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The Relationship Between Arachidonic Acid Release and Catecholamine Secretion from Cultured Bovine Adrenal Chromaffin Cells

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Abstract: Increased arachidonic acid release occurred during activation of catecholamine secretion from cultured bovine adrenal medullary chromaffin cells. The nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP) caused an increased release of preincubated [^{3}H]arachidonic acid over a time course which corresponded to the stimulation of catecholamine secretion. Like catecholamine secretion, the DMPP-induced [^{3}H]arachidonic acid release was calcium-dependent and was blocked by the nicotinic antagonist mecamylamine. Depolarization by elevated K^+, which induced catecholamine secretion, also stimulated arachidonic acid release. Because arachidonic acid release from cells probably results from phospholipase A_2 activity, our findings indicate that phospholipase A_2 may be activated in chromaffin cells during secretion. **Key Words:** Arachidonic acid—Catecholamine secretion—Chromaffin cells—Phospholipase A_2. **Frye R. A. and Holz R. W.** The relationship between arachidonic acid release and catecholamine secretion from cultured bovine adrenal chromaffin cells. *J. Neurochem.* **43**, 146–150 (1984).

Prepackaged hormones and neurotransmitters are usually released from cells via exocytosis. During exocytosis a rise in cytosolic [Ca^{2+}] triggers fusion of the secretory vesicle membrane and the plasma membrane. Phospholipase A_2, which may be activated by a rise in cytosolic [Ca^{2+}] (Van den Bosch, 1980), causes the release of *cis*-unsaturated fatty acids (including arachidonic acid) which are potent membrane fusogens (Creutz, 1981). Activation of phospholipase A_2 may be a necessary step in the chain of events linking elevated cytosolic [Ca^{2+}] to the vesicle membrane-plasma membrane fusion event. Although we have recently found that putative phospholipase A_2 inhibitors block secretion from cultured chromaffin cells, they also block calcium uptake into the cells which is necessary to stimulate secretion (Frye and Holz, 1983); thus, the effects of these inhibitors are difficult to interpret. In the present study we have investigated whether phospholipase A_2 activation (measured by release of preincorporated [^{3}H]arachidonic acid) occurs during the stimulation of catecholamine secretion from cultured bovine adrenal medullary chromaffin cells.

MATERIALS AND METHODS

Cell preparation

Cells disaggregated from bovine adrenal medullae (Fenwick et al., 1978; Livett et al., 1979) were added to 16-mm diameter uncoated culture wells (Costar, Cambridge, MA) at a density of 500,000 cells per well in 1 ml of Eagle's Minimum Essential Medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 10 μ*M* cytosine arabinoside (to inhibit fibroblast proliferation), gentamycin (50 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (2.5

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Abbreviations used: AA, Arachidonic acid; Ach, Acetylcholine; BSA, Bovine serum albumin; DG, Diacylglyceride; DMPP, 1,1-Dimethyl-4-phenylpiperazinium; HEPES, *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Mec, Mecamylamine; Meth, Methacholine; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PI/PS, Phosphatidylinositol/phosphatidylserine; PL, Phospholipid; PSS, Physiological salt solution; TG/CE, Triacylglyceride/cholesterol ester.

μg/ml) (Squibb, Princeton, NJ). After 4 days at 34° in 5% CO_2/95% air, chromaffin cells formed monolayers containing approximately 20 nmol catecholamine per well. The incubation medium was replaced after 4 days and experiments were performed on days 5–10. Cultures were incubated in cytosine arabinoside-free medium for 1 day before each experiment.

[^{3}H]Arachidonic acid labeling

Cells were incubated for 4 h with 0.2 ml per well tissue culture medium without cytosine arabinoside which contained 0.5 μCi [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (100 μCi/mmol) (Amersham, Chicago, IL) and 0.5% (wt/vol) fatty acid free bovine serum albumin (BSA) (Sigma, St. Louis, MO). Wells were then incubated twice in 0.2 ml medium plus BSA without arachidonate for 10 min and then in 0.4 ml of the same medium for 1 h. Cells were then incubated in 1.0 ml of physiological salt solution (PSS) containing 145 m*M* NaCl, 5.6 m*M* KCl, 2.2 m*M* $CaCl_2$, 15 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4), and 0.5% (wt/vol) fatty acid free BSA for 30 min.

