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Arachidonic acid release and prostaglandin $F_{2\alpha}$ formation induced by anandamide and capsaicin in PC12 cells

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Abstract

Anandamide, an endogenous agonist of cannabinoid receptors, activates various signal transduction pathways. Anandamide also activates vanilloid VR_1 receptor, which was a nonselective cation channel with high Ca^{2+} permeability and had sensitivity to capsaicin, a pungent principle in hot pepper. The effects of anandamide and capsaicin on arachidonic acid metabolism in neuronal cells have not been well established. We examined the effects of anandamide and capsaicin on arachidonic acid release in rat pheochromocytoma PC12 cells. Both agents stimulated [^{3}H]arachidonic acid release in a concentration-dependent manner from the prelabeled PC12 cells even in the absence of extracellular $CaCl_2$. The effect of anandamide was neither mimicked by an agonist nor inhibited by an antagonist for cannabinoid receptors. The effects of anandamide and capsaicin were inhibited by phospholipase A_2 inhibitors, but not by an antagonist for vanilloid VR_1 receptor. In PC12 cells preincubated with anandamide or capsaicin, [^{3}H]arachidonic acid release was marked and both agents were no more effective. Co-addition of anandamide or capsaicin synergistically enhanced [^{3}H]arachidonic acid release by mastoparan in the absence of $CaCl_2$. Anandamide stimulated prostaglandin $F_{2\alpha}$ formation. These findings suggest that anandamide and capsaicin stimulated arachidonic acid metabolism in cannabinoid receptors- and vanilloid VR_1 receptor-independent manner in PC12 cells. The possible mechanisms are also discussed.

Keywords: Anandamide; Capsaicin; Arachidonic acid; Prostaglandin $F_{2\alpha}$; Phospholipase A_2; PC12 cell

1. Introduction

Anandamide (*N*-arachidonoyl ethanolamine) was isolated from the brain as an endogenous agonist of cannabinoid receptors, and biosynthetic pathways for anandamide are present in various tissues (Hillard and Campbell, 1997; Howlett and Mukhopadhyay, 2000). The cannabinoid CB_1 receptors are expressed primarily in the central nervous system (CNS) and the cannabinoid CB_2 receptors are expressed by cells of the immune system (Hillard and Campbell, 1997; Howlett and Mukhopadhyay, 2000). PC12 cells, a neuroendocrine cell line derived from rat pheochromocytoma, have been used as a neuron model. It was shown that PC12 cells produced anandamide (Bisogno et al., 1998) and that the addition of anandamide at 1–10 μM induced apoptosis of PC12 cells via activation of cannabinoid CB_1 receptor (Sarker et al., 2000). It was reported that several pharmacological effects of anandamide were not mediated via cannabinoid receptors. For example, anandamide at concentrations greater than 10 μM could elicit an increase in the intracellular-free Ca^{2+} concentration ($[Ca^{2+}]_i$) levels in Chinese hamster ovary cells, irrespective of whether the cells expressed cannabinoid CB_1 or CB_2 receptors (Felder et al., 1992, 1993). Anandamide interacted with L-type Ca^{2+} channels (Shimasue et al., 1996) and potentiated the growth in hematopoietic cell lines via a cannabinoid receptors-independent pathway (Derocq et al., 1998). Recently, it was established that anandamide acted as a partial or full agonist for capsaicin-sensitive vanilloid VR_1 receptor (Zygmunt et al., 1999; Smart et al., 2000; Olah et al., 2001).

Capsaicin, one of the flavoring ingredients in chilli peppers, excites subpopulation of sensory neurons (Szallasi and Blumberg, 1999; Caterina and Julius, 2001). Caterina et al. (1997) reported the cloning of vanilloid VR_1 receptor, which was a nonselective cation channel with high Ca^{2+} permeability and activated by capsaicin, other vanilloid compounds, low pH and noxious heat. Vanilloid VR_1 receptor

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was found to express in various tissues including the CNS (Szallasi and Blumberg, 1999; Caterina and Julius, 2001). In human embryonic kidney 293 cells expressing the rat vanilloid VR_1 receptor, both capsaicin and anandamide showed the maximal increase in the $[Ca^{2+}]_i$ levels at 1 and 10 μM, respectively (Sprague et al., 2001). In PC12 cells, however, capsaicin at concentrations greater than 3 μM, which alone showed no effects, inhibited an increase in the $[Ca^{2+}]_i$ levels and dopamine release induced by acetylcholine (Nakazawa et al., 1994). Choi and Kim (1999) also reported that capsaicin inhibited an increase in $[Ca^{2+}]_i$ by blocking store-operated Ca^{2+} entry in PC12 cells. Thus, it has not been established whether the pharmacological effects of capsaicin were due to the activation of vanilloid VR_1 receptor in PC12 cells.

Phospholipase A_2, the rate-limiting enzyme in arachidonic acid metabolism, catalyzes the hydrolysis of phospholipids at the *sn*-2 position to produce lysophospholipids and polyunsaturated fatty acids including arachidonic acid that are the precursors for prostaglandins, thromboxanes and a variety of other eicosanoids (Leslie, 1997; Balsinde et al., 1999). Arachidonic acid and its metabolites such as prostaglandins regulate many neuronal cell functions (Shimizu and Wolfe, 1990; Lukiw and Bazan, 2000). It was reported that activation of cannabinoid receptors by anandamide released arachidonic acid in various cells including neuronal cells (Diaz et al., 1994; Wartmann et al., 1995; Hunter and Burstein, 1997; Chan et al., 1998). However, Felder et al. (1992, 1993) reported that cannabinoid agonists including anandamide stimulated arachidonic acid release through both cannabinoid receptor- and non-receptor-mediated pathways in the same cell. In PC12 cells, it has not been determined whether anandamide activates arachidonic acid release via cannabinoid receptors and/or via other receptors such as vanilloid VR_1 receptor. In addition, the effect of capsaicin on arachidonic acid metabolism has not been well established in neuronal cells including PC12 cells. Here, we report that anandamide stimulated arachidonic acid release via capsaicin-sensitive receptors and/or sites, but not via cannabinoid CB_1 receptor and vanilloid VR_1 receptor, in an extracellular $CaCl_2$-independent manner in PC12 cells. The effects of anandamide and capsaicin were inhibited by phospholipase A_2 inhibitors, and anandamide stimulated prostaglandin $F_{2\alpha}$ formation in PC12 cells. The possible involvement of Ca^{2+}-independent mechanisms in phospholipase A_2 activation induced by anandamide and capsaicin in PC12 cells are discussed.

