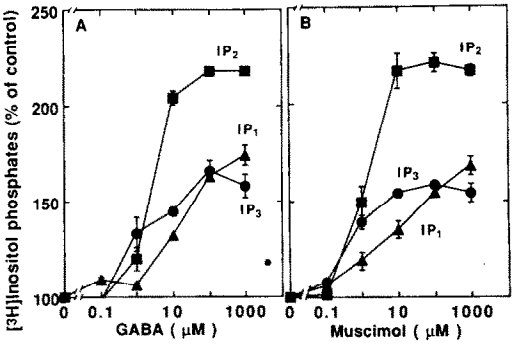

Fig. 4. Dose dependency of the effect of GABA or muscimol on [^{3}H]IP accumulation in chromaffin cells. [^{3}H]Inositol-labeled cells were incubated for 2 min at 37° with the indicated concentrations of GABA (A) or muscimol (B). IP_1 (▲), IP_2 (■) and IP_3 (●) formed were determined as described in Materials and Methods. Values shown are the mean ± SE of triplicate experiments.

especially [^{3}H]IP_3 and [^{3}H]IP_2 (Fig. 4) and catecholamine release (Fig. 1B). To elucidate whether GABA-stimulated [^{3}H]IP formation was mediated by the activation of $GABA_A$ receptor-coupled Cl^- channel, we examined the effects of bicuculline, picrotoxin, diazepam and pentobarbital on GABA-stimulated [^{3}H]IP formation. As shown in Table 2, bicuculline and picrotoxin inhibited it by about 90%. In contrast, diazepam and pentobarbital markedly potentiated it. These results confirm that GABA-stimulated [^{3}H]IP formation is due to the activation of $GABA_A$ receptor-coupled Cl^- channels.

To explore the participation of GABA-stimulated phosphoinositide metabolism in GABA-induced catecholamine release in the presence of ouabain,

Table 2. Effect of bicuculline, picrotoxin, diazepam or pentobarbital on GABA- or muscimol-induced [^{3}H]IP formation

Addition	Total [^{3}H]IPs increased* (%)	
	GABA	Muscimol
None†	100 ± 6.5	100 ± 7.1
Bicuculline (100 μM)	11 ± 5.6	9.0 ± 4.2
Picrotoxin (100 μM)	7.3 ± 4.4	5.1 ± 3.7
None‡	100 ± 4.2	100 ± 5.9
Diazepam (10 μM)	137 ± 5.6	142 ± 6.0
Pentobarbital (10 μM)	165 ± 5.6	160 ± 5.0

After [^{3}H]inositol-labeled cells were preincubated for 10 min at 37° with 100 μM bicuculline or 100 μM picrotoxin, the cells were further incubated for 2 min at 37° with 10 μM GABA or 10 μM muscimol. [^{3}H]Inositol-labeled cells were incubated for 2 min at 37° with 10 μM diazepam or 10 μM pentobarbital in the presence of 1 μM GABA or 1 μM muscimol. Total [^{3}H]IPs formed were determined as described in Materials and Methods.

* The increase in total [^{3}H]IPs induced by secretagogue alone is taken as 100%. Values shown are the mean ± SE of triplicate experiments.

† The percentage of total [^{3}H]IPs induced by 10 μM GABA and 10 μM muscimol was 168 ± 11 and 170 ± 12% of the control.

‡ The percentage of total [^{3}H]IPs induced by 1 μM GABA and 1 μM muscimol was 118 ± 5 and 136 ± 8% of the control.

we next examined the effects of neomycin, a phosphatidylinositol-specific phospholipase C inhibitor [31, 32], on GABA-stimulated [^{3}H]IP formation and catecholamine release. Neomycin inhibited both GABA- and muscimol-induced catecholamine release in the presence of ouabain with a half-maximal concentration of 0.5 mM (Fig. 5A), but it had no effect on the basal and ouabain-induced release (data not shown). Neomycin also inhibited GABA- or muscimol-stimulated [^{3}H]IP formation with a half-maximal concentration of 0.5 mM (Fig. 5B), a value equivalent to that for the inhibition of catecholamine release. These results demonstrate that GABA-induced catecholamine release in the presence of ouabain is triggered by phosphoinositide metabolism.

