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EVIDENCE THAT PROSTAGLANDINS ACTIVATE CALCIUM CHANNELS TO ENHANCE BASAL AND STIMULATION-EVOKED CATECHOLAMINE RELEASE FROM BOVINE ADRENAL CHROMAFFIN CELLS IN CULTURE

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Abstract—The effects of prostaglandins (PGs) on catecholamine (CA) secretion and Ca^{2+} fluxes were studied in a primary culture of bovine chromaffin cells. PGD_2, $PGF_{2\alpha}$ and PGE_2 induced CA release from cultured bovine chromaffin cells in a concentration dependent manner (0.03–3 μM). PGD_2, $PGF_{2\alpha}$ and PGE_2 at 3 μM elicited maximum CA release of 0.043 ± 0.001, 0.059 ± 0.008, 0.062 ± 0.002 $\mu g/10^6$ cells, respectively. Three micromolar of PGD_2, $PGF_{2\alpha}$ and PGE_2 enhanced CA release induced by acetylcholine (ACh) in a degree of 186 ± 10, 206 ± 6, $150 \pm 4\%$ of control respectively. PGs also enhanced CA release induced by 20 mM K^+, veratridine and A23187. In Ca^{2+}-free medium, PGs failed to affect basal and caffeine (50 mM)-induced CA release. $PGF_{2\alpha}$ increased ^{45}Ca uptake and showed additive effect with ACh on ^{45}Ca uptake. Nicardipine (0.1–10 μM) suppressed CA release and ^{45}Ca uptake induced by $PGF_{2\alpha}$, while diltiazem and verapamil failed to affect these responses to $PGF_{2\alpha}$. BAY K 8644 (1 μM) potentiated CA release and ^{45}Ca uptake evoked by $PGF_{2\alpha}$. These results suggest that PGs enhance basal and stimulation-evoked CA release from chromaffin cells possibly through facilitation of Ca^{2+} influx. The mechanisms of action of PGs in adrenal medulla are discussed.

It has been well documented that the neurotransmission in peripheral nervous system is modulated by prostaglandins (PGs). Recent study suggested that PGs may modulate catecholamine (CA) release in adrenal medulla, which is homologous organ with sympathetic ganglion. Karaplis and Powell [1, 2] observed specific binding of PGEs to plasma membrane prepared from bovine adrenal medulla. Ramwell *et al.* [3] detected the efflux of PG-like material from perfused cat adrenal gland when stimulated by ACh. However, functional relevance of PGs to CA release is rarely known, and reports concerning this problem seem controversial. For example, there are some differences among species in the modulation of CA release by PGs in adrenal medulla. Administration of PGE_2 into lumboadrenal artery of anesthetized dogs caused CA release [4]. Miele [5] observed no effect of PGs on CA release in perfused cat adrenal glands. On the other hand, PGs reduced basal and evoked CA release in rat and rabbit adrenals [6, 7].

In addition, the report which suggest the mechanisms of action of PGs in adrenomedullary CA secretion is rare. The only report by Gutman and Boonyaviroj [6] suggested that PGs may suppress CA release by lowering intracellular Ca^{2+} levels through inhibition of adenylate cyclase in rat adrenals. In adrenal medulla, extracellular Ca^{2+} is essential to CA secretion induced by ACh [8]. The greater part of CA release induced by ACh is mediated through voltage dependent Ca^{2+} channels [9, 10]. Shimizu *et al.* [11] reported that $PGF_{2\alpha}$ stimulated contraction in dog mesenteric artery. The $PGF_{2\alpha}$-induced contractions are less sensitive to Ca^{2+} channel blocker, verapamil ($EC_{50} > 10$ μM), than high K^+-evoked response ($EC_{50} = 10$ μM) [11]. This difference suggests that $PGF_{2\alpha}$ may activate Ca^{2+} channel other than voltage dependent Ca^{2+} channel.

In the present study, to elucidate the role of PGs in adrenal CA secretion the effects of PGs on basal and stimulation-evoked CA release were examined in a primary culture of bovine adrenal chromaffin cells. Further to clarify the nature of the action of PGs on chromaffin cells, we investigated the effect of PGs on ^{45}Ca uptake in association with CA release.

