

Regulation of calcium influx and catecholamine secretion in chromaffin cells by a cytochrome P450 metabolite of arachidonic acid

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Abstract These studies were designed to determine the role of arachidonic acid metabolites in catecholamine secretion from adrenal chromaffin cells. Inhibitors of the cytochrome P450-dependent metabolism of arachidonic acid were shown to interfere with stimulus-secretion coupling in cultured chromaffin cells. Ketoconazole (10 μM), clotrimazole (20 μM), and piperonyl butoxide (50 μM) inhibited carbachol-dependent catecholamine secretion by 44%, 83%, and 100%, respectively; histamine-dependent secretion by 25%, 60%, and 81%, and secretion induced by 59 mM KCl depolarization by 25%, 55%, and 89%. Uptake of $^{45}\text{Ca}^{2+}$ into the cells in response to carbachol was inhibited 63% by ketoconazole, 86% by clotrimazole, and 95% by piperonyl butoxide; KCl-dependent uptake was inhibited 7%, 56%, and 85%, respectively. However, cytochrome P450 inhibitors did not inhibit catecholamine secretion when cells were stimulated with the calcium ionophores ionomycin or lasalocid. These results indicated the involvement of a cytochrome P450 product in controlling Ca^{2+} influx in response to membrane depolarization. Cells prelabeled with [^3H]arachidonic acid formed a ^3H -labeled metabolite which comigrated with authentic 5,6-epoxyeicosatrienoic (5,6-EET) acid on reverse phase and normal phase HPLC. Pretreatment with clotrimazole inhibited the production of this ^3H -labeled metabolite. Addition of synthetic 5,6-EET (1 nM) to cells pretreated with piperonyl butoxide resulted in catecholamine secretion. These data suggest a role for a cytochrome P450 metabolite of arachidonic acid in agonist-stimulated catecholamine secretion.—Hildebrandt, E., J. P. Albanesi, J. R. Falck, and W. B. Campbell. Regulation of calcium influx and catecholamine secretion in chromaffin cells by a cytochrome P450 metabolite of arachidonic acid. *J. Lipid Res.* 1995. **36**: 2599-2608.

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Chromaffin cells of the adrenal medulla secrete catecholamines in response to acetylcholine, histamine, bradykinin, angiotensin II, and prostaglandin E_2 . As in sympathetic neurons, the release of secretory vesicles is exocytotic and is triggered by influx of extracellular calcium (Ca^{2+}) rather than release of Ca^{2+} from intracel-

lular stores (1). Binding of acetylcholine to nicotinic receptors initiates sodium (Na^+) entry via the receptor-associated ion channel. The resultant change in membrane potential activates further Na^+ entry via voltage-dependent fast Na^+ channels. Na^+ channel activity contributes to the firing of action potentials, which may serve to amplify the depolarization signal within the cell or to recruit neighboring cells. Ultimately, depolarization opens voltage-dependent Ca^{2+} channels. L-type channels are clearly involved in Ca^{2+} influx, and there is evidence for the participation of other, less well-characterized voltage-dependent Ca^{2+} channels and of receptor-associated ion channels (1–4). Although this overall sequence of events is well established, the regulatory mechanisms that govern the opening and closing of specific Ca^{2+} channels in response to activation of a given receptor are not presently understood.

The following observations indicate an essential role for a metabolite of arachidonic acid in controlling Ca^{2+} influx in the chromaffin cell. 1) Arachidonic acid (AA) is released from cellular phospholipids when chromaffin cells are stimulated to secrete (5). Inhibitors of phospholipase A_2 or diglyceride lipase, which prevent AA release, also inhibit Ca^{2+} uptake and catecholamine secretion (6–8). 2) Addition of exogenous AA to cultured chromaffin cells induces secretion and enhances acetylcholine-stimulated secretion (8). 3) Nordihydroguaiaretic acid (NDGA), 3-amino-1-trifluoromethyl-7-phenylpyrazoline (BW755C), and 2,3,5-trimethyl-6-

Abbreviations: AA, arachidonic acid; AA861, 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadienyl)-1,4-benzoquinone; BW755C, 3-amino-1-trifluoromethyl-7-phenylpyrazoline; NDGA, nordihydroguaiaretic acid; Pbutox, piperonyl butoxide; SKF525A, 2-diethylaminoethyl-2,2-diphenyl-n-pentanoate; 5,6-EET, 5,6-epoxyeicosatrienoic acid.

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(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone (AA861), inhibitors of the lipoxygenase metabolism of AA, were shown to inhibit both AA-dependent secretion and secretagogue-stimulated uptake of Ca^{2+} (8, 9).

In order to more clearly define the involvement of AA metabolites in the events that trigger secretion, we evaluated a panel of inhibitors for their effects on catecholamine secretion by chromaffin cells in response to several different experimental stimuli. Cells were treated with compounds that inhibit AA metabolism via the cyclooxygenase-, lipoxygenase-, or cytochrome P450-mediated pathways and were then stimulated by receptor activation, depolarization or ionophores that bypass Ca^{2+} channels. These studies revealed that inhibitors of the cytochrome P450 pathway of arachidonic acid metabolism, like the lipoxygenase inhibitors, inhibit catecholamine secretion by interfering with signalling at a step subsequent to depolarization but which precedes Ca^{2+} entry. We then tentatively identified a cytochrome P450 metabolite of AA formed in the cells as 5,6-epoxyeicosatrienoic (EET) acid and showed that this metabolite induces catecholamine secretion when added to chromaffin cells.