Effects of various Ca^{2+} channel blockers on GABA-induced catecholamine release and [^{3}H]IP formation

To further explore the possibility of the involvement of Ca^{2+} channels in the actions of GABA, we examined the effects of various Ca^{2+} channel blockers on GABA-induced accumulation of [^{3}H]IPs and catecholamine release in the presence of ouabain. As shown in Fig. 6, ω-conotoxin had no significant effect on catecholamine release. Verapamil and diltiazem showed very weak inhibition, the level of which was less than 20% at 10 μM. On the other hand, the dihydropyridine blocker nicardipine reduced the GABA-induced release in the presence of ouabain in a concentration-dependent manner and almost completely inhibited

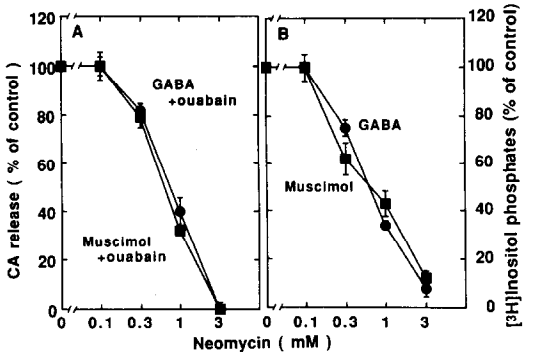

Fig. 5. Dose dependency of neomycin effect on GABA- or muscimol-induced catecholamine release and accumulation of IPs. (A) After cells were preincubated for 10 min at 37° with the indicated concentrations of neomycin, they were further incubated for 30 min at 37° with 10 μM GABA and 100 μM ouabain (●), or 10 μM muscimol and 100 μM ouabain (■). Catecholamine release was determined as described in Materials and Methods, and is expressed as described in the legend for Fig. 2. (B) After [^{3}H]inositol-labeled cells were preincubated for 10 min at 37° with the indicated concentrations of neomycin, they were further incubated for 2 min at 37° with 10 μM GABA (●) or 10 μM muscimol (■), and [^{3}H]IPs formed were determined as described in Materials and Methods. The data are expressed as a percentage of the accumulation induced by secretagogue in the absence of neomycin. Values shown are the mean ± SE of triplicate experiments.

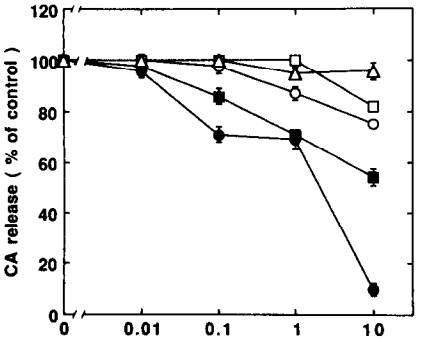

Fig. 6. Dose dependency of the effect of various Ca^{2+} channel blockers on GABA-induced catecholamine release. After cells were preincubated for 10 min at 37° with the indicated concentrations of nicardipine (●), nifedipine (■), verapamil (○), diltiazem (□) or ω-conotoxin (△), they were further incubated for 30 min at 37° with 10 μM GABA and 100 μM ouabain. Catecholamine release was determined as described in Materials and Methods, and is expressed as described in the legend for Fig. 2. Values shown are the mean ± SE of triplicate experiments.

it at 10 μM; but another dihydropyridine blocker, nifedipine, was less effective. Table 3 shows the effects of these Ca^{2+} channel blockers and removal of Ca^{2+} from the medium on accumulation of [^{3}H]IPs. Removal of extracellular Ca^{2+} almost completely inhibited the accumulation of [^{3}H]IPs induced by GABA or muscimol. At 1 μM, nicardipine

