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SHORT COMMUNICATION

Phospholipase A_2 Inhibitors Block Catecholamine Secretion and Calcium Uptake in Cultured Bovine Adrenal Medullary Cells

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SUMMARY

Phospholipase A_2 is a calcium-dependent enzyme which produces membrane fusogens. The possibility that it may be involved in exocytosis of catecholamine from primary dissociated cultures of bovine adrenal medullary cells was investigated by studying the effects on catecholamine secretion and $^{45}Ca^{2+}$ uptake of three phospholipase A_2 inhibitors: *p*-bromophenacyl bromide (BPB), Upjohn Compound 1002, and mepacrine. The three compounds completely inhibited catecholamine secretion induced by the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP), elevated K^+, and Ba^{2+}. The inhibition of nicotinic agonist-induced secretion by mepacrine may have been caused by direct nicotinic antagonist activity of the drug. The phospholipase inhibitors also inhibited $^{45}Ca^{2+}$ uptake into the cells stimulated by DMPP and elevated K^+. Inhibition of $^{45}Ca^{2+}$ uptake and catecholamine secretion exhibited identical dose-response curves. Other effects of the inhibitors were also investigated. Compound 1002 had no effect on $^{45}Ca^{2+}$ efflux from the cells in the presence of either normal or reduced Na^+ concentrations. BPB inhibited DMPP-stimulated phosphorylation of tyrosine hydroxylase which, like exocytosis, is dependent on a rise in cytosolic Ca^{2+}. The data suggest that phospholipase A_2 inhibitors block catecholamine secretion from intact chromaffin cells by blocking Ca^{2+} influx.

Hormones and neurotransmitters contained in secretory vesicles are released into the extracellular space by exocytosis. Exocytosis of catecholamine from adrenal medullary chromaffin cells is Ca^{2+}-dependent (1–4) and is initiated by increases in the cytosolic Ca^{2+} concentrations. An enzyme which may be involved in exocytosis is phospholipase A_2 (phosphatide 2-acylhydrolase, EC 3.1.1.4). Phospholipase A_2 is activated by Ca^{2+}, possibly via calmodulin (5, 6). The products which are formed, lysophosphatides and free fatty acid, are membrane fusogens (7, 8) which may induce fusion between the secretory vesicle membrane and the plasma membrane.

If phospholipase A_2 plays a key role in exocytosis then phospholipase A_2 inhibitors should block exocytosis. Phospholipase A_2 inhibitors have been shown to block exocytosis in many model systems, including gonadotropin release from anterior pituitary cells (9), histamine release from basophils and mast cells (10–12), lysosomal enzyme release from neutrophils (13), serotonin release from platelets (14), acrosome reaction in sperm (15), and insulin release from perfused pancreas (16) and isolated islets (17).

Using cultured bovine adrenal medullary chromaffin cells we have investigated the effects of three phospholipase A_2 inhibitors, BPB[2] (18), Upjohn Compound 1002 (19), and mepacrine (20, 21) on catecholamine secretion elicited by three different stimuli: (a) the nicotinic agonist DMPP, (b) elevated K^+-induced depolarization, and (c) replacement of medium Ca^{2+} with Ba^{2+}. The effects of the phospholipase A_2 inhibitors on $^{45}Ca^{2+}$ uptake stimulated by DMPP and elevated K^+ were also investigated.

Cells disaggregated from bovine adrenal medullae (22, 23) were added to 16-mm diameter uncoated culture wells (Costar, Cambridge, Mass.) at a density of 500,000 cells/well in 1 ml of Eagle's MEM (GIBCO, Grand Island, N. Y.) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 10 μM cytosine arabinoside (to inhibit fibroblast proliferation) gentamycin (50 μg/ml), and Fungizone (2.5 μg/ml) (Squibb, Princeton, N. J.). After 4 days at 34° in 5% CO_2/95% air, the chromaffin cells had formed monolayers. The incubation medium was replaced after 4 days and experiments were performed on days 5–10. Experiments were performed at 25° in PSS containing 142 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 15 mM 4-

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[2] The abbreviations used are: BPB, *p*-bromophenacyl bromide; Compound 1002, 1-(benzylmethylamino)-3-(α,α,α-trifluoro-*m*-tolyl)oxy-2-propanol; DMPP, 1,1-dimethyl-4-phenylpiperazinium; MEM, minimal essential medium; PSS, physiological salt solution.

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(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), and 0.5 mM ascorbic acid. Elevated K^+ PSS contained 56.0 mM KCl and 91.6 mM NaCl. Ba^{2+} PSS contained 2.2 mM $BaCl_2$ and 0 mM $CaCl_2$. The drugs BPB and Compound 1002 were added from a concentrated solution in dimethyl sulfoxide. Control solutions contained an equivalent amount of dimethyl sulfoxide (0.5%, v/v).