METHODS

Isolation and culture of chromaffin cells

Bovine adrenal glands were perfused with collagenase to dissociate the tissue. Chromaffin cells were isolated on Percoll gradients (10), washed, and resuspended at a density of 2×10^6 cells/ml in DME/F12 containing 10% fetal calf serum. After differential plating (11), the puri-

fied cells were plated onto collagen-coated polystyrene multiwell plates. Cultures were maintained in a humidified 37°C incubator under 5% CO_2 in air. After 3 days, the medium was changed to serum-free DME/F12 supplemented with 5 mg/ml insulin and 0.23 mM sodium ascorbate. Media also contained 100 U/ml penicillin G, 100 mg/ml streptomycin sulfate, 100 mg/ml gentamicin, 2.5 mg/ml amphotericin B, and 5 mg/ml each of uridine, fluorodeoxyuridine, and cytosine arabinoside.

Catecholamine secretion assay

Monolayer cultures (5×10^5 cells/16-mm well) were washed with antibiotic-free DME/F12 media and incubated for 2 h with 2 mCi/ml [^3H]norepinephrine at 37°C (12). The monolayers were then washed with six to eight changes of buffer 1 (145 mM NaCl, 5.6 mM KCl, 0.5 mM MgCl_2 , 2.2 mM CaCl_2 , 5.6 mM glucose, 0.25 mM ascorbate, 2.5 mg/ml bovine serum albumin, and 15 mM HEPES brought to pH 7.4 with NaOH). Inhibitors were added to preincubation solutions from ethanolic stock solutions. Controls received identical additions of ethanol, not exceeding 0.1% of the volume. Secretion was initiated by replacing the preincubation buffer with fresh buffer containing the inhibitor (or vehicle) plus a secretagogue. At the end of the stimulation period, the buffer was collected for measurement of the [^3H]catecholamine released. Radioactivity remaining in the cells was measured after solubilization with 1% Triton X-100. When the secretagogue was 59 mM KCl, the NaCl concentration was reduced by 50 mM. The buffer was supplemented with 0.2 mM Ca-EDTA in ionophore experiments. Sodium-deficient buffer was

TABLE 1. Effect of lipoxygenase inhibitors and cytochrome P450 inhibitors on secretion of catecholamines from bovine adrenal chromaffin cells

Pretreatment	Catecholamine Secreted (% of control) Stimulated by	
	Carbachol	KCl
NDGA (50 μM)	56 ± 1 (n = 4) ^a	72 ± 5 (n = 10) ^a
BW755C (50 μM)	66 ± 4 (n = 3) ^a	86 ± 3 (n = 10) ^a
AA861 (50 μM)	22 ± 3 (n = 4) ^a	41 ± 1 (n = 3) ^a
Ketoconazole (10 μM)	56 ± 2 (n = 4) ^a	75 ± 1 (n = 3) ^a
Clotrimazole (20 μM)	17 ± 1 (n = 4) ^a	45 ± 6 (n = 4) ^a
Piperonyl butoxide (50 μM)	0 ± 7 (n = 4) ^a	11 ± 5 (n = 6) ^a
Indomethacin (50 μM)	97 ± 4 (n = 4)	97 ± 2 (n = 4)
Metyrapone (50 μM)	88 ± 6 (n = 4)	105 ± 6 (n = 5)

After preincubation of cells for 15 min with the inhibitor, catecholamine release was measured in incubations in the presence of 0.05 mM carbachol for 15 min or with 59 mM KCl for 8 min. In the controls, net catecholamine release averaged $10 \pm 2\%$ of total cell content per 15 min (3- to 4-fold above basal) in response to carbachol stimulation (n = 12, three experiments), and $8.2 \pm 0.7\%$ per 8 min (2- to 6-fold above basal) upon KCl stimulation (n = 23, seven experiments). Values for net catecholamine release in each treatment group have been normalized to controls, and are expressed as mean \pm standard error.

^aSignificantly decreased compared to the control at $P < 0.01$.

made by substituting 0.29 M sucrose for NaCl in buffer 1 and by buffering with a pH 7.4 mixture of HEPES free acid and Tris base.

For each inhibitor tested, catecholamine release was measured in identical incubations in the presence and absence of secretagogue. Basal values were subtracted, and the resulting values for net release were expressed as percent of the non-inhibited control values measured within the same experiment. The data represent summarized results from multiple incubations from different cell preparations. The N values represent the number of wells used per treatment. Statistical comparisons were made using a one-way analysis of variance followed by a Student's *t*-test.

Calcium uptake assay

Uptake of $^{45}\text{CaCl}_2$ (2 $\mu\text{Ci}/\text{ml}$) was measured at various incubation time under the same conditions used for secretion measurements. Incubations were terminated by six rapid washes with Ca^{2+} -free buffer 1 containing 2 mM LaCl_3 and 2 mM EGTA (13). The cells were then solubilized in 1% Triton, and radioactivity was determined.