2. Experimental procedures

2.1. Materials

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (215 Ci/mmol, 7.96 TBq/mmol) was purchased from Amersham (Buckinghamshire, UK). Anandamide, capsaicin, ruthenium red, capsazepine, ionomycin, *p*-bromophenacyl bromide, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis acetoxymethyl ester (BAPTA-AM) and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO, USA). Mastoparan and R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]-pyrrolo-[1,2,3,-de]-1,4-benzoxazin-yl]-(1-napthalenyl)-methanone mesylate (WIN-55212-2) were purchased from Bachem (Bubendorf, Switzerland) and Tocris (Ballwin, MO, USA), respectively. *N*-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide (SR141716A) and methyl arachidonyl fluorophosphonate were obtained from RBI (Natick, MA, USA) and Calbiochem (San Diego, CA, USA), respectively. 1,6-*bis*(Cyclohexyloximinocarbonylamino)hexane (RHC-80267) was purchased from Alexis (San Diego, CA, USA). Capsaicin and anandamide were dissolved in a minimum of ethanol, and WIN-55212-2 was dissolved in a minimum of dimethyl sulphoxide. These agents were diluted with buffer when used. An enzyme immunoassay kit of prostaglandin $F_{2\alpha}$ was purchased from Cayman (Cat No. 516011, Ann Arbor, MI, USA). The vehicle containing ethanol or dimethyl sulphoxide (the final concentration was less than 1%) had no effect on arachidonic acid release and prostaglandin $F_{2\alpha}$ formation.

2.2. Cell culture and measurement of [^{3}H]arachidonic acid release

PC12 cells were cultured on collagen-coated dishes in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 5% horse serum, and [^{3}H]arachidonic acid release from prelabeled PC12 cells was determined as described previously (Thang et al., 2000; Mori et al., 2001). In brief, PC12 cells on dishes were incubated with Dulbecco's modified Eagle's medium (0.2% serum) and 0.67 μCi/ml (24.7 kBq/ml) of [^{3}H]arachidonic acid for 24 h. The labeled cells were detached from dishes by pipetting. The cells were washed and suspended in a modified Tyrode HEPES buffer (137 mM NaCl, 5 mM KCl, 5 mM glucose, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES (pH 7.4)). Cell suspensions (30–50 μg protein) were incubated with the indicated agents for 30 min at 37 °C in the presence of 0.1% fatty acid-free bovine serum albumin (Sigma A-7511). The total volume was 200 μl and the reaction was terminated by the addition of 500 μl of ice-cold, Ca^{2+}-, Mg^{2+}-free Tyrode buffer containing 5 mM EDTA and EGTA followed by centrifugation (5000 × *g*, 30 s) at 4 °C. The ^{3}H content of the supernatant was estimated by liquid scintillation spectrometry. Values were calculated as percentages relative to the total incorporation of [^{3}H]arachidonic acid.

2.3. Measurement of $PGF_{2\alpha}$ formation in PC12 cells

Confluent PC12 cells on 22-mm dishes (12-well plates) were incubated with the indicated agents for 30 min at 37 °C in the Tyrode HEPES buffer (pH 7.4) containing 0.1%

fatty acid-free albumin. The contents of prostaglandin $F_{2\alpha}$ in the buffer after centrifugation (1000 × *g*, 30 s, 4 °C) were determined using an enzyme immunoassay kit. Although 300 μM (104 μg/ml) anandamide solution without PC12 cells cross-reacted with the kit slightly, the cross-reactivity of anandamide was low. In addition, the samples after stimulation with 300 μM anandamide were assayed with a 10-fold dilution; thus, the contaminated 30 μM anandamide did not show a significant cross-reactivity.

2.4. Statistics

Values are means ± S.E.M. for the indicated numbers (over three) of independent experiments performed in triplicate assays. In the case of multiple comparisons, the significance of differences was determined using one-way analysis of variance followed by Dunnett's or Tukey's test. For pairwise comparisons, Student's two-tailed *t*-test was used. *P* values at <0.05 were considered to be significant.

3. Results

3.1. Anandamide- and capsaicin-stimulated [^{3}H]arachidonic acid release in the presence or absence of extracellular $CaCl_2$ in PC12 cells

First, the effects of anandamide and capsaicin on [^{3}H]arachidonic acid release from the prelabeled PC12 cells were investigated in the presence of 2 mM extracellular $CaCl_2$ (Fig. 1). The addition of anandamide stimulated [^{3}H]arachidonic acid release in a concentration-dependent manner; the effect induced by anandamide appeared to be maximal at 300 μM (Panel A). Capsaicin stimulated [^{3}H]arachidonic acid release in a concentration-dependent manner; the effects induced by 100 and 200 μM capsaicin were small but significant, and 300 μM capsaicin markedly stimulated the release (Panel B). Anandamide and capsaicin from 0.1 to 30 μM showed no effect. Since the solubility of capsaicin in the buffer was low, we could not examine the effect of capsaicin at concentrations greater than 300 μM. The vehicle containing ethanol did not stimulate [^{3}H]arachidonic acid release. The addition of 300 μM PMSF (an inhibitor of fatty acid amide hydrolase) had no effect on anandamide- and capsaicin-induced arachidonic acid releases in PC12 cells (data not shown).