In experiments in which both catecholamine secretion and $^{45}Ca^{2+}$ uptake were measured, cells were preincubated for 15 min in the presence or absence of phospholipase A_2 inhibitor. The preincubation solution was removed and replaced with 0.36 ml of test solution containing the same concentration of phospholipase A_2 inhibitor, $^{45}Ca^{2+}$ (2 μCi/ml), and secretory stimulus where indicated. After 2 min the test incubation solution was removed from the cells and added to a test tube containing 40 μl of 50% trichloroacetic acid. The cells were rapidly rinsed three times with 1.0 ml of PSS (0–4°) and 0.8 ml of 5% trichloroacetic acid was added to the well to release intracellular $^{45}Ca^{2+}$ and catecholamine. Catecholamine content in the test incubation solution and in a 0.4-ml aliquot of the 5% trichloroacetic acid cell extract was measured by fluorometric assay (24). The remaining 0.4 ml of the 5% trichloroacetic acid cell extract was added to 4 ml of ACS scintillation counting solution (Amersham, Chicago, Ill.) to determine $^{45}Ca^{2+}$ uptake. Experiments in which Ba^{2+} was the stimulus utilized 15-min rather than 2-min incubations with secretagogue.

Because mepacrine interfered with the fluorometric catecholamine assay, an alternative radiometric method (25) for measuring catecholamine secretion was used in experiments involving mepacrine. Cells were incubated for 1 hr in MEM containing [^{3}H]norepinephrine (0.5 μCi/ml) and 0.5 mM sodium ascorbate and then subjected to three 10-min rinse periods in PSS. Experiments were conducted in the fashion outlined above except that separate wells were used to determine secretion and $^{45}Ca^{2+}$ uptake. Mepacrine caused substantial quenching which was adjusted for by internal standardization.

Cells were incubated in PSS (0.25 ml) containing [^{33}P] phosphate (60 μCi, carrier-free). After 30 min the solution was removed and 0.36 ml of PSS with or without 100 mM BPB was added. After 15 min the solution was replaced with a test solution containing the same concentration of BPB with or without DMPP. After 5 min the test solution was aspirated and the cellular proteins were denatured and solubilized by addition of 0.25 ml of stop solution [3% sodium dodecyl sulfate, 2% mercaptoethanol, 5% glycerol, 62 mM Tris-Cl (pH 6.7), and a small amount of bromphenol blue]. The solution was incubated at 90° for 3 min, and 0.1 ml was analyzed for phosphoprotein by sodium dodecyl sulfate/polyacrylamide (6.9%) slab gel electrophoresis and subsequent autoradiography (26). Molecular weights were determined by electrophoresis of standard proteins which were visualized by Coomassie blue staining. Areas from densitometer tracings were measured after subtracting background density in neighboring regions of the tracing.

$^{45}CaCl_2$ and L-[7-^{3}H]norepinephrine (3.8 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.); (^{32}P)Phosphate was from ICN (Irvine, Calif.); BPB was from Aldrich (Milwaukee, Wisc.); mepacrine and DMPP were from Sigma (St. Louis, Mo.); Compound 1002 was kindly provided by Dr. Donald Wallach of the Upjohn Company (Kalamazoo, Mich.).

The phospholipase A_2 inhibitors BPB, Compound 1002, and mepacrine inhibited catecholamine secretion and $^{45}Ca^{2+}$ uptake stimulated by the nicotinic agonist, DMPP, and by the depolarizing stimulus, 56 mM K^+; the inhibitors also blocked catecholamine secretion elicited by 2.2 mM Ba^{2+} (Fig. 1). BPB and Compound 1002 blocked secretion and $^{45}Ca^{2+}$ uptake elicited by all three types of stimuli at similar concentrations (Fig. 1A and B). However, mepacrine blocked DMPP-stimulated secretion and $^{45}Ca^{2+}$ uptake more potently than 56 mM K^+- and Ba^{2+}-stimulated events (Fig. 1C). The nicotinic blocking action of mepacrine (27) probably accounts for the more potent effects of the drug on DMPP-stimulated events. The approximate IC_{50} values for the inhibition of