In experiments in which the distribution of labeled cell lipids was determined, the lipids of the cell monolayer were dissolved in 1 ml of ice-cold methanol which was added to the well immediately following the removal of the incubation solution. The labeled lipids in the methanol extracts were fractionated by one-dimensional, sequential, ascending TLC. The plates were first run for 15 min in chloroform/methanol/water (95:35:6). The plates were then run for 25 min in hexane/diethylether/ethanol/NH_3 (75:20:5:0.5). The lipids were separated with the following R_f values: phosphatidylinositol/phosphatidylserine (0.05), phosphatidylcholine (0.10), phosphatidylethanolamine (0.25), arachidonic acid (0.45), diacylglyceride (0.60), and triacylglyceride/cholesterol ester (0.80).

[^{3}H]Norepinephrine uptake into cells

Cells were incubated for 4 h in 0.2 ml incubation medium containing 0.5 μCi/ml 1-[7-^{3}H]norepinephrine (New England Nuclear, Boston, MA) and 0.5 m*M* ascorbate, and were then incubated three times for 10 min with PSS without BSA.

[^{3}H]Norepinephrine secretion and stimulation of [^{3}H]arachidonic acid release

Cells prelabeled with [^{3}H]norepinephrine or [^{3}H]arachidonic acid were rinsed with 0.4 ml PSS plus 0.5% BSA immediately prior to addition of the test solution. Experiments were performed at 25° with 0.4 ml PSS containing 0.5% BSA with or without 1,1-dimethyl-4-phenylpiperazinium (DMPP) or elevated K^+ (56.0 m*M* KCl and 95 m*M* NaCl). Following the incubation the test solutions were removed from the [^{3}H]norepinephrine-labeled cells and added to scintillation vials for determination of [^{3}H]norepinephrine secretion. [^{3}H]Arachidonic acid in lipid extracts (Billah and Lapetina, 1982) of test solutions from [^{3}H]arachidonic acid-labeled cells was separated by TLC with silica gel HL plates (Analtech, Newark, DE) with petroleum ether/diethyl ether/acetic acid (70:30:1). The released radioactivity which comigrated with authentic arachidonic acid on the TLC plate was scraped and counted as released [^{3}H]arachidonic acid. Radioactivity remaining in cells in both [^{3}H]arachidonic acid- and [^{3}H]norepinephrine-labeled wells was solubilized in 0.4 ml of 1% Triton X-100 and quantitated by liquid scintillation counting. Results of experiments are expressed as the percent of total radioactivity which was released from the cells during the test period and are expressed as the mean ± the standard error of the mean. Statistical significance was determined by Student's *t* test.

RESULTS

Secretory stimuli cause [^{3}H]arachidonic acid release

Bovine adrenal chromaffin cell cultures incorporated [^{3}H]arachidonic acid into cellular lipid pools. Nearly 80% of the incorporated label was esterified into the phospholipid pool with the remainder esterified into neutral lipids (Table 1); only a small fraction (0.15%) of the cell-associated label was present as free [^{3}H]arachidonic acid. In the resting state the prelabeled cells released [^{3}H]arachidonic acid-labeled products including neutral lipid, free arachidonic acid, and phospholipid. Upon stimulation with the nicotinic agonist DMPP, the release of labeled free arachidonic acid increased significantly, while the release of labeled neutral lipid and phospholipid was not significantly altered (Fig. 1). DMPP did not alter the amount of [^{3}H]arachidonic acid or other labeled lipids associated with the cells (Table 1). The data indicate that the nicotinic agonist-induced secretion of catecholamine is associated with a net deacylation of [^{3}H]arachidonic acid from lipid precursors and release of [^{3}H]arachidonic acid into the medium.