MATERIALS AND METHODS

Perfusion of bovine adrenal glands. Bovine adrenal glands were perfused retrogradely via central vein with a modified Krebs–Ringer phosphate solution (KRP: NaCl 154 mM, KCl 5.6 mM, $CaCl_2$ 1.3 mM, $MgSO_4$ 1.1 mM, Na_2HPO_4 2.2 mM, NaH_2PO_4 0.9 mM, glucose 11.1 mM, pH 7.4, saturated with 100% O_2) according to the method of Robinson [12] as modified by Tsujimoto *et al.* [13]. Perfusion pressure was 60 cm of water, and temperature was 30°. Perfusion rate was 3–10 ml/15 sec. Samples of perfusate for CA assay (see below) were collected every 15 sec.

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† Abbreviations used: PG, prostaglandin; CA, catecholamine; ACh, acetylcholine; DMEM, Dulbecco's Modified Eagle Medium.

Isolation and culture of bovine chromaffin cells. Chromaffin cells were isolated by the method of Fenwick *et al.* [14] with the modification [15], but in this study, we use neutral protease (1.2 U/ml) from *Bacillus polymyxa* (EC 3.4.24.4) to digest the tissue instead of collagenase.

CA release and ^{45}Ca uptake in cultured chromaffin cells. In the study where chromaffin cells were used, all the methods were described by Morita *et al.* [15, 16]. Briefly, cells were washed three times with 3 ml of KRP and kept at room temperature for 2 hr. Then, after preincubation for 3 min at 37°, cells were incubated for 6 min with KRP-containing stimulants. Then the 0.5 ml of samples were collected for CA assay. For the assay of ^{45}Ca uptake, ^{45}Ca (4 μCi/ml) was added to the incubation medium. After the samples for CA assay were collected, cells were washed 5 times with ice-cold KRP. Then the cells were harvested and the radioactivity in the cells was determined by liquid scintillation spectrometry.

When concentration of KCl was changed or caffeine was added to KRP, NaCl was reduced to maintain isotonicity. To Ca^{2+}-free KRP, EDTA (1 mM) was added. When ACh was used, eserine (10 μM) was added to KRP. CA was assayed fluorometrically by the method of Euler and Lishajko [17] with epinephrine as a standard. All data were expressed as the mean ± SE of triplicate determinations. Statistical analysis of significance of the difference of unbiased means was carried out with the Student's *t*-test.

Materials. The sources of materials used were as follows: acetylcholine hydrochloride from Daiichi Seiyaku, Tokyo, Japan; caffeine from Katayama Chemical, Osaka, Japan; diltiazem hydrochloride, nicardipine hydrochloride and eserine salicylate from Sigma, St. Louis, MO; neutral protease (Dispase, Grade II, EC 3.4.24.4) and fetal calf serum from Boehringer Manheim GmbH, F.R.G.; DMEM from Nissui Pharmaceutical, Tokyo, Japan; epinephrine from Merck, Darmstadt, F.R.G.; verapamil hydrochloride, a gift from Eisai, Tokyo, Japan; BAY K 8644 a gift from Bayer AG, Wuppertal, F.R.G.; PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2, gifts from Ono Pharmaceutical, Osaka, Japan; $^{45}CaCl_2$ (1.15–1.55 GBq/ml) from New England Nuclear, Boston, MA; Penicillin G and Streptomycin sulphate from Meiji Seika, Tokyo, Japan.

RESULTS

Treatment with PGD_2, $PGF_{2\alpha}$, PGE_2 (1 μM) for 1 min increased CA output from perfused bovine adrenal glands. Basal CA output just prior to stimulation by PGs was 6.10 ± 1.24 μg/15 sec. At the peak of the responses to PGD_2, $PGF_{2\alpha}$ and PGE_2 increases in CA output were 2.45 ± 0.74, 3.48 ± 2.21, 7.64 ± 3.05 μg/15 sec respectively (mean ± SE, basal CA output has been subtracted).

PGD_2, $PGF_{2\alpha}$ and PGE_2 also induced CA release from cultured bovine chromaffin cells in a concentration dependent manner (Fig. 1a). These PGs also enhanced ACh-evoked CA release concentration-dependently in cultured bovine chromaffin cells (Fig. 1b). The rank order of potency that PGs enhanced ACh-evoked CA release and that PGs evoked CA by themselves were somewhat different