It was reported that PC12 cells expressed cannabinoid CB_1 receptors (Sarker et al., 2000; Wang et al., 2000). However, the addition of 100 μM WIN-55212-2, a selective and potent agonist for cannabinoid receptors (Pertwee, 2001), had no effect on [^{3}H]arachidonic acid release; the value was 0.5 ± 0.2% ($n=3$), which was similar to that with vehicle. Treatment with 20 μM SR141716A, a selective antagonist for cannabinoid CB_1 receptors (Pertwee, 2001),

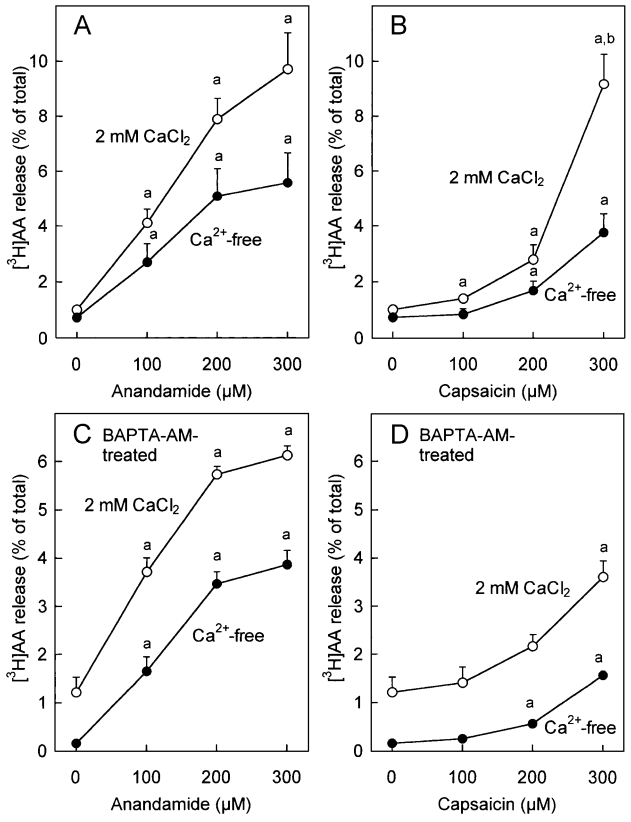

Fig. 1. Anandamide- and capsaicin-induced [^{3}H]arachidonic acid release in PC12 cells. The labeled PC12 cells were detached from dishes and washed three times by centrifugation with the $CaCl_2$-free Tyrode HEPES buffer. In Panels C and D, the cells were further pretreated with 20 μM BAPTA-AM for 10 min in the absence of extracellular $CaCl_2$, and washed by centrifugation with the $CaCl_2$-free buffer. For measurement of [^{3}H]arachidonic acid release, cells were incubated for 30 min with the indicated concentrations of anandamide (Panels A and C) and capsaicin (Panels B and D) in the presence (○) or absence (●) of 2 mM $CaCl_2$. EGTA (0.2 mM) was further added to the assay mixture. Values are means ± S.E.M. for four independent experiments done in triplicate. [a]$P<0.05$, significantly different from the value without anandamide or capsaicin. [b]$P<0.05$, significantly different from the net increase by capsaicin in the absence of $CaCl_2$.

did not inhibit anandamide- and capsaicin-stimulated [^{3}H]arachidonic acid releases (data not shown). Capsazepine and ruthenium red were shown to be a competitive and a noncompetitive antagonist for vanilloid VR_1 receptor, respectively (Szallasi and Blumberg, 1999). However, treatment with 10 and 100 μM capsazepine for 10 min rather enhanced than inhibited capsaicin-stimulated [^{3}H]arachidonic acid release in PC12 cells. Treatment with 100 μM ruthenium red for 1 h inhibited anandamide- and capsaicin-stimulated [^{3}H]arachidonic acid release, but the treatment also inhibited basal [^{3}H]arachidonic acid release without stimulants markedly (data not shown).

In the extracellular $CaCl_2$-free buffer containing 0.2 mM EGTA, the addition of anandamide significantly stimulated [^{3}H]arachidonic acid release, although the effects were less than those in the presence of $CaCl_2$ (Fig. 1, Panel A). Similarly, 200 and 300 μM capsaicin

significantly stimulated [^{3}H]arachidonic acid release in the absence of $CaCl_2$, although the effect was much less than that in the presence of $CaCl_2$ (Panel B). Next, we treated PC12 cells with 20 μM BAPTA-AM (a cell-permeable chelator of Ca^{2+}) for 10 min in the absence of extracellular $CaCl_2$, to further reduce $[Ca^{2+}]_i$ levels in PC12 cells (Panels C and D). In BAPTA-AM-treated PC12 cells, basal [^{3}H]arachidonic acid release in the absence of $CaCl_2$ was markedly less than that in the presence of 2 mM $CaCl_2$. The addition of anandamide from 100 to 300 μM and capsaicin at 200–300 μM significantly stimulated [^{3}H]arachidonic acid release from the BAPTA-AM-treated PC12 cells in the absence of $CaCl_2$. These findings suggest that anandamide- and capsaicin-stimulated [^{3}H]arachidonic acid releases were not dependent on an extracellular $CaCl_2$, although the releases in the absence of $CaCl_2$ were less than those in the presence of $CaCl_2$.