Labeling of phospholipids with [^3H]arachidonic acid and identification of metabolites

Chromaffin cell cultures (10 \times 10⁶ per 10-cm dish) were washed with serum-free medium, incubated 90 min in 4 ml of medium containing 0.9 mCi of [^3H]arachidonic acid, and washed twice with buffer 2 (149 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1.8 mM CaCl_2 , 10 mM HEPES, pH 7.4, 5.6 mM glucose, and 2.5 mg/ml fatty acid-free bovine serum albumin). Some cells were pre-

treated with clotrimazole (50 μM). The cells were incubated for 10 min with 4 ml buffer 2. The cells were scraped from the dish, and the suspension was mixed with 0.68 ml ice-cold ethanol. The lysates were sonicated, brought to pH 3 with acetic acid, and centrifuged. The extracts were applied to Bondelut C18 extraction cartridges (Varian, Palo Alto, CA). After washing the cartridges with 5 ml each of 15% ethanol, water, and petroleum ether, AA metabolites were eluted with 2 ml ethyl acetate (14), evaporated to dryness, taken up in 50% CH_3CN in water, and separated by reverse phase HPLC on a 4.6 \times 250 mm (5 μ) Nucleosil C18 column (Phenomenex, Rancho Palos Verdes, CA) at 1 ml/min using a 40 min linear gradient from $\text{H}_2\text{O}-\text{CH}_3\text{CN}-\text{HOAc}$ 500:499:1 to $\text{CH}_3\text{CN}-\text{HOAc}$ 999:1 (solvent system 1) (15). The column eluate was collected in 0.2-ml fractions and analyzed for radioactivity by liquid scintillation spectrometry. In a second set of experiments, cells were preincubated for 10 min with buffer 2 containing indomethacin (50 μM) and then incubated for 10 min in 4 ml of buffer 2 containing indomethacin. The cells were scraped from the dish, treated with ethanol, sonicated, and extracted as described above. The extract was analyzed by HPLC using solvent system 1. The column eluate was collected in 0.2-ml fractions and aliquots were analyzed for radioactivity. Radioactive peaks comigrating with the EETs (29.5–32 min) were pooled, extracted into cyclohexane-ethyl acetate 1:1, evaporated under nitrogen, redissolved in CH_2Cl_2 , and separated by normal phase HPLC on a 4.6 \times 250 mm (5 μ) Nucleosil silica column (Phenomenex) at 2 ml/min using an isocratic solvent of hexane-isopropanol-acetic acid 995:4:1 (solvent system 2) (15). The column eluate was collected in 0.3-ml fractions and analyzed for radioactivity. In a third

TABLE 2. Effect of cytochrome P450 and lipoxygenase inhibitors on catecholamine secretion stimulated by calcium ionophores

Pretreatment	Catecholamine Secreted (% of control) Stimulated by	
	Lasalocid	Ionomycin
Ketoconazole (10 μM)	102 \pm 6 (n = 8)	85 \pm 7 (n = 8)
Clotrimazole (20 μM)	126 \pm 11 (n = 8)	100 \pm 8 (n = 8)
Piperonyl butoxide (50 μM)	97 \pm 6 (n = 8)	215 \pm 34 (n = 4) ^b
NDGA (50 μM)	110 \pm 3 (n = 8) ^b	84 \pm 8 (n = 8)
BW755C (50 μM)	114 \pm 7 (n = 8)	
AA861 (50 μM)	86 \pm 5 (n = 8) ^a	

After preincubation of cells for 15 min with inhibitor, catecholamine release was measured in incubations in the presence of 50 μM lasalocid for 15 min or 20 μM ionomycin for 60 min. In the controls, net catecholamine release averaged 16.1 \pm 0.6% per 15 min (8- to 20-fold above basal; n = 16, four experiments) in response to lasalocid, and 6.2 \pm 0.5% per 60 min (2-fold above basal; n = 16, four experiments) in response to ionomycin. Values for net catecholamine release in each treatment group have been normalized to controls, and are expressed as mean \pm standard error.

^aSignificantly decreased compared to the control at $P < 0.001$.

^bSignificantly increased compared to the control at $P < 0.05$.

set of experiments, cells were preincubated for 10 min with buffer 2 containing indomethacin, then in 4 ml buffer 2 containing indomethacin and 0 or 0.3 mM carbachol. After 5 min, the cells were scraped, treated with methanol, and extracted as described above. The extract was analyzed by HPLC using solvent system 1. The column eluate was collected in 0.2-ml fractions and analyzed for radioactivity.

Materials

[³H]arachidonic acid (100 Ci/mmol, Amersham, Arlington Heights, IL) was purified before use by reverse phase HPLC as described above for the separation of metabolites, extracted into cyclohexane-ethyl acetate, evaporated to dryness, and dissolved in redistilled ethanol. Epoxyeicosatrienoic acids were synthesized as previously reported (16). Other compounds were obtained from the following sources: [³H]l-norepinephrine and ⁴⁵CaCl₂ (New England Nuclear, Boston, MA), AA861 (Takeda Chemical Industries, Osaka, Japan), analytical grade piperonyl butoxide (Serva Biochemicals, Paramus, NJ), SKF525A (2-(diethylamino) ethyl α -phenyl- α -propylbenzene acetate Biomol, Plymouth Meeting, PA), carbachol (Aldrich, Milwaukee, WI), fatty acid-free bovine serum albumin (Hazleton, Lenexa, KS), fetal calf serum and penicillin/streptomycin (Gibco BRL, Gaithersburg, MD). Other secretagogues, inhibitors, and tissue culture reagents were purchased from Sigma (St. Louis, MO). Metyrapone was a gift from Ciba-Geigy (Summit, NJ).