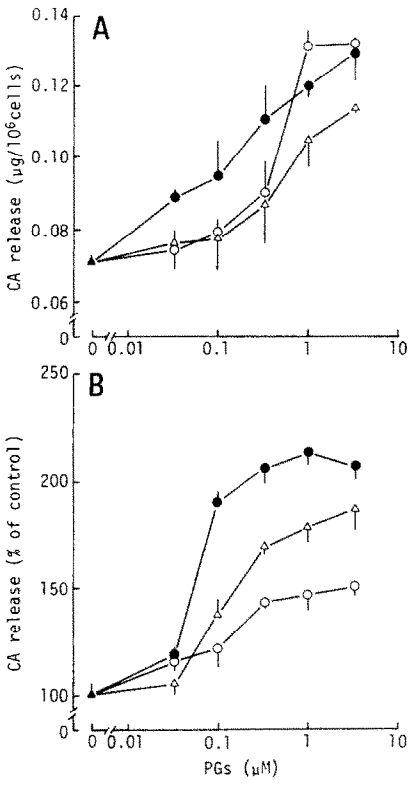

Fig. 1. Effects of PGs on basal and ACh-evoked CA release. (A) PGs-evoked CA release from cultured bovine chromaffin cells. After preincubation for 3 min, cells were incubated with or without PGs for 6 min. Points and bars represent mean ± SEM of triplicate determinations. Symbols: ▲, basal value; ○, PGE_2; △, PGD_2; ●, $PGF_{2\alpha}$. (B) Enhancement of ACh (5 μM)-evoked CA release by PGs. After preincubation for 3 min, cells were incubated for 6 min in KRP containing ACh with or without PGs. Basal or PGs-evoked CA release has been subtracted from each responses and the net increases in CA release were expressed as % of control (▲, ACh alone, 0.261 ± 0.009 μg/10^6 cells). Other symbols mean as follows: ○, ACh + PGE_2; △, ACh + PGD_2; ●, ACh + $PGF_{2\alpha}$.

from each other. The maximum potentiations of ACh-evoked CA release by PGs were observed at 1 μM of $PGF_{2\alpha}$ (210% of control), and 3 μM of PGD_2 (180%) and of PGE_2 (160%). PGI_2 had no effect on basal and ACh-evoked CA release (data not shown). $PGF_{2\alpha}$ also enhanced 20 mM K^+-, veratridine- and A23187-induced CA release. On the other hand, it failed to affect basal and caffeine-induced CA release in Ca^{2+} free KRP (Table 1). These results indicate that the actions of PGs are dependent on extracellular Ca^{2+} and suggest that PGs may alter Ca^{2+} influx.

To confirm this idea, we measured ^{45}Ca uptake in cultured chromaffin cells. Figure 2 shows that ACh and $PGF_{2\alpha}$ increased ^{45}Ca uptake respectively. The concomitant administration of them produced nearly additive increase in ^{45}Ca uptake. To investigate whether the effects of PGs were mediated through Ca^{2+} channel, we examined the effects of Ca^{2+} antagonists, verapamil, diltiazem and nicardipine, and Ca^{2+} agonist, BAY K 8644, on CA release and ^{45}Ca

Table 1. Effects of $PGF_{2\alpha}$ on the basal and stimulation-evoked CA release from cultured bovine chromaffin cells in normal or Ca^{2+}-free medium

Condition	CA release ($\mu g/10^6$ cells)		Net evoked CA release ($\mu g/10^6$ cells)	
	(−) $PGF_{2\alpha}$	(+) $PGF_{2\alpha}$	(−) $PGF_{2\alpha}$	(+) $PGF_{2\alpha}$
(A) In normal KRP:				
Basal	0.075 ± 0.003	0.128 ± 0.004*		
20 mM K^+			0.077 ± 0.007	0.235 ± 0.020*
Veratridine (15 μM)			0.118 ± 0.014	0.739 ± 0.016*
A23187 (1 μM)			0.051 ± 0.007	0.159 ± 0.007*
(B) In Ca^{2+}-free KRP:				
Basal	0.135 ± 0.004	0.136 ± 0.011^{NS}		
Caffeine (50 mM)			0.059 ± 0.010	0.066 ± 0.013^{NS}

After preincubation for 3 min, cells were stimulated by various secretagogues with or without $PGF_{2\alpha}$ for 6 min. Net evoked CA release represents CA release from which basal CA release in the presence or absence of $PGF_{2\alpha}$ has been subtracted. Values are mean ± SEM of triplicate determinations.

* Significantly different from the value in the absence of $PGF_{2\alpha}$ at $P < 0.05$.