3.2. Enhancement of ionomycin- and mastoparan-stimulated [^{3}H]arachidonic acid release by anandamide and capsaicin in PC12 cells

Next, we investigated the effects of anandamide and capsaicin on ionomycin- and mastoparan-stimulated [^{3}H]arachidonic acid release from PC12 cells. As previously reported (Murayama et al., 1995; Mori et al., 2001), addition of 5 μM ionomycin alone stimulated [^{3}H]arachidonic acid release in the presence of 2 mM $CaCl_2$ (Fig. 2). The addition of 100 μM anandamide appeared to enhance the release by ionomycin. The addition of 100 and 200 μM capsaicin enhanced ionomycin-stimulated [^{3}H]arachidonic acid release (Panel B); the release stimulated by ionomycin in the presence of capsaicin was synergistically higher compared with the estimated additive value by combination of the two agents. The [^{3}H]arachidonic acid release induced by the combination of anandamide (200 or 300 μM) or 300 μM capsaicin in the presence of 5 μM ionomycin was similar to those without ionomycin.

Mastoparan, a wasp venom peptide, stimulated [^{3}H]arachidonic acid release by activation of cytosolic phospholipase A_2 in the absence of $CaCl_2$ from PC12 cells (Thang et al., 2000). In addition, in the present study, mastoparan stimulated [^{3}H]arachidonic acid release in a concentration-dependent manner, and the ED_{50} value of mastoparan was 17.03 ± 0.29 μM ($n=3$) (Fig. 3). The ED_{50} values of mastoparan in the presence of 100 μM anandamide and 200 μM capsaicin were 11.87 ± 0.85 and 8.80 ± 0.96 μM ($n=3$), respectively; both values were significantly lower ($P<0.05$) than that with mastoparan alone. The maximal releases of [^{3}H]arachidonic acid induced by the combination of mastoparan and anandamide/capsaicin were similar to that induced by 30 μM mastoparan alone.

Previously, we reported that the addition of Na_3VO_4 alone stimulated [^{3}H]arachidonic acid release and that

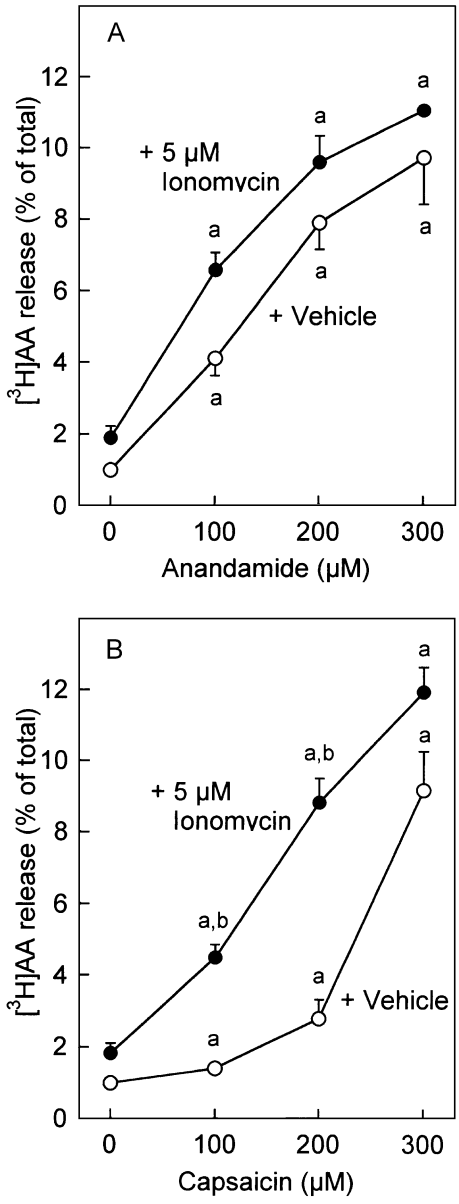

Fig. 2. Enhancement of ionomycin-stimulated [^{3}H]arachidonic acid release by anandamide and capsaicin in PC12 cells. The labeled PC12 cells were incubated for 30 min with the indicated concentrations of anandamide (Panel A) and capsaicin (Panel B) in the presence of 2 mM $CaCl_2$. The assay mixture was further supplemented with vehicle (○) or 5 μM ionomycin (●). Values are means ± S.E.M. for four independent experiments done in triplicate. [a]$P<0.01$, significantly different from the value without anandamide or capsaicin. [b]$P<0.01$, significantly different from the estimated additive value by capsaicin and ionomycin.

Na_3VO_4 synergistically enhanced ionomycin- and mastoparan-induced [^{3}H]arachidonic acid release (Mori et al., 2001). Interestingly, neither anandamide nor capsaicin enhanced 5 mM Na_3VO_4-stimulated [^{3}H]arachidonic acid release (Table 1). These findings suggest that anandamide and capsaicin showed stimulatory effects on ionomycin- and mastoparan-, but not Na_3VO_4-, stimulated arachidonic acid releases in PC12 cells.