Depolarization induced by 56 m*M* K^+ stimulated calcium-dependent catecholamine secretion (Holz et al., 1982) and also stimulated [^{3}H]arachidonic acid release (Fig. 2). In the present experiments the amount of elevated K^+-induced secretion was approximately half the DMPP-induced secretion (Fig. 2B). Similarly, the elevated K^+-induced release of [^{3}H]arachidonic acid was smaller than that induced by DMPP (Fig. 2A). Because the DMPP-stimulated release of [^{3}H]arachidonic acid was larger and more readily studied, the following experiments focus on the relationship between cholinergic agonist-induced [^{3}H]arachidonic acid release and catecholamine secretion.

It is unlikely that the DMPP-induced [^{3}H]-arachidonic acid release is due to a stimulated release of phospholipase A_2 from the chromaffin cells and subsequent action of the released enzyme on the cell from the extracellular medium. Solutions containing 10 μ*M* DMPP which had been incubated for 5 min with unlabeled chromaffin cells (i.e., solutions which would have contained the putative released phospholipase A_2) did not stimulate [^{3}H]arachidonic acid release when added back to [^{3}H]arachidonic acid-labeled cells (in the presence of 5 μ*M* mecamylamine to block the DMPP stimulation, results not shown).

TABLE 1. *[^{3}H]Arachidonate distribution in lipids of chromaffin cell cultures*

	NE secretion	AA medium	AA cell	PC cell	PE cell	PI/PS cell	TG/CE cell	DG cell
0 DMPP	2.5 (±0.2)	0.16 (±0.01)	0.15 (±0.02)	47.2 (±0.6)	27.9 (±0.7)	5.0 (±0.2)	18.6 (±1.2)	1.03 (±0.05)
10 μ*M* DMPP	14.2[a] (±0.9)	0.22[a] (±0.01)	0.15 (±0.01)	45.3 (±0.7)	26.5 (±0.6)	4.4 (±0.1)	22.3 (±1.1)	1.20 (±0.04)

Chromaffin cell cultures which were preincubated with [^{3}H]arachidonic acid (see Materials and Methods) were incubated for 5 min with or without 10 μ*M* DMPP. Radioactivity in the medium and in the cells was fractionated by TLC and the radioactivity comigrating with free arachidonic acid (AA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol/phosphatidylserine (PI/PS), triacylglyceride/cholesterol ester (TG/CE), and diacylglyceride (DG) was scraped and counted; results are expressed as the percentage of total cpm per well. There were 140,000 cpm of labeled lipid per well. In a parallel experiment, [^{3}H]norepinephrine secretion was determined with cells preincubated with [^{3}H]norepinephrine. There were 4 wells per group.

[a] $p < 0.01$ vs respective control.

Time course of DMPP-stimulated [^{3}H]arachidonic acid release and [^{3}H]norepinephrine secretion

There is a continual and approximately linear basal release of [^{3}H]arachidonate with time (Fig. 3A). DMPP stimulated the rate of [^{3}H]arachidonate release between 0 and 2 min when the catecholamine secretory rate was maximal (Fig. 3B). A continued enhanced, but slower, rate of [^{3}H]arachidonate release caused by DMPP is evident at the later time points when the rate of DMPP-induced catecholamine secretion had decreased.