RESULTS

Catecholamine secretion is blocked by inhibitors of cytochrome P450-dependent arachidonic acid metabolism

Table 1 summarizes the effects of a number of inhibitors of selected pathways of AA metabolism on catecholamine secretion. Cultured chromaffin cells were pretreated with the inhibitor, then stimulated either with the cholinergic agonist carbachol or by direct membrane depolarization with 59 mM KCl, and catecholamine secretion was measured. The depolarization attained at this KCl concentration is great enough to activate voltage-dependent Ca²⁺ channels directly, bypassing the participation of both receptor-associated and voltage-sensitive Na⁺ channels (17). Under either stimulation protocol, NDGA, BW755C, and AA861, which block lipoxygenation of AA, inhibited the secretory response. These data are in agreement with earlier reports (8, 9). In addition, we found that both carbachol-dependent and KCl-dependent secretion were markedly

inhibited when the cells were treated with ketoconazole, clotrimazole, or piperonyl butoxide (Pbutox), compounds which inhibit the cytochrome P450-dependent metabolism of AA (18–20). Secretion was similarly inhibited when depolarization was achieved with 50 mM veratridine (data not shown). Cytochrome P450 inhibitors have reproducibly inhibited secretion in every cell preparation tested. The extent of inhibition was maximal with preincubations as brief as 2 min (data not shown), indicating a rapid onset.

Metyrapone, an inhibitor of certain forms of cytochrome P450 involved in steroidogenesis (20), had no effect (Table 1). The cyclooxygenase inhibitor indomethacin also failed to inhibit secretion. As the secretory response was not affected by blockade of prostaglandin formation with indomethacin, the cytochrome P450-like enzymes thromboxane synthase and prostacyclin synthase could not represent the targets for the P450 inhibitors (21). Nitric oxide synthase is also a cytochrome P450-like enzyme (22). Inhibitors of nitric oxide synthase, L-nitroarginine (L-NA) (30 μ M) and L-N-monomethyl arginine (L-NMMA) (100 μ M) failed to inhibit catecholamine release stimulated by 59 mM KCl. The secretion with L-NA was 128 \pm 11% (N = 4) of the KCl-stimulated response and L-NMMA was 120 \pm 6% (N = 4).

Neither lipoxygenase nor cytochrome P450 inhibitors had any effect on the amount of ³H-labeled catecholamine released into the buffer in the absence of secretagogue. During the 15-min pretreatment period, control cells released 3.0 \pm 0.3% (SEM, n = 24) of the cell ³H content, while the inhibitor-treated groups (each n = 12) released 3.2 \pm 0.2% (NDGA), 3.3 \pm 0.3% (BW755C), 3.0 \pm 0.2% (AA861), 2.4 \pm 0.2% (ketoconazole), 2.9 \pm 0.4%

TABLE 3. Effect of cytochrome P450 inhibitors on carbachol-stimulated Ca²⁺ uptake in adrenal chromaffin cells

Pretreatment	Net ⁴⁵ Ca ⁺ Uptake (% of control)
	Stimulated by Carbachol
Ketoconazole (10 μ M)	37 \pm 17 (n = 8) ^a
Clotrimazole (20 μ M)	14 \pm 6 (n = 8) ^a
Piperonyl butoxide (50 μ M)	5 \pm 8 (n = 8) ^a

After preincubation of cells for 16 min with inhibitor, uptake of ⁴⁵CaCl₂ was measured in 2-min incubations in the presence of 0.05 mM carbachol. Basal values were determined for each treatment and subtracted. In the controls, net Ca²⁺ uptake averaged 1.4 \pm 0.4 fmol/cell (2-fold above basal, n = 8) upon carbachol stimulation, and 1.4 \pm 0.3 fmol/cell (3-fold above basal, n = 12) in response to KCl (59 mM) (a positive control). Values for net Ca²⁺ uptake in each treatment group have been normalized to the controls, and are expressed as mean \pm standard error.

^aValues significantly decreased compared to the control at *P* < 0.003.