NS No significant difference between the values in the absence and presence of $PGF_{2\alpha}$.

uptake induced by $PGF_{2\alpha}$ or ACh. These three Ca^{2+} channel blockers reduced ACh-induced CA release and ^{45}Ca uptake in a concentration dependent fashion (Fig. 3). In contrast, CA release and ^{45}Ca uptake induced by $PGF_{2\alpha}$ were reduced only by nicardipine in a concentration dependent manner, but not by diltiazem and verapamil (Fig. 4). BAY K 8644 increased ^{45}Ca uptake although it could not evoke CA release. BAY K 8644 enhanced $PGF_{2\alpha}$-evoked CA release and ^{45}Ca uptake (Fig. 5).

DISCUSSION

The present study shows that PGs enhanced basal and evoked CA release from cultured bovine adrenal chromaffin cells, and that these effects of PGs were dependent on extracellular Ca^{2+}, suggesting that enhancement by PGs of CA release may be via facilitation of Ca^{2+} influx. Further, the increase in CA release by PGs accompanied ^{45}Ca uptake, both of which were reduced by nicardipine, a dihydropyridine Ca^{2+} channel blocker. These results suggest that PGs increase CA release by activating Ca^{2+} channels.

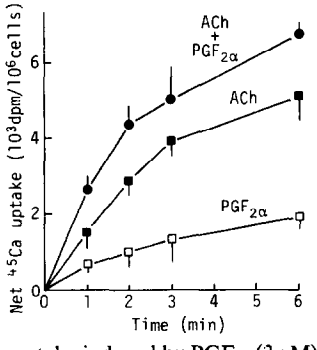

Fig. 2. ^{45}Ca uptake induced by $PGF_{2\alpha}$ (3 μM), ACh (5 μM) and PGF_2 + ACh. After preincubation for 3 min, cells were incubated in KRP containing $^{45}CaCl_2$ (4 μCi/ml). See Materials and Methods on details. Points and bars represent mean ± SEM of triplicate determinations.

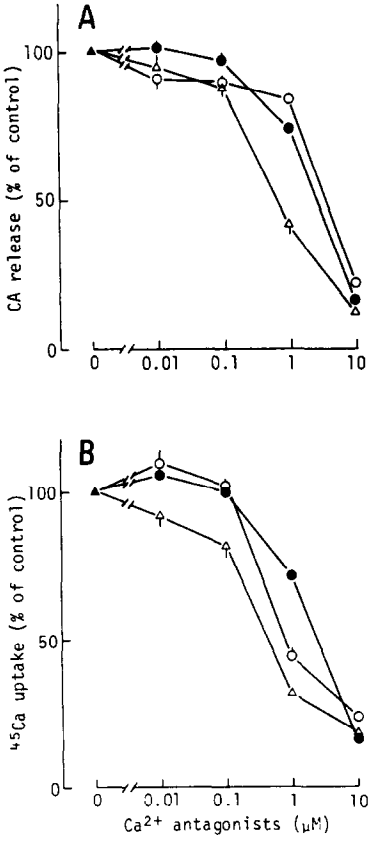

Fig. 3. Effects of Ca^{2+} antagonists on ACh (100 μM)-induced CA release (A) and ^{45}Ca uptake (B) in cultured bovine chromaffin cells. After preincubation for 3 min, cells were incubated for 6 min in KRP containing $^{45}CaCl_2$ and ACh with or without Ca^{2+} antagonists. Values are mean ± SEM of triplicate determinations, represented as % of control response in the absence of Ca^{2+} antagonists. Net CA release evoked by 100 μM ACh was 1.971 ± 0.008 $\mu g/10^6$ cells and net ^{45}Ca uptake evoked by 100 μM ACh was 19762 ± 281 dpm/10^6 cells. Symbols: ▲, control (ACh); ○, diltiazem + ACh; ●, verapamil + ACh; △, nicardipine + ACh.