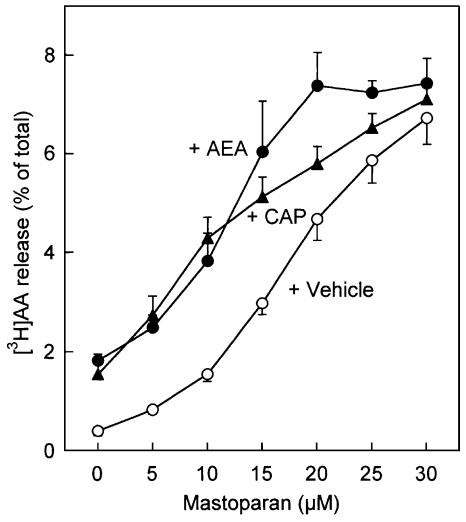

Fig. 3. Enhancement of mastoparan-stimulated [^{3}H]arachidonic acid release by anandamide and capsaicin in PC12 cells. The labeled PC12 cells were incubated for 30 min with the indicated concentrations of mastoparan in the absence of extracellular $CaCl_2$. The assay mixture was further supplemented with vehicle (○), 100 μM anandamide (AEA, ●) and 200 μM capsaicin (CAP, ▲). Values are means ± S.E.M. for four independent experiments done in triplicate. The [^{3}H]arachidonic acid release induced by 10 μM mastoparan in the presence of 200 μM capsaicin was significantly synergistic compared with the estimated value by a combination of mastoparan and capsaicin ($P<0.05$).

3.3. Irreversible effects of anandamide and capsaicin on [^{3}H]arachidonic acid release in PC12 cells

Next, we measured [^{3}H]arachidonic acid release from anandamide-treated PC12 cells (Table 2). The release from PC12 cells preincubated with 300 μM anandamide for 30 min and washed by centrifugation was significantly higher than that from the nontreated (control) PC12 cells. The [^{3}H]arachidonic acid release from anandamide-treated cells was similar to the release stimulated with 300 μM anandamide from the control cells. The addition of 300 μM anandamide or capsaicin did not show an additive stimulatory effect on the release from the anandamide-treated cells. The effect of capsaicin was also irreversible, as the [^{3}H]arachidonic acid release from the 300 μM capsaicin-treated PC12 cells was markedly higher than that from the control cells. The addition of 300 μM anandamide or capsaicin did not stimulate [^{3}H]arachidonic acid release any more from the capsaicin-treated PC12 cells. Although 20 μM mastoparan slightly stimulated [^{3}H]arachidonic acid release from the anandamide- and capsaicin-treated PC12 cells, the effects were limited and not significant.

Table 1
Not enhancement of Na_3VO_4-stimulated [^{3}H]arachidonic acid release by anandamide and capsaicin in PC12 cells

Addition	[^{3}H]Arachidonic acid release (% of total)	
	None	5 mM Na_3VO_4
None	1.0 ± 0.1	1.4 ± 0.2
Anandamide (100 μM)	4.1 ± 0.5[a]	3.3 ± 0.5[a]
Anandamide (200 μM)	7.9 ± 0.7[a]	6.3 ± 0.8[a]
Capsaicin (300 μM)	9.2 ± 0.5[a]	7.1 ± 0.4[a]

The labeled PC12 cells were incubated for 30 min with vehicle, 100 and 200 μM anandamide, 300 μM capsaicin and/or 5 mM Na_3VO_4 in the presence of 2 mM $CaCl_2$. Values are means ± S.E.M. for four independent experiments done in triplicate.

[a] $P<0.01$, significantly different from the value without agents.

Table 2
Irreversible stimulation of [^{3}H]arachidonic acid release by anandamide and capsaicin in PC12 cells

Addition	[^{3}H]Arachidonic acid release (% of total)		
	Control	Anandamide-treated	Capsaicin-treated
None	1.3 ± 0.2	5.9 ± 0.8[a]	5.8 ± 0.6[a]
Anandamide	5.9 ± 1.0[a]	6.1 ± 0.5	4.5 ± 0.5
Capsaicin	3.5 ± 0.6[a]	4.0 ± 0.2	5.0 ± 0.6
Mastoparan	5.4 ± 0.8[a]	6.5 ± 0.4	7.4 ± 0.4

The labeled PC12 cells were incubated with vehicle (control), 300 μM anandamide or 300 μM capsaicin for 30 min in the absence of $CaCl_2$. The cells were washed twice with the buffer without agents, and then incubated for 30 min for measurement of [^{3}H]arachidonic acid release in the absence of $CaCl_2$. The assay mixture was further supplemented with vehicle, 300 μM anandamide, 300 μM capsaicin or 20 μM mastoparan. The total incorporated amounts of [^{3}H]arachidonic acid in the cells treated with anandamide and capsaicin were similar to that in the control cells. Values are means ± S.E.M. for three independent experiments done in triplicate.

[a] $P<0.05$, significantly different from the control value without agents.

3.4. Effects of phospholipase A_2 inhibitors on anandamide- and capsaicin-stimulated [^{3}H]arachidonic acid release in PC12 cells

Previously, we reported that treatment with 50 μM *p*-bromophenacyl bromide, a nonspecific covalent-modifying phospholipase A_2 agent (Balsinde et al., 1999), inhibited

Table 3
Effects of phospholipase A_2 inhibitors on anandamide- and capsaicin-stimulated [^{3}H]arachidonic acid release in PC12 cells

Treatments	[^{3}H]Arachidonic acid release (% of control)		
	Control	50 μM BPB	20 μM MAFP
Anandamide (300 μM)	100	49.6 ± 2.6[a]	61.7 ± 1.0[a]
Capsaicin (300 μM)	100	43.6 ± 5.2[a]	44.8 ± 0.7[a]
Mastoparan (20 μM)	100	52.7 ± 7.5[a]	not determined

The labeled PC12 cells were preicubated with vehicle (control), 50 μM *p*-bromophenacyl bromide (BPB) or 20 μM methyl arachidonyl fluorophosphonate (MAFP) or vehicle for 10 min. The washed cells were stimulated with the indicated agents for 30 min in the absence of $CaCl_2$. The same concentrations of phospholipase A_2 inhibitors were further added to the assay mixture. Values are normalized as percentages of [^{3}H]arachidonic acid release induced by the indicated agents from the nontreated (control) cells. Values are means ± S.E.M. for four independent experiments done in triplicate. The absolute values of [^{3}H]arachidonic acid release (% of total) were 0.6 ± 0.2 (vehicle), 5.5 ± 0.3 (anandamide), 3.4 ± 0.3 (capsaicin) and 6.0 ± 0.8 (mastoparan), respectively. The basal [^{3}H]arachidonic acid releases without the stimulants in the cells treated with phospholipase A_2 inhibitors were similar to that in the control cells.