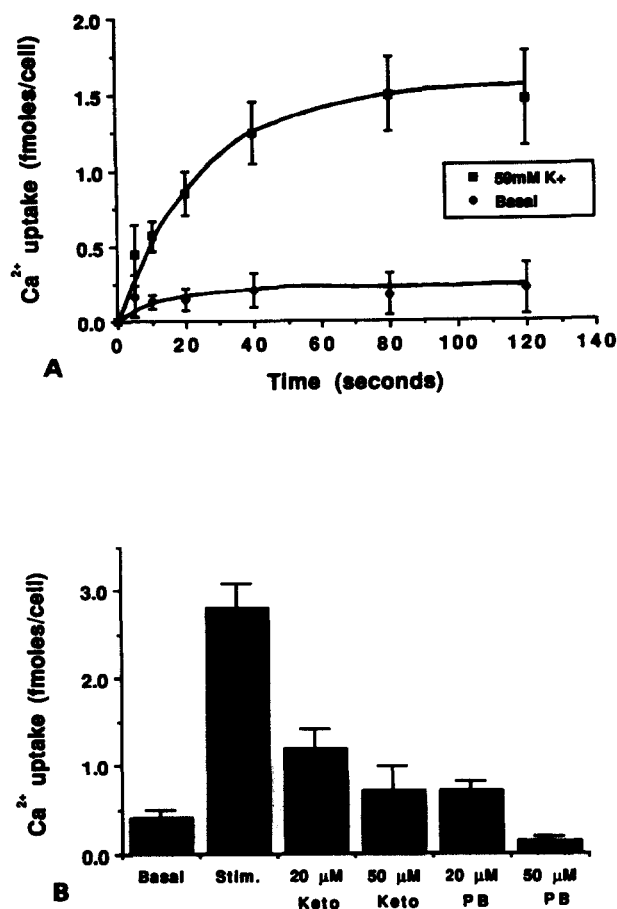


Fig. 1. Uptake of ^{45}Ca into bovine adrenal chromaffin cells. **A:** Effect of KCl on $^{45}\text{Ca}^{2+}$ uptake. Cells were incubated for various times with $2\ \mu\text{Ci}/\text{ml}$ of $^{45}\text{CaCl}_2$ in the presence and absence of $59\ \text{mM}$ KCl. Each value represents the mean \pm SD for $n = 4$. **B:** Effect of ketoconazole (KETO) and piperonyl butoxide (PB) on KCl-induced $^{45}\text{Ca}^{2+}$ uptake. Cells were preincubated with the inhibitors for 16 min. They were then incubated for 2 min with $^{45}\text{CaCl}_2$ in the presence of $59\ \text{mM}$ KCl. Each value represents the mean \pm SD for $n = 4$.

(clotrimazole), and $2.9 \pm 0.2\%$ (Pbutox). Thus, decreases in catecholamine release observed during the subsequent stimulation period did not arise from detachment of cells or leakage of catecholamines during the inhibitor pretreatment.

Cytochrome P450 inhibitors interfere with Ca^{2+} entry

Loss of responsiveness of the cells to KCl depolarization indicated that the cytochrome P450 inhibitors did not affect the cholinergic receptor or the fast Na^+ channel, but a later step in the secretory response. The question of whether the block occurred before or after Ca^{2+} entry was approached using the calcium ionophores lasalocid and ionomycin to bypass Ca^{2+} channels (Table 2). Ionophore-dependent secretion was not in-

hibited by ketoconazole, clotrimazole, Pbutox, NDGA, or BW755C. AA861 gave a small decrease in ionophore-dependent secretion. In two cases, NDGA and Pbutox, ionophore-dependent secretion was actually enhanced; the significance of that effect is unclear. Nevertheless, the overall absence of inhibitory effects ruled out the possibilities that the cytochrome P450 inhibitors were cytotoxic, or that they interfered with the process of exocytosis subsequent to Ca^{2+} influx. Instead, a signaling step involving Ca^{2+} entry must be affected.

In a second experimental approach, the effect of inhibitors on $^{45}\text{Ca}^{2+}$ uptake was measured during stimulation of cells with either carbachol (Table 3) or KCl (Fig. 1). The uptake of ^{45}Ca increased with time and was maximal after 40 sec (Fig. 1A). The uptake was significantly greater in the presence of KCl. This stimulated uptake was inhibited in a concentration-related manner by ketoconazole and Pbutox (Fig. 1B). Ketoconazole inhibited the uptake by 67% and 88% at 20 and $50\ \mu\text{M}$, respectively, while Pbutox inhibition was 88 and 100% at 20 and $50\ \mu\text{M}$, respectively. Carbachol also stimulated $^{45}\text{Ca}^{2+}$ uptake, and this uptake was markedly inhibited by exposure to ketoconazole, clotrimazole, or Pbutox (Table 3). Thus, the step affected by the action of these inhibitors was a voltage-dependent Ca^{2+} channel.

Inhibitors of cytochrome P450 on histamine-induced secretion

Histamine is another important neurotransmitter that induces secretion in chromaffin cells by activating Ca^{2+} influx, but the histaminergic response may involve Ca^{2+} channels having somewhat different properties than the voltage-dependent channels involved in the cholinergic response (4, 23). It was therefore of interest to determine whether the response of chromaffin cells to histamine could be inhibited by lipoxygenase and cytochrome P450 inhibitors. Histamine-dependent catecholamine secretion, like carbachol-dependent secretion, was inhibited by ketoconazole, clotrimazole, Pbutox, NDGA, and AA861 (Table 4).

Identification of a cytochrome P450 metabolite formed by chromaffin cells prelabeled with [^3H]arachidonic acid

Cells that were incubated for 90 min with [^3H]AA incorporated 16–24% of the fatty acid into cellular phospholipid. Control experiments showed that [^3H]AA, but none of its metabolites, were esterified into the phospholipid pool during this brief prelabeling period. The prelabeled cells released free [^3H]AA (35 min), and labeled metabolites eluting between 3 and 5.5 min and 28 and 30 min on reverse phase HPLC (Fig. 2A). The early metabolites (3–5.5 min) comigrated with

TABLE 4. Effect of cytochrome P450 and lipoxygenase inhibitors on histamine-dependent catecholamine secretion from adrenal chromaffin cells

Pretreatment	Catecholamine Secreted (% of control)
Ketoconazole (10 μM)	75 \pm 2 (n = 4) ^a
Clotrimazole (20 μM)	40 \pm 2 (n = 4) ^a
Piperonyl butoxide (50 μM)	19 \pm 3 (n = 4) ^a
NDGA (50 μM)	68 \pm 3 (n = 4) ^a
AA861 (50 μM)	60 \pm 2 (n = 4) ^a

After preincubation of cells for 15 min with inhibitor, catecholamine release was measured in 30-min incubations in the presence of 25 μM histamine. In the controls, net catecholamine release averaged 12.8 \pm 0.2% of total cell content (3- to 6-fold above basal; n = 8, two experiments). Values for net catecholamine release in each treatment group have been normalized to controls, and are expressed as mean \pm standard error.