Table 3. Effect of extracellular Ca^{2+} on GABA- or muscimol-induced [^{3}H]IP accumulation

	Total [^{3}H]IPs (% of control)	
Addition	GABA	Muscimol
None	100 ± 7.2	100 ± 9.1
$-CaCl_2$ + EGTA (0.1 mM)	7.4 ± 5.4	4.3 ± 2.6
Verapamil (1 μM)	87 ± 5.4	98 ± 6.7
Diltiazem (1 μM)	102 ± 3.0	102 ± 5.2
Nifedipine (1 μM)	60 ± 6.1	67 ± 5.9
Nicardipine (1 μM)	39 ± 5.7	52 ± 7.0
ω-Conotoxin (1 μM)	98 ± 2.1	100 ± 3.3

After [^{3}H]inositol-labeled cells were preincubated for 10 min at 37° with various Ca^{2+} channel blockers or in Ca^{2+}-free buffer containing 0.1 mM EGTA, the cells were further incubated for 2 min at 37° with 10 μM GABA or 10 μM muscimol. Total [^{3}H]IPs formed were determined as described in Materials and Methods. The percentage of total [^{3}H]IPs induced by 10 μM GABA and 10 μM muscimol was 172 ± 12 and 175 ± 9%, respectively, and used as the control. The data are expressed as a percentage of the accumulation induced by secretagogue alone. Values shown are the mean ± SE of triplicate experiments.

was the most efficient in inhibition of [^{3}H]IP accumulation. Nifedipine, verapamil and diltiazem were much less potent in this order. ω-Conotoxin had no effect. A rough correlation was observed between the effects of these Ca^{2+} channel blockers on [^{3}H]IP accumulation and catecholamine release.

DISCUSSION

The present study is the first demonstration that GABA can cause phosphoinositide breakdown with the resultant formation of IP_3, IP_2 and IP_1 in a time- and dose-dependent manner and then induce catecholamine release in the presence of ouabain from bovine chromaffin cells. It is evident that these effects induced by GABA or muscimol in chromaffin cells are mediated via activation of $GABA_A$ receptor-coupled Cl^- channels because they were prevented by the $GABA_A$ receptor antagonist bicuculline and by the Cl^- channel blocker picrotoxin, and they were conversely potentiated by diazepam and pentobarbital (Fig. 2; Tables 1 and 2). It has been found that Ca^{2+} entry either through a receptor-gated ion channel or through a voltage-sensitive Ca^{2+} channel induces an increase in cytosolic Ca^{2+}, which in turn activates Ca^{2+}-sensitive phospholipase C [33]. The formation of IPs in bovine adrenal chromaffin cells by activation of nicotinic acetylcholine receptors or by treatment with high K^+ is a case in point [30]. GABA was recently reported to cause Ca^{2+} entry into chromaffin cells following activation of $GABA_A$ receptors [21]. The author concluded that activation of the $GABA_A$ receptor induces Cl^- channel current, which elicits membrane depolarization sufficiently to activate the voltage-sensitive Ca^{2+} channel. Collectively, we can deduce that GABA-evoked Ca^{2+} entry causes the accumulation of IPs in chromaffin cells. In agreement

with this idea, GABA- or muscimol-induced accumulation of IPs was absolutely dependent on extracellular Ca^{2+} (Table 3).