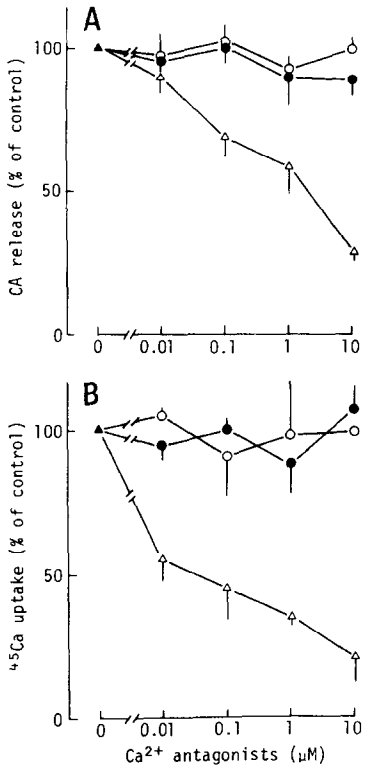

Fig. 4. Effects of Ca^{2+} antagonists on $PGF_{2\alpha}$ (3 μM)-induced CA release (A) and ^{45}Ca uptake (B) in cultured bovine chromaffin cells. Experimental design as Fig. 3, but here cells were stimulated by $PGF_{2\alpha}$ instead of ACh. Values are mean ± SEM of triplicate determinations, represented as % of control response in the absence of Ca^{2+} antagonists. Net CA release evoked by 3 μM $PGF_{2\alpha}$ was 0.058 ± 0.006 $\mu g/10^6$ cells and net ^{45}Ca uptake evoked by 3 μM $PGF_{2\alpha}$ was 1976 ± 55 dpm/10^6 cells. Symbols: ▲, control ($PGF_{2\alpha}$); ○, diltiazem + $PGF_{2\alpha}$; ●, verapamil + $PGF_{2\alpha}$; △, nicardipine + $PGF_{2\alpha}$.

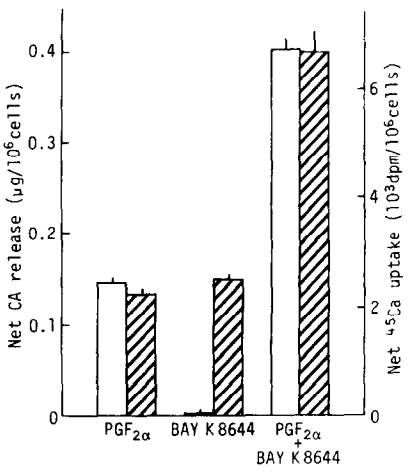

Fig. 5. Effect of BAY K 8644 (1 μM) on CA release and ^{45}Ca uptake induced by $PGF_{2\alpha}$ (3 μM). After preincubation for 3 min, cells were incubated for 6 min in ^{45}Ca containing medium with or without drugs. Basal CA release (0.069 ± 0.004 $\mu g/10^6$ cells) and ^{45}Ca uptake (6775 ± 140 dpm/10^6 cells) have been subtracted from each response. Open and hatched columns represent net CA release and net ^{45}Ca uptake respectively.

The effects of PGs on CA release in cultured chromaffin cells did not result from enzymatic digestion and culture procedure, since PGs increased CA output also in perfused bovine adrenal glands.

Karaplis and Powell [1, 2] demonstrated specific binding of PGEs to plasma membrane of bovine adrenal medulla using radioligand binding assay technique. They showed that $PGF_{2\alpha}$ and PGD_2 have 240- and 1300-fold lower binding affinity than PGE_2 [1]. In the present study, however, PGD_2 and $PGF_{2\alpha}$ increased CA release, and $PGF_{2\alpha}$ and PGD_2 enhanced ACh-evoked CA release more than PGE_2 did. One possible interpretation for these differences is that the efficacy of PGD_2 and $PGF_{2\alpha}$ leading to the process subsequent to their receptor activation may be higher than that of PGE_2. Malet *et al.* [18] showed low or non-significant binding of PGD_2 to rat brain synaptic membrane. On the other hand, Shimizu *et al.* [19] found that PGD_2 bound specifically to rat brain synaptic membrane in an Na^+-dependent manner. In other tissues GTP [20] and Ca^{2+} [21] influenced receptor binding for PGs. In adrenal medulla, therefore, it may be that the unknown factors participate in specific binding of $PGF_{2\alpha}$ and PGD_2.

The enhancement of PGs of basal and evoked CA release is dependent on external Ca^{2+}, because of the following reasons. First, PGs failed to affect basal and caffeine-evoked CA release in Ca^{2+}-free medium. Second, PGs potentiated high K^+- and veratridine-evoked CA release which are thought to be mediated by Ca^{2+} influx through voltage-sensitive Ca^{2+} channels [9, 10]. Third, increase in CA release caused by PGs accompanied ^{45}Ca uptake. Finally ^{45}Ca uptake and CA release induced by $PGF_{2\alpha}$ were markedly potentiated by BAY K 8644 which is suggested to prolong the opening time of Ca^{2+} channels in chromaffin cells [22]. Thus, it is suggested that PGs facilitate Ca^{2+} influx to elicit CA release and to enhance CA release evoked by ACh, high K^+ or veratridine.