^aValues significantly decreased compared to the control at $P < 0.001$.

the prostaglandins whereas the latter metabolites (28–30 min) comigrated with the epoxyeicosatrienoic acids (EETs). Inclusion of clotrimazole (50 μM) during the prelabeling, washing, and incubation steps had no effect on [³H]AA incorporation or release, but diminished the formation of the ³H-labeled metabolite that comigrated with the EETs, indicating its formation was cytochrome P450-dependent (Fig. 2B). The ³H-labeled metabolite comigrating with the prostaglandins was not altered by clotrimazole. These results were obtained reproducibly in replicate experiments. Separate analysis of the buffer and the cell layer showed that the ³H-labeled metabolites were retained within the cells, with only 7% released into the buffer over 10 min.

Additional studies were performed to identify the EETs produced by chromaffin cells. In these experiments, the cells were pretreated with indomethacin to inhibit synthesis of prostaglandins and maximize the production of EETs. In indomethacin-treated cells, only two radioactive peaks were observed, one comigrating with the EETs (29.5–32 min) and the other comigrating with AA (35 min). No ³H-labeled metabolites comigrating with the prostaglandins were observed. The radioactive peak eluting at 29.5–32 min was collected from reverse phase HPLC (solvent system 1) (Fig. 3A) and rechromatographed on normal phase HPLC (solvent system 2) (Fig. 3B). A single radioactive peak was observed on normal-phase HPLC that eluted at 18 min. It comigrated with the authentic 5,6-epoxyeicosatrienoic acid (5,6-EET) standard. This compound has been previously characterized as a cytochrome P450 metabolite of AA in other systems (24).

Another group of indomethacin-treated cells was pre-labeled with [³H]AA and incubated in the presence and absence of carbachol. Control cells produced a ³H-la-

beled metabolite that comigrated with the EETs and released [³H]AA (Fig. 4A). The release of both was increased with carbachol treatment. Carbachol caused a 1.6-fold increase in the release of [³H]AA and a 1.7-fold increase in the [³H]EET. These increases were obtained reproducibly in replicate experiments.

Addition of 5,6-epoxyeicosatrienoic acid to piperonyl butoxide-treated cells results in catecholamine secretion

We tested the ability of the synthetic 5,6-EET to restore secretion in chromaffin cells under cytochrome P450 blockade. We found that 5,6-EET alone induced catecholamine secretion. Addition of 1×10^{-9} M 5,6-EET increased secretion by Pbutox-pretreated cells from 2.4

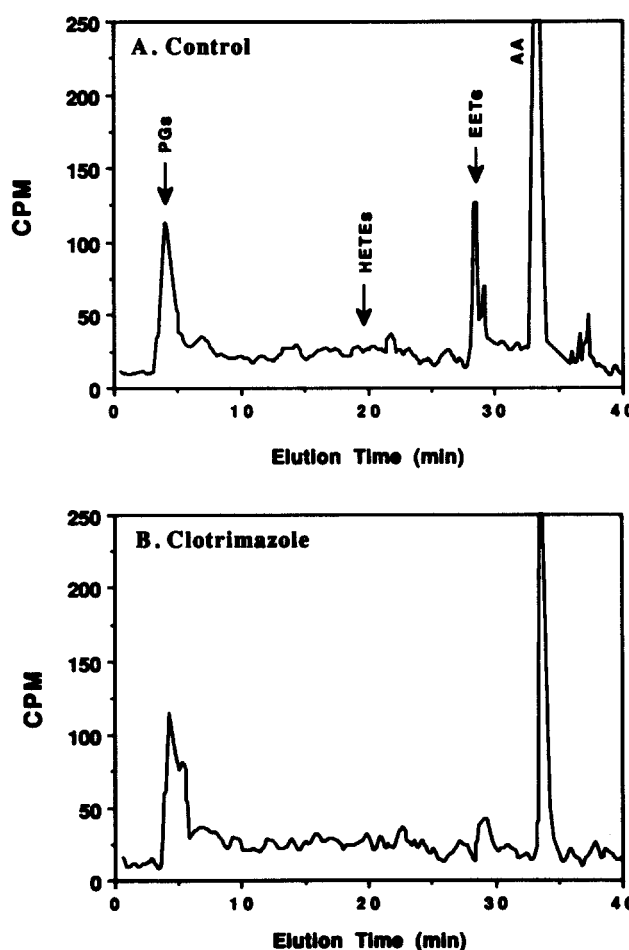


Fig. 2. The metabolism of [³H]arachidonic acid by pre-labeled chromaffin cells. Cells were pretreated with vehicle or 50 μM clotrimazole and the formation of [³H]arachidonic acid-labeled metabolites was determined. The cells were extracted and the metabolites were resolved by reverse-phase HPLC using solvent system 1. Panel A indicates control and panel B after clotrimazole. Elution times of standard eicosanoids are shown by arrows above the chromatogram. (PG, prostaglandin; HETE, hydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid).