The IP formation by GABA or muscimol was significantly inhibited by dihydropyridine derivatives, especially nicardipine at a 1 μM concentration; but it was fairly insensitive to verapamil and diltiazem and to ω-conotoxin (Table 3), which are known to be blockers of the voltage-sensitive L- and N-type Ca^{2+} channels, respectively. Nicardipine also inhibited the GABA-induced catecholamine release in the presence of ouabain (Fig. 6). Consistent with our results, it was previously reported that only nicardipine (0.1–10 μM) inhibited $PGF_{2\alpha}$-induced $^{45}Ca^{2+}$ influx and catecholamine release in bovine chromaffin cells, whereas diltiazem, verapamil and nicardipine suppressed acetylcholine-induced catecholamine release and $^{45}Ca^{2+}$ uptake in a similar dose-dependent manner [7]. The higher potency of nicardipine in the inhibition of secretory responses to non-nicotinic agonists might be because it has a greater affinity for Ca^{2+} channels activated by them than nifedipine, verapamil and diltiazem. At high doses, however, these agents not only inhibit Ca^{2+} channels but also Na^+ and K^+ channels, and interact with central and peripheral α_2-adrenergic and muscarinic receptors *in vitro* as well [34]. Therefore we cannot rule out the possibility that the effect of nicardipine reported here was nonspecific.

In many secretory systems exocytosis is associated with a receptor-mediated phosphoinositide metabolism that produces two second messengers, i.e. IP_3 and 1,2-diacylglycerol [11, 12]. We previously reported that PGE_2 caused phosphoinositide breakdown via pertussis toxin-insensitive GTP-binding protein [9, 35], stimulated the Na^+,H^+-antiport by activation of protein kinase C resulting from phosphoinositide metabolism [10], and induced a gradual catecholamine release from bovine chromaffin cells in the presence of ouabain [36]. The increase in intracellular Na^+ by both inhibition of Na^+,K^+-ATPase by ouabain and activation of the Na^+,H^+-antiport by PGE_2 may lead to the redistribution of Ca^{2+} through a Na^+,Ca^{2+}-exchange system or by other mechanisms on the plasma membrane, which may be the initial trigger of PGE_2-stimulated catecholamine release from bovine adrenal chromaffin cells [36]. Thus, synergistic effect of PGE_2 and ouabain on catecholamine release and involvement of protein kinase C in the release have been clarified in a series of studies in our laboratory.

The GABA-induced rise in cytosolic free Ca^{2+} was reportedly near the threshold of about 300 nM required for evoking detectable catecholamine release, and thus only a marginal secretory response was observed (Fig. 1). In analogy with PGE_2-induced catecholamine release, several lines of evidence suggest that the stimulation of phosphoinositide metabolism is involved in the release induced by GABA. (i) GABA-induced catecholamine release was synergistically enhanced by ouabain (Fig. 1). (ii) Neomycin, an aminoglycoside antibiotic, binds strongly to polyphosphoinositides and thereby inhibits phosphoinositide-specific phospholipase C [31, 32]. We previously demonstrated that neomycin inhibited PGE_2-induced accumulation of IPs and catecholamine release in the presence of ouabain in bovine chromaffin cells [10]. Similarly, GABA-induced phosphoinositide metabolism and catecholamine release were inhibited by neomycin (Fig. 5). (iii) GABA- or muscimol-induced catecholamine release in the presence of ouabain was inhibited by the protein kinase C inhibitor staurosporine (data not shown). (iv) We also observed that the release was inhibited by the inhibitors of the Na^+,H^+-antiport amiloride and ethylisopropylamiloride and that the latter was 100-fold more potent than the former for inhibiting the release (data not shown). Although PGE_2 and GABA may stimulate phosphoinositide metabolism through different mechanisms mentioned above, we consider the mechanism of GABA-induced catecholamine release in the presence of ouabain to be similar to that of PGE_2-induced release involving stimulation of the Na^+,H^+-antiport by protein kinase C [10, 36].

The adrenal glands are one of the rich sources of endogenous ouabain-like compounds [37], and an endogenous ouabain-like compound was extracted and partially purified from bovine adrenal gland [38]. The existence of an endogenous ouabain-like compound in bovine adrenal gland and the ability of GABA to induce catecholamine release in the presence of ouabain suggest that the $GABA_A$ receptor channels on chromaffin cells may be involved in controlling catecholamine release *in vivo* via stimulation of phosphoinositide metabolism.

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