[a] $P<0.01$, significantly different from the value without phospholipase A_2 inhibitors.

Table 4
Increase in prostaglandin $F_{2\alpha}$ formation by anandamide in the medium of PC12 cell culture

Increase in prostaglandin $F_{2\alpha}$ (pg/dish/30 min)	
None	15.3 ± 1.8
Anandamide (100 μM)	69.8 ± 10.0[a]
Anandamide (300 μM)	568.3 ± 75.7[a]

PC 12 cells on 22-mm dishes were incubated in the Tyrode HEPES buffer containing 0.1% bovine serum albumin (fatty acid-free grade) for 30 min with vehicle or anandamide. The content of prostaglandin $F_{2\alpha}$ in the buffer without PC12 cells was under the detection limit (<8 pg/ml). Values are means ± S.E.M. for four independent experiments done in triplicate.

[a] $P<0.01$, significantly different from the value without anandamide.

mastoparan-stimulated [^{3}H]arachidonic acid release in PC12 cells (Thang et al., 2000). The treatment with 50 μM *p*-bromophenacyl bromide significantly inhibited not only 20 μM mastoparan- but also 300 μM anandamide- and capsaicin-stimulated [^{3}H]arachidonic acid release in PC12 cells (Table 3). Methyl arachidonyl fluorophosphonate is a potent inhibitor of cytosolic phospholipase A_2 (Balsinde et al., 1999). The treatment with 20 μM methyl arachidonyl fluorophosphate partially, but significantly, inhibited 300 μM anandamide- and capsaicin-stimulated [^{3}H]arachidonic acid release. The higher concentrations of methyl arachidonyl fluorophosphate did not show a significantly higher inhibitory effect (data not shown).

3.5. Increase in prostaglandin $F_{2\alpha}$ formation by anandamide in PC12 cells

Arachidonic acid is metabolized to prostaglandins and other metabolites in cells, and then released to the culture medium. Next, we measured the prostaglandin $F_{2\alpha}$ formation in the medium for 30 min after stimulation of PC12 cells with anandamide in the presence of $CaCl_2$ (Table 4). The addition of 100 and 300 μM anandamide markedly stimulated prostaglandin $F_{2\alpha}$ formation. In preliminary experiments, 300 μM capsaicin did not stimulate prostaglandin $F_{2\alpha}$ formation in PC12 cells. The reasons are not clear at present.

4. Discussion

4.1. Characteristics of arachidonic acid release by anandamide in PC12 cells

Anandamide has been established as an endogenous eicosanoid with moderate affinity for both of the cannabinoid CB_1 and CB_2 receptors, although anandamide is functionally ineffective at cannabinoid CB_2 receptors (Howlett and Mukhopadhyay, 2000; Pertwee, 2001). It was reported that cannabinoids and anandamide stimulated arachidonic acid release and its metabolites from various cells (Diaz et al., 1994; Felder et al., 1992, 1993; Wartmann et al., 1995; Hunter and Burstein, 1997; Chan et al., 1998). Wartmann et al. (1995) reported the involvement of cannabinoid receptors coupled to pertussis toxin-sensitive GTP-binding proteins on activation of cytosolic phospholipase A_2 induced by anandamide in WI-38 human fetal lung fibroblasts. In neuronal cells, cannabinoids such as anandamide stimulated arachidonic acid release via activation of cannabinoid receptors (Hunter and Burstein, 1997; Chan et al., 1998). In the present study, we showed that anandamide at concentrations greater than 100 μM stimulated arachidonic acid release (Fig. 1) and prostaglandin $F_{2\alpha}$ formation (Table 4) in PC12 cells. Although anandamide at 1–10 μM induced apoptosis via cannabinoid CB_1 receptors in PC12 cells (Sarker et al., 2000; Wang et al., 2000), arachidonic acid release induced by anandamide did not appear to be due to activation of cannabinoid CB_1 receptors. The reasons were: (1) high concentrations greater than 100 μM of anandamide were required for [^{3}H]arachidonic acid release, (2) WIN-55212-2 (100 μM), an agonist for cannabinoid receptors, did not stimulate the release and (3) SR141716A, an antagonist for cannabinoid CB_1 receptor, did not inhibit the effect of anandamide.

As mentioned in Section 1, it was reported that anandamide showed various pharmacological effects including an increase of $[Ca^{2+}]_i$ levels in cannabinoid receptor-independent manner. In addition, Δ^9-tetrahydrocannabinol produced an increase in the $[Ca^{2+}]_i$ levels at least partially via cannabinoid receptor-independent mechanisms in DDT1MF-2 smooth muscle cells (Filipeanu et al., 1997). However, the effect of anandamide on [^{3}H]arachidonic acid release was observed in the BAPTA-AM-treated PC12 cells in the absence of $CaCl_2$. Kiss (1999) reported that anandamide stimulated phospholipase D activity and thus produced phosphatidic acid in PC12 cells, although it has not been determined whether the effect of anandamide was mediated by cannabinoid receptors or not. Phosphatidic acid can be degraded to 1,2-diacylglycerol, and thus hydrolysis of 1,2-diacylglycerol by diacylglycerol lipase can stimulate arachidonic acid release. Burstein et al. (1994) reported that cannabinoid-induced arachidonic acid release occurred through activation of phospholipase D, diacylglycerol lipase and protein kinase C in mouse peritoneal cells. However, we previously reported that the addition of phorbol 12-myristate 13-acetate, which activated phospholipase D activity in PC12 cells (Kiss, 1999), showed no effect on [^{3}H]arachidonic acid release in PC12 cells (Murayama et al., 1995). In addition, treatment with 10 μM diacylglycerol lipase inhibitor (RHC-80267) did not inhibit arachidonic acid release induced by anandamide (data not shown).