However, our study on the effect of $PGF_{2\alpha}$ on ACh-evoked CA release in association with Ca^{2+} uptake revealed the lack of a strict correlation between the amount of ^{45}Ca taken up by chromaffin cells and CA release. It follows from Fig. 2 that concomitant treatment with $PGF_{2\alpha}$ and ACh caused supraadditive increase in CA release whereas only additive ^{45}Ca uptake was observed. A possible explanation for these results might be provided by assuming that ^{45}Ca efflux is accelerated in this condition, resulting in the only apparent amount of ^{45}Ca taken up by chromaffin cells. Another interpretation of these results is that additive increase in Ca^{2+} influx may be sufficient to cause much greater increase in CA release, as suggested by Nachshen and Sanchez-Armass [23] in synaptosome, that small changes in the inward Ca^{2+} current or in the resting Ca^{2+} level in the cytosol of the nerve terminals may have disproportionately large effects on transmitter release. This may also explain our results that $PGF_{2\alpha}$ enhanced CA release evoked by A23187, a Ca^{2+} ionophore,

which bypasses the event via Ca^{2+} channels. These interpretations for the present problems are highly speculative, and further studies are necessary to determine the precise mechanism(s) underlying the facilitatory modulation by PGs of evoked CA release from adrenal chromaffin cells.

PGs have been reported to act as a Ca^{2+} ionophore [24–26]. In this study, however, the possibility that PGs might partly act as an ionophore in bovine chromaffin cells seems to be low, since the reported concentration of PGs to exhibit the ionophoretic action is higher than that of PGs actions on CA release and ^{45}Ca uptake observed here.

It is interesting that verapamil and diltiazem failed to affect the responses induced by PGs whereas nicardipine did suppress. In contrast, ACh-evoked CA release and ^{45}Ca uptake were inhibited by all Ca^{2+} antagonists tested. Recent findings show that there are different types of Ca^{2+} channels in the same cell [27–31] and that different stimuli and conditions open different Ca^{2+} channels [27–29]. The sensitivity of Ca^{2+} channels to Ca^{2+} antagonists varies according to types of Ca^{2+} channels [27, 30], and membrane potential [32, 33]. Shimizu *et al.* demonstrated that sensitivities of high K^+- and $PGF_{2\alpha}$-evoked contraction to Ca^{2+} channel blocker, verapamil, are different in dog mesenteric artery [11]. Our observations presented here indicate that there are differences of sensitivities to Ca^{2+} antagonists in CA release and ^{45}Ca uptake induced by PGs and ACh. Recently Boarder *et al.* [34] demonstrated that maximal stimulation of CA release with nicotine was not stereospecifically inhibited by nicardipine in cultured bovine chromaffin cells, suggesting more than one route of Ca^{2+} entry after activation of nicotinic ACh receptor. On the contrary Ceña *et al.* [35] showed the blockade of nicotine-evoked CA release by nitrendipine in cultured bovine chromaffin cells although higher concentration is needed to block nicotine-evoked CA release than that evoked by excess K^+. Our observation in the case of ACh is consistent with the latter. The reason why these discrepancies occur is not known at present. However, it is possible that PGs may activate a Ca^{2+} channel other than that activated by ACh to elicit and modulate CA release from chromaffin cells.

In addition to the sensitivity of PGs-induced ^{45}Ca uptake and CA release only to nicardipine, supra-additive increases in ^{45}Ca uptake associated with CA release were observed when PGs and BAY K 8644 were simultaneously added. Thus it is probable that PGs may act at the site of Ca^{2+} entry which is sensitive to dihydropyridines. BAY K 8644 by itself, however, failed to affect CA release even though it caused same degree of ^{45}Ca uptake as that caused by $PGF_{2\alpha}$. This may suggest another possibility of different sites of the action of PGs and BAY K 8644 on Ca^{2+} entry, and consequently resulting in the synergistic effect of the two on CA release at downstream of Ca^{2+} entry. Although this possibility cannot be ruled out, our finding of supra-additive increase in ^{45}Ca uptake caused by PGF_2 and BAY K 8644 can support our hypothesis. Further work is needed to pursue this hypothesis and to assess the extent of their assumed participation in CA release.

In conclusion, the present studies demonstrate that PGs enhance basal and evoked CA release from bovine chromaffin cells possibly through facilitation of Ca^{2+} influx. Further we provide the evidence suggesting that PGs may activate the Ca^{2+} channel, which is different from that activated by ACh.

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