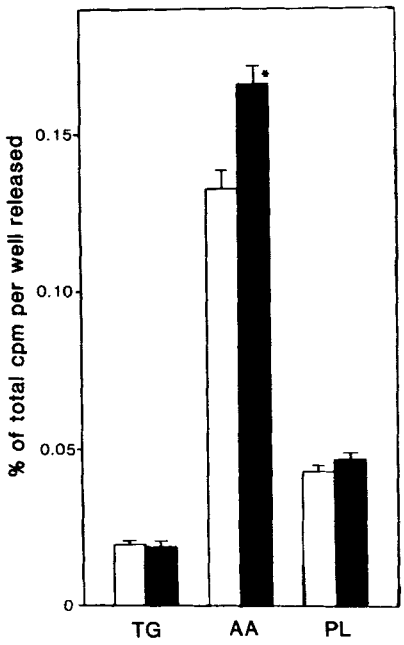

FIG. 1. Lipid distribution of radioactivity released from chromaffin cells prelabeled with [^{3}H]arachidonic acid. Cells were treated for 5 min with (filled columns), or without (open columns) 10 μ*M* DMPP. Following incubation with the cells the solutions were centrifuged (25,000 × *g*, 10 min) to pellet any cells which may have detached from the monolayer; the supernatants were extracted and the labeled lipids were separated by TLC and the radioactivity comigrating with other triacylglycerides (TG), free arachidonic acid (AA), and phospholipid (PL) was scraped and counted. The [^{3}H]arachidonic acid-labeled wells contained 200,000 cpm per well. There were six wells per group. *$p < 0.01$.

Pharmacology and calcium dependency of DMPP stimulation of [^{3}H]arachidonic acid release

Cholinergic stimulation of catecholamine secretion from bovine chromaffin cells is mediated by nicotinic cholinergic receptors. [^{3}H]Arachidonic acid release is also mediated by nicotinic cholinergic receptors (Fig. 4). Acetylcholine, the natural agonist of both nicotinic and muscarinic cholinergic receptors, significantly increased [^{3}H]arachidonic acid release. The specific nicotinic agonist DMPP caused significant stimulation of [^{3}H]arachidonic acid release, while the muscarinic agonist methacholine did not. The DMPP stimulation of

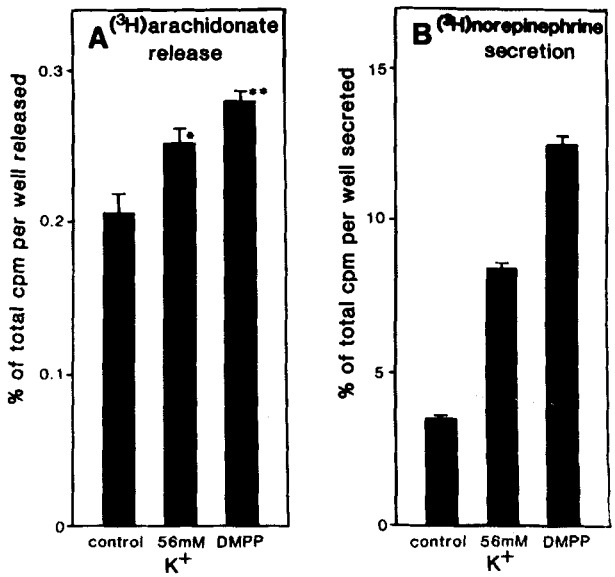

FIG. 2. Effects of 56 m*M* K^+, DMPP, on [^{3}H]arachidonic acid release **(A)** and on [^{3}H]norepinephrine secretion **(B)**. Cells prelabeled with [^{3}H]arachidonic acid or with [^{3}H]norepinephrine were incubated for 3 min with or without 56 m*M* K^+ or 10 μ*M* DMPP in the presence of 200 μ*M* arachidonic acid. The [^{3}H]arachidonic acid-labeled wells contained 70,000 cpm per well. There were four wells per group. *$p < 0.05$, **$p < 0.01$ vs respective control.