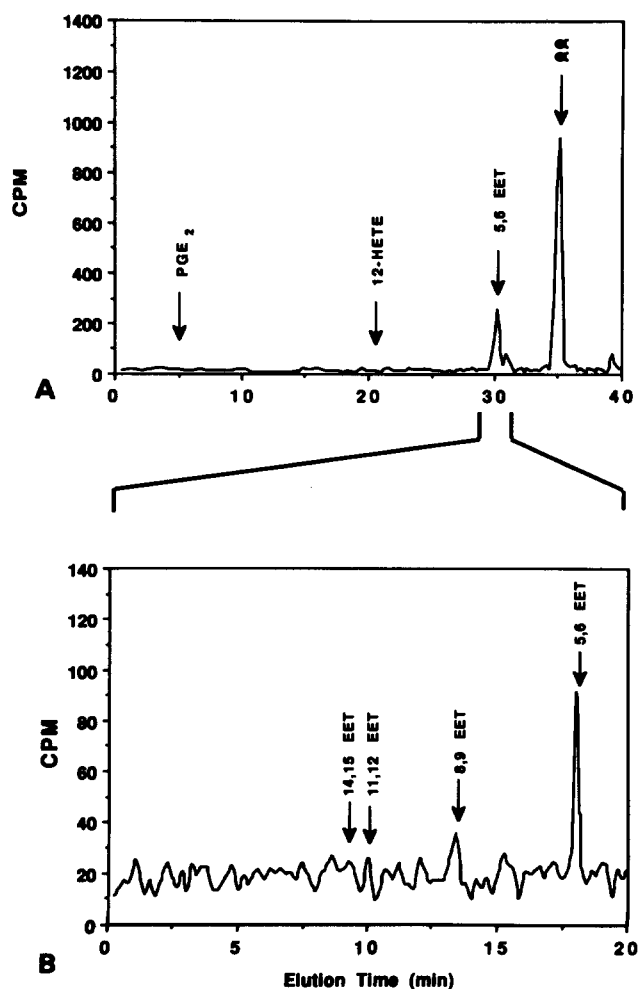


Fig. 3. [³H]arachidonic acid release and ³H-labeled metabolite formation by prelabeled chromaffin cells. Labeled metabolites that were formed in indomethacin-treated cells from [³H]arachidonic acid were extracted and separated on a C18 reverse-phase HPLC using solvent system 1 (panel A). Fractions (0.2 min) were collected, and aliquots were measured for radioactivity. The peak eluting at 35 min comigrated with arachidonic acid and the peak eluting at 29.5 to 32 min comigrated with the EETs. Elution times of standards are indicated by arrows above the chromatogram. Metabolites eluting in fractions 88–92 on reverse-phase HPLC (panel A) were collected and separated by normal phase HPLC using solvent system 2 (panel B). Fractions (0.3 min) were collected and measured for radioactivity. Elution times of standard eicosanoids are shown by arrows above the chromatogram.

$\pm 0.1\%$ per 15 min ($n=11$) to $2.9 \pm 0.2\%$ ($n=11$). This stimulation of secretion by 5,6-EET was observed in four out of four independent experiments ($P < 0.05$). Similar experiments were conducted in Na^+ -free buffer to reduce $\text{Na}^+/\text{Ca}^{2+}$ exchange and enhance accumulation of Ca^{2+} . Cells were pretreated 10 min with 50 mM Pbutox in Na^+ -free buffer, then catecholamine secretion was measured in the absence or presence of 5,6-EET. Secretion was increased from $2.9 \pm 0.1\%$ per 15 min ($n = 5$) to $3.6 \pm 0.2\%$ ($n = 5$) ($P < 0.005$) by the addition of 1×10^{-9} M 5,6-EET.

DISCUSSION

We have found that inhibitors of the cytochrome P450-dependent metabolism of arachidonic acid inhibit catecholamine secretion by chromaffin cells. Inhibition is observed when cells are stimulated through cholinergic or histaminergic receptors or by direct depolarization, but not when stimulated with Ca^{2+} ionophore. Cytochrome P450 inhibitors also inhibit Ca^{2+} uptake in response to carbachol or KCl depolarization. For both catecholamine release and Ca^{2+} uptake, clotrimazole and piperonyl butoxide were more effective inhibitors than ketoconazole. Consistent with these findings, Capdevila and coworkers (18) reported that clotrimazole was more potent than ketoconazole in inhibiting cytochrome P450 metabolism of AA. Thus, inhibition of cytochrome P450 may prevent formation of a second messenger that controls Ca^{2+} influx through voltage-dependent channels.

Further evidence to support this hypothesis is provided by the reports that Ca^{2+} uptake and catecholamine secretion by chromaffin cells are inhibited by agents that prevent AA release from phospholipids (5, 7, 8). However, pharmacological experiments alone provide insufficient proof, because nonspecific actions of these inhibitors are possible (18, 25, 26). We obtained more direct support by demonstrating that chromaffin cells produce a clotrimazole-sensitive metabolite of AA. The metabolite comigrated with 5,6-EET on HPLC. We were further able to show partial restoration of Pbutox-inhibited catecholamine secretion by addition of 5,6-EET in a concentration of 10^{-9} M. The magnitude of the stimulation by 5,6-EET was smaller than the inhibition by Pbutox. This may be due to the instability of 5,6-EET in aqueous solution or binding of the eicosanoid to albumin in the buffer. Also, the endogenously synthesized 5,6-EET was found to be cell associated with only 7% being released into the media. This finding suggests that it may exert its effect intracellularly. If this is the case, exogenously (extracellularly) added 5,6-EET may have limited access to the intracellular site of action and therefore may have limited activity. Alternatively, 5,6-EET may be an incomplete agonist for catecholamine release and may only exert its full stimulatory effect in the presence of another agonists as histamine or carbachol.

Chromaffin cells respond to a number of secretagogues (angiotensin II, histamine, bradykinin) and pharmacological agents (caffeine, thapsigargin) with transient release of Ca^{2+} from intracellular stores followed by influx of extracellular Ca^{2+} (1). Thus, these cells exhibit the characteristic features of "capacitative" Ca^{2+} regulation, as described by Putney (27). According to this model, depletion of an intracellular Ca^{2+} pool triggers Ca^{2+} influx through plasma membrane channels.