The cannabinoid receptor-independent effects induced by anandamide may be due to the structural similarities between arachidonic acid and anandamide and its hydrophobicity. Anandamide and arachidonic acid in higher concentrations over 10 μM promoted an increase in membrane fluidity in synaptosomes (Bloom et al., 1997). In addition, capsaicin is a hydrophobic agent because the structure of capsaicin is an acylamide derivative of homo-

vanillic acid (8-methyl-*N*-vanillyl-6-noneamide). However, [^{3}H]arachidonic acid releases induced by anandamide and capsaicin decreased by inhibitors of phospholipase A_2, as described below. In addition, anandamide and capsaicin showed stimulatory effects on [^{3}H]arachidonic acid releases induced by ionomycin and mastoparan, but not by Na_3VO_4. These findings suggest that the effects of anandamide and capsaicin were not due to membrane perturbation and the exchange with fatty acids in the *sn*-2 position of phospholipids.

4.2. Stimulation of arachidonic acid release by anandamide and capsaicin in vanilloid VR_1 receptor-independent manner in PC12 cells

Anandamide was shown to act as an agonist for capsaicin-sensitive vanilloid VR_1 receptor in the dorsal root ganglia neurons and in cells expressing recombinant vanilloid VR_1 receptor (Zygmunt et al., 1999; Smart et al., 2000; Olah et al., 2001). Previously, Nakazawa et al. (1994) and Choi and Kim (1999) reported the pharmacological effects of capsaicin in PC12 cells. Since not only anandamide but also capsaicin stimulated [^{3}H]arachidonic acid release from PC12 cells (Fig. 1), it is possible that anandamide stimulates the release via activation of capsaicin-sensitive vanilloid VR_1 receptor. However, the pharmacological characteristics of capsaicin (and anandamide) response in the present study were quite different from those of classical vanilloid VR_1 receptor-mediated responses (Szallasi and Blumberg, 1999; Caterina and Julius, 2001). Firstly, capsaicin required higher concentrations greater than 100 μM to stimulate [^{3}H]arachidonic acid release in PC12 cells, although the ED_{50} values of capsaicin in the vanilloid VR_1 receptor-mediated responses in the sensory neurons were lower than 1 μM (Szallasi and Blumberg, 1999; Caterina and Julius, 2001). Secondly, the effects of capsaicin and anandamide in PC12 cells were, at least partially, independent of extracellular $CaCl_2$. Capsaicin and anandamide stimulated [^{3}H]arachidonic acid release from BAPTA-AM-treated PC12 cells, and enhanced the release induced by mastoparan in the absence of $CaCl_2$. Thirdly, capsazepine, an antagonist for vanilloid VR_1 receptor, did not inhibit but enhanced capsaicin-stimulated [^{3}H]arachidonic acid release. Although treatment with 100 μM ruthenium red inhibited the effects induced by capsaicin and anandamide, the inhibitory effect of ruthenium red does not appear to be due to the inhibition of vanilloid receptors because the basal [^{3}H]arachidonic acid release was inhibited markedly by ruthenium red. In addition, ruthenium red is known to show various effects including changes in intracellular Ca^{2+} homeostasis (Henzi and MacDermott, 1992). Fourth, the effects of capsaicin and anandamide on [^{3}H]arachidonic acid release were not modified in acid pH; the releases in the buffers at pH 5.0 and 6.5 were similar to that in the normal buffer (pH 7.4, data not shown). The responses of vanilloid VR_1 receptor by capsaicin and anandamide in cells expressing recombinant vanilloid VR_1 receptor were enhanced in the low pH buffer (Smart et al., 2000; Olah et al., 2001; Sprague et al., 2001). These findings suggest that anandamide and capsaicin stimulated [^{3}H]arachidonic acid release via a distinct manner from the classical vanilloid VR_1 receptor-mediated mechanism in PC12 cells. Pharmacological evidence suggested the existence of vanilloid receptor subtypes with distinct characteristics (Ács et al., 1997), and homologs of vanilloid VR_1 receptor were cloned (Suzuki et al., 1999; Caterina et al., 1999). Other subtypes of vanilloid receptor or anandamide- and capsaicin-sensitive target protein(s) may exist with coupling mechanisms to arachidonic acid release in PC12 cells.

4.3. Subtypes of phospholipase A_2 for arachidonic acid release by anandamide and capsaicin in PC12 cells

More than 10 different groups of phospholipase A_2 enzymes have been described (Leslie, 1997; Balsinde et al., 1999). The majority of these enzymes, secretory phospholipase A_2s, appeared not to be regulated by receptor activation, whereas two groups, cytosolic phospholipase A_2 (group IV) and Ca^{2+}-independent phospholipase A_2 (group VI), are subject to regulation by receptor stimulation by extracellular signals. Secretory phospholipase A_2s require Ca^{2+} at millimolar concentrations for its activation. Due to Ca^{2+}-independency, the effects induced by anandamide and capsaicin were not due to activation of secretory phospholipase A_2s in PC12 cells, although the enzymes were expressed in PC12 cells (Matsuzawa et al., 1996; Ohsawa et al., in press).