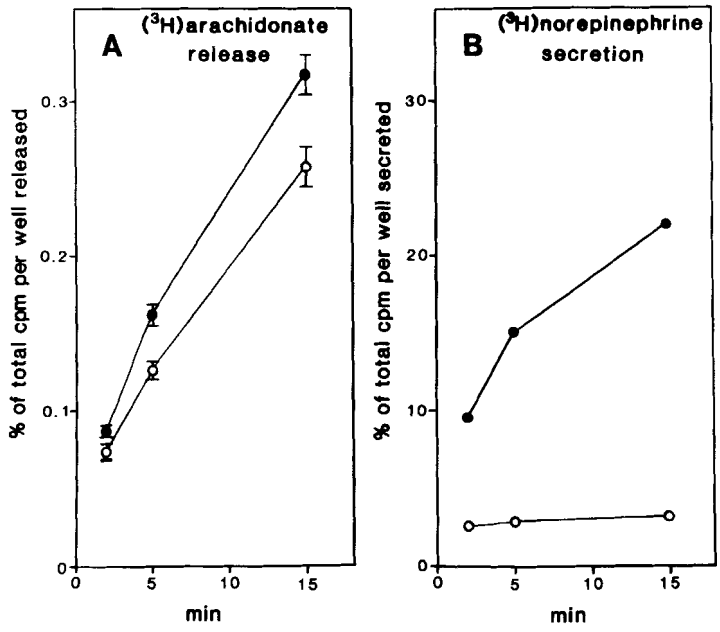

FIG. 3. Time course of DMPP-stimulated [^{3}H]arachidonic acid release **(A)** and [^{3}H]norepinephrine secretion **(B)**. Cells prelabeled with [^{3}H]arachidonic acid or [^{3}H]norepinephrine were treated for various times with (●) or without (○) 10 μ*M* DMPP. The [^{3}H]arachidonic acid-labeled wells contained 70,000 cpm per well. There were four wells per point. Standard error of the mean bars smaller than symbols were omitted.

[^{3}H]arachidonic acid release was blocked by the nicotinic antagonist mecamylamine.

Omission of Ca^{2+} from the physiological salt solution resulted in loss of the DMPP-induced stimulation of both [^{3}H]arachidonic acid release and [^{3}H]norepinephrine secretion (Fig. 5).

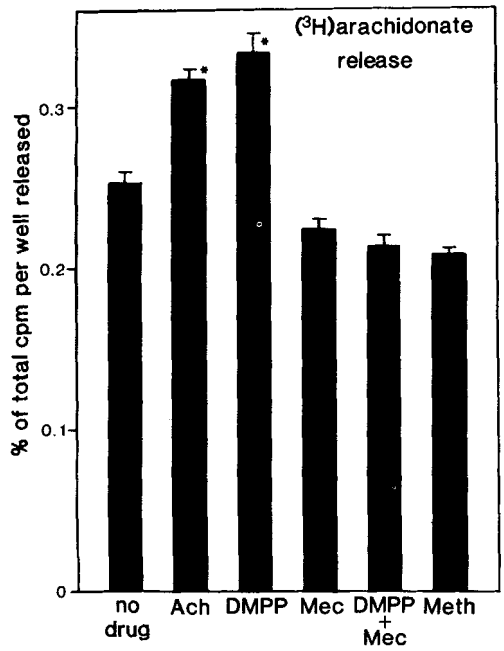

FIG. 4. The effect of cholinergic drugs on [^{3}H]arachidonic acid release from chromaffin cells. After chromaffin cells were prelabeled with [^{3}H]arachidonic acid, cells were incubated for 15 min with no drug, 0.3 m*M* acetylcholine (Ach), 10 μ*M* DMPP, 5 μ*M* mecamylamine (Mec), or 0.3 m*M* methacholine (Meth). The acetylcholinesterase inhibitor BW 284 C51 (0.5 μ*M*) was present together with acetylcholine. The [^{3}H]arachidonic acid-labeled wells contained 180,000 cpm per well. There were four wells per group. *$p < 0.01$.

DISCUSSION

Increased arachidonic acid release from chromaffin cells occurred coincident with catecholamine secretion induced by nicotinic stimulation or by elevated K^+-induced depolarization. The stimulation of arachidonic acid release correlated qualitatively with the stimulation of secretion in regard to time course, calcium dependency, and cholinergic pharmacology. [^{3}H]Arachidonic acid release from the cells did not result from phospholipase A_2 that may have been released into the medium. The data suggest that phospholipase A_2 is activated intracellularly in conjunction with catecholamine secretion and are consistent with phospholipase A_2 playing a role in secretion. Other investigators in a preliminary report observed similar results (Hotchkiss et al., 1981).