To date, the mechanism of communication between the intracellular pool and plasma membrane channels has not been established. On the basis of evidence obtained in thymocytes, platelets, and Ehrlich ascites cells, Alvarez, Montero, and Garcia-Sancho (28) proposed that cytochrome P450 may generate the second messenger that signals intracellular pool depletion, and activates entry of extracellular Ca^{2+} . Our data are consistent with a similar role for cytochrome P450 in regulation of Ca^{2+} influx in the chromaffin cell.

We propose the following scheme. Upon receptor activation, hydrolysis of phospholipids releases AA. The signal to initiate release of AA could be provided by Ca^{2+} influx through the receptor-associated ion channel, Ca^{2+} release from intracellular stores, or by some other transduction mechanism such as G-protein- or protein kinase-mediated phospholipase activation. Both Ca^{2+} -dependent and -independent pathways for AA release from phospholipids occur in chromaffin tissue: these include the phospholipase C/diglyceride lipase pathway (29) and phospholipase A_2 pathway (30, 31). AA is

rapidly converted via cytochrome P450 to an active metabolite, possibly 5,6-EET, that modulates the gating properties of voltage-dependent Ca^{2+} channels in favor of the open state. The additional postulate that formation of 5,6-EET depends upon depletion of intracellular Ca^{2+} stores is an interesting corollary to be explored.

There is precedent for such a model. Epoxides of AA play essential roles in stimulus-secretion coupling in hypothalamic neurons (32) and in pituitary cells (19, 33, 34) and have been shown to control release of Ca^{2+} from intracellular stores (26, 34–36). Most notably, 5,6-EET was found to enhance Ca^{2+} influx through voltage-dependent channels in proximal tubule epithelial cells (37). We have no direct evidence that 5,6-EET increases Ca^{2+} influx in chromaffin cells.

In the present studies, the effects of the lipoxygenase inhibitors NDGA, AA861, and BW 755c on catecholamine secretion were very similar to the effects of the cytochrome P450 inhibitors. One explanation is that these lipoxygenase inhibitors may also inhibit cytochrome P450 (15). A second explanation is that participation of both a lipoxygenase metabolite and a cytochrome P450 metabolite are required in the signalling cascade. A third possibility is that the formation of the metabolite of AA involves the sequential action of both lipoxygenase and cytochrome P450. These alternatives remain to be explored; however, our data on the metabolism of [^3H]AA would argue against the latter possibilities.

There is disagreement as to the signalling events linking histamine receptor activation in chromaffin cells to Ca^{2+} -dependent exocytosis. It was recently suggested that voltage-dependent channels do not mediate histamine-dependent Ca^{2+} uptake in these cells (unpublished results cited in refs. 1 and 38). To the contrary, however, others have demonstrated the essential participation of dihydropyridine-sensitive Ca^{2+} channels in the histaminergic secretory response (4, 23). The latter view is more consistent with the results reported here. Our observation that histamine-dependent secretion was inhibited by both lipoxygenase and cytochrome P450 inhibitors suggests that the sequence of events is similar to that elicited by carbachol. Specifically, stimulation of histamine receptors leads to membrane depolarization, release of AA, and cytochrome P450-dependent formation of 5,6-EET which promotes Ca^{2+} entry through voltage-dependent channels. Indeed, an analogous transduction mechanism occurs in *Aplysia* neurons, where histaminergic stimulation activates AA release from phospholipid and is converted via lipoxygenase to metabolites that control gating of K^+ channels (39). ■

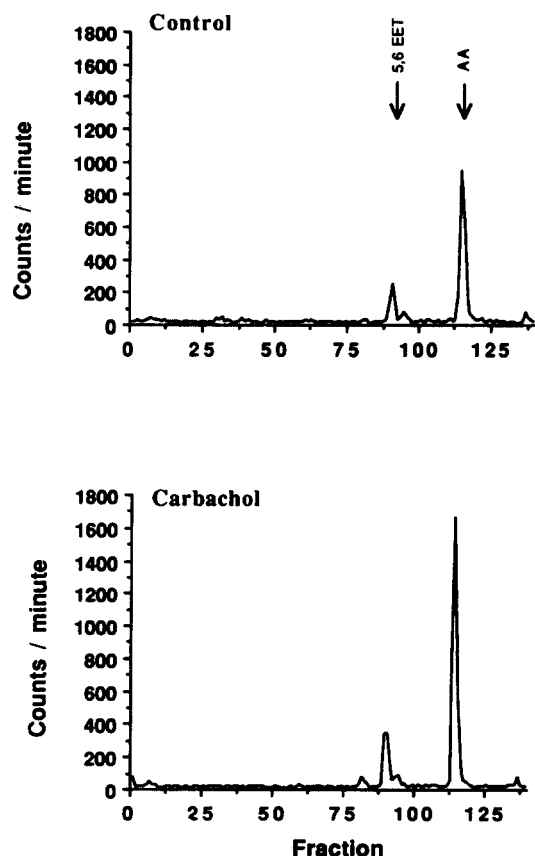


Fig. 4. Effect of carbachol on the release of [^3H]arachidonic acid and formation of ^3H -labeled metabolites. Chromaffin cells prelabeled with [^3H]arachidonic acid were treated with vehicle or 0.3 mM carbachol. The cells were extracted and the metabolites were resolved by reverse-phase HPLC using solvent system 1. Elution times of standard eicosanoids are shown by arrows above the chromatogram.

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