Cytosolic phospholipase A_2 is regulated by Ca^{2+} at approximately micromolar concentrations and its phosphorylation by various kinases; both were regulated by receptor activation (Leslie, 1997; Balsinde et al., 1999; Hirabayashi and Shimizu, 2000). In the present study, anandamide- and capsaicin-stimulated [^{3}H]arachidonic acid releases in the presence of $CaCl_2$ were greater than those without $CaCl_2$ (Fig. 1). The addition of 100 and 200 μM capsaicin synergistically enhanced ionomycin-stimulated [^{3}H]arachidonic acid release in the presence of $CaCl_2$ (Fig. 2). Both anandamide and capsaicin shifted the concentration-dependent curve of mastoparan, which activated cytosolic phospholipase A_2 in PC12 cells (Thang et al., 2000), to the left without changing the maximal response in the absence of $CaCl_2$ (Fig. 3). Treatment with methyl arachidonyl fluorophosphonate, a potent inhibitor of cytosolic phospholipase A_2 (Balsinde et al., 1999), decreased [^{3}H]arachidonic acid release stimulated by anandamide and capsaicin (Table 3). Treatment with *p*-bromophenacyl bromide, which inhibited mastoparan-stimulated [^{3}H]arachidonic acid release probably via cytosolic phospholipase A_2 activation (Thang et al., 2000), also inhibited the releases by anandamide and capsaicin in PC12 cells. These findings suggest that anandamide and capsaicin stimulate arachidonic acid release via cytosolic phospholipase A_2 activation in PC12 cells.

Although a major function of Ca^{2+}-independent phospholipase A_2 is to mediate phospholipids remodeling, the enzymes are subjected to activation by the receptor-mediated signaling system and may play other roles (Winstead et al., 2000). In the present study, the effects of capsaicin and anandamide on [^{3}H]arachidonic acid release were Ca^{2+}-independent at least in part. Methyl arachidonyl fluorophosphonate was shown to inhibit both cytosolic and Ca^{2+}-independent phospholipase A_2s, and *p*-bromophenacyl bromide is a nonspecific inhibitor of phospholipase A_2 (Balsinde et al., 1999). Thus, the possible involvement of Ca^{2+}-independent phospholipase A_2 on anandamide- and capsaicin-stimulated arachidonic acid release in PC12 cells remains to be determined.

4.4. Problems to be solved and possible mechanisms for activation of cytosolic phospholipase A_2 by anandamide and capsaicin in PC12 cells

In conclusion, the present findings showed that anandamide and capsaicin at concentrations greater than 100 μM stimulated arachidonic acid release in PC12 cells, in cannabinoid CB_1 receptor- and vanilloid VR_1 receptor-independent manner. As mentioned in Section 1, it was reported that anandamide and capsaicin at higher concentrations caused various pharmacological effects in cannabinoid receptors- and/or vanilloid VR_1 receptor-independent manner (Felder et al., 1992, 1993; Nakazawa et al., 1994; Shimasue et al., 1996). Capsaicin at concentrations greater than 30 μM inhibited the activities of Ca^{2+} channels for capacitative Ca^{2+} entry in PC12 cells (Choi and Kim, 1999) and Jurkat T-cells (Fischer et al., 2001). The existence of the transport systems and the metabolism (or degradation) pathways for anandamide has been shown in some cells including PC12 cells (Bisogno et al., 1998; Hwang et al., 2000; De Petrocellis et al., 2001), although the systems and/or pathways for capsaicin have not been well established. These may explain the pharmacological effects induced by higher concentrations of anandamide and capsaicin, although the addition of 300 μM PMSF (an inhibitor of fatty acid amide hydrolase) had no effect on anandamide-induced arachidonic acid in PC12 cells. Research in the transport and metabolic systems of anandamide and capsaicin will be needed to further explore and validate the biological effects of anandamide and capsaicin.

The addition of anandamide and capsaicin did not show an additive stimulatory effect either on the anandamide- or capsaicin-treated PC12 cells (Table 2). Co-addition of 300 μM capsaicin did not show an additive effect on 300 μM anandamide-stimulated [^{3}H]arachidonic acid release (data not shown). These findings suggest that there is common and/or similar process in arachidonic acid release by anandamide and capsaicin in PC12 cells. Recently, Huwiler et al. (2001) reported that some ceramides having long-chain fatty acids bound tightly to the Ca^{2+}-dependent lipid binding domain of cytosolic phospholipase A_2 and activated the activity in vitro. Since anandamide has an arachidonyl-moiety and capsaicin has an acyl chain in their structure, both agents may react with the lipid binding domain in cytosolic phospholipase A_2 and stimulate arachidonic acid release in PC12 cells.

Another possibility is the regulation of cytosolic phospholipase A_2 activity by phosphorylation by anandamide and capsaicin. Previously, we reported that Na_3VO_4, a general inhibitor of protein tyrosine phosphatases, stimulated [^{3}H]arachidonic acid release and prostaglandin $F_{2\alpha}$ formation, and enhanced ionomycin- and mastoparan-stimulated [^{3}H]arachidonic acid release via activation of cytosolic phospholipase A_2 in PC12 cells (Mori et al., 2001). Like Na_3VO_4, both anandamide and capsaicin stimulated [^{3}H]arachidonic acid release from BAPTA-AM-treated PC12 cells, and enhanced mastoparan-stimulated [^{3}H]arachidonic acid release in the absence of $CaCl_2$ (Figs. 1 and 3). Anandamide and capsaicin did not enhance Na_3VO_4-induced [^{3}H]arachidonic acid release (Table 1). Thus, it is probable that anandamide and capsaicin activate cytosolic phospholipase A_2 in a similar pathway induced by Na_3VO_4 in PC12 cells. Further studies concerning the subtypes of phospholipase A_2 and its signal transduction pathway(s) for arachidonic acid release and the physiological responses stimulated by anandamide and capsaicin in PC12 cells are currently in progress in our laboratory.

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