In a previous study (Fisher et al., 1981) we demonstrated that muscarinic agonists but not nicotinic agonists or elevated K^+ increased phosphatidylinositol turnover. The increased arachidonic acid release observed in the present study was associated with nicotinic stimulation and elevated K^+ but not with muscarinic stimulation. Thus, arachidonic acid release was not associated with stimulation of phosphatidylinositol metabolism.

The stimulated arachidonic acid release, which represented only a 20–40% increase over basal release, may not reflect the total amount of arachidonic acid release by phospholipase A_2. Arachidonic acid reincorporation via fatty acyl-CoA syn-

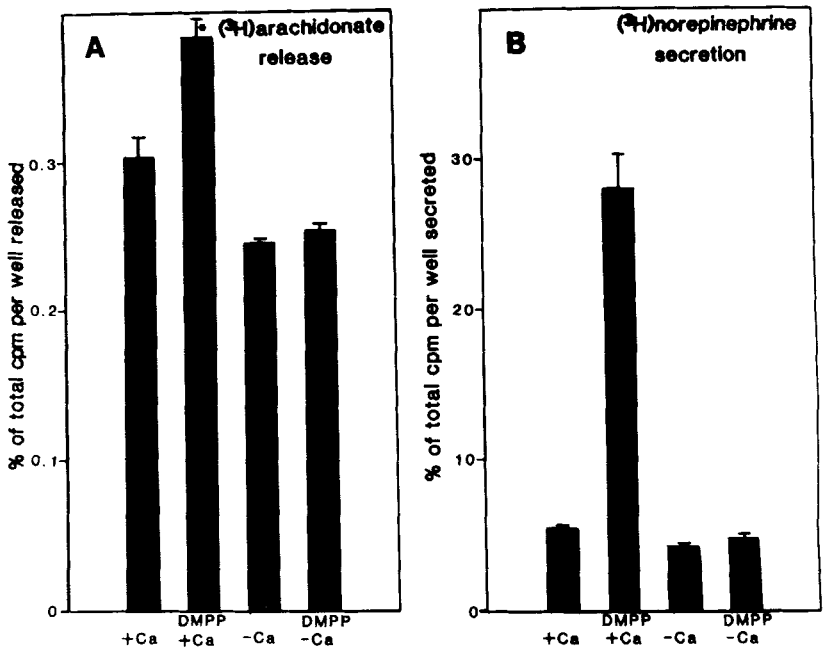

FIG. 5. Calcium dependence of DMPP-stimulated [^{3}H]arachidonic acid release **(A)** and [^{3}H]norepinephrine secretion **(B)**. Cells prelabeled with [^{3}H]arachidonic acid or with [^{3}H]norepinephrine were incubated for 15 min with or without 10 μM DMPP in the presence or absence of 2.2 mM Ca^{2+}. The [^{3}H]arachidonic acid-labeled wells contained 90,000 cpm per well. There were four wells per group. *$p < 0.02$ vs 0.0 DMPP + Ca^{2+}.

thetase followed by acyl-CoA:lysophospholipid acyltransferase (Lands and Crawford, 1976) may prevent a large proportion of the [^{3}H]arachidonic acid released within the cell from exiting from the cell. Indeed, we found that addition of 200 μM nonradioactive arachidonic acid approximately doubled the release of [^{3}H]arachidonic acid in the presence or absence of 10 μM DMPP or 56 mM K^+, possibly by competition for the reincorporation pathway (data not shown).

The association of arachidonic acid release with exocytosis has been found in many systems (Naor and Catt, 1981; McGivney et al., 1981) in addition to chromaffin cells. Phospholipase A_2 may play a direct role in the process of exocytosis since it is apparently activated by the same triggering mechanism (increase in cytosolic free calcium level) and the enzyme's products are effective membrane fusogens (Creutz, 1981). Further work is required to demonstrate a direct causal relationship between phospholipase A_2 activation and exocytosis.

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