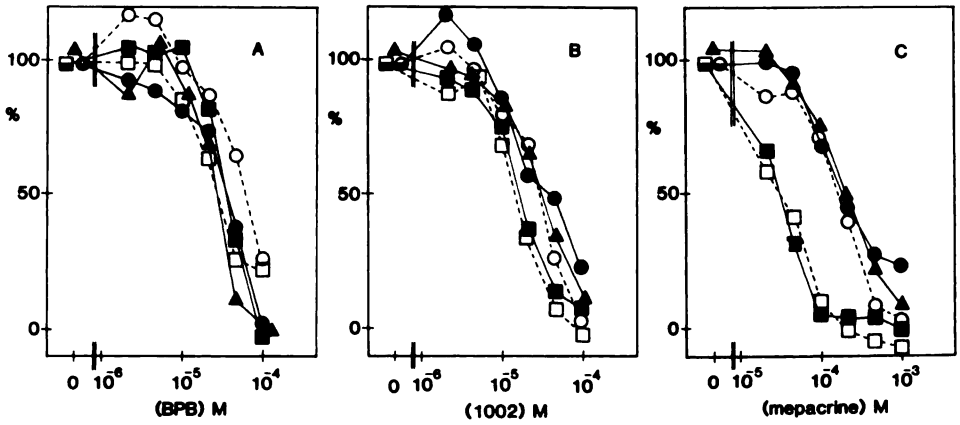

FIG. 1. *Dose-response curves for the inhibition of catecholamine secretion and $^{45}Ca^{2+}$ uptake by the phospholipase A_2 inhibitors BPB (A), Compound 1002 (B), and mepacrine (C)*

The inhibitors were present during a 15-min preincubation as well as during the test period. *Filled symbols* represent percentage of normal stimulated catecholamine secretion elicited by 10 μM DMPP (■), 56 mM K^+ (●), and 2.2 mM Ba^{2+} (▲). In the absence of inhibitors, 15–20% of the total catecholamine was released by DMPP and elevated K^+ and 40% was released by Ba^{2+}. *Open symbols* represent percentage of normal stimulated $^{45}Ca^{2+}$ (0.8–1.2 nmoles/2 min) uptake elicited by 10 μM DMPP (□) and 56 mM K^+ (○). Points represent the means of triplicate determinations. Standard errors of the mean were usually less than 5% and are not shown.

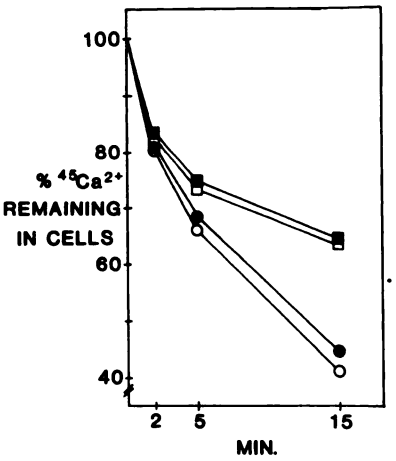

FIG. 2. *Lack of effect of the phospholipase A_2 inhibitor Compound 1002 on efflux of $^{45}Ca^{2+}$ from chromaffin cells*

After a 5-min incubation in 56 mM K^+ PSS containing $^{45}Ca^{2+}$ (2 μCi/ml) to label stores which take up calcium during secretion, cells were rapidly rinsed three times with PSS. The cells were then incubated with (●, ■) or without (○, □) 100 μM Compound 1002 for the times indicated; the efflux incubation medium contained 153 mM NaCl (○, ●) or 153 mM choline chloride (□, ■), 5.6 mM KCl, 0.5 mM $MgCl_2$, 5.6 mM glucose, 7.5 mM sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid and 0.5 mM ascorbic acid. The choline chloride-containing solution also contained 50 μM mecamylamine to block nicotinic effects of choline (28). Points represent the means of quadruplicate determinations; error bars were smaller than symbols and were omitted.

secretion and $^{45}Ca^{2+}$ uptake elicited by all three types of stimuli were BPB (32 μM), Compound 1002 (22 μM), and mepacrine (180 μM, except for the DMPP-stimulated events in which the IC_{50} was 22 μM).

Because these agents blocked $^{45}Ca^{2+}$ uptake, the effect of Compound 1002 on efflux of $^{45}Ca^{2+}$ from preloaded cells was measured. Na^+-Ca^{2+} exchange has been implicated in Ca^{2+} efflux. Therefore, we studied the effect of replacement of medium NaCl with choline chloride on $^{45}Ca^{2+}$ efflux in the presence and absence of Compound 1002. Na^+ replacement with choline$^+$ resulted in significant inhibition of $^{45}Ca^{2+}$ efflux (Fig. 2); isoosmotic replacement of NaCl with sucrose resulted in identical inhibition (data not shown). At a concentration (100 μM) at which secretion and $^{45}Ca^{2+}$ uptake were maximally inhibited, the phospholipase A_2 inhibitor Compound 1002 had no appreciable effect on efflux of preloaded $^{45}Ca^{2+}$ from the cells either in the presence or absence of Na^+ (Fig. 2).

The effects of BPB were investigated on the Ca^{2+}-dependent phosphorylation of a 60,000-dalton cytosolic protein recently identified as tyrosine hydroxylase[3] (29, 30). The phosphorylation occurs upon nicotinic cholinergic stimulation of chromaffin cells and is dependent upon medium Ca^{2+}. DMPP caused a 5-fold increase in phosphorylation of this protein which was completely inhibited by BPB (100 μM) (Table 1). Hence BPB inhibited not only secretion but also another intracellular Ca^{2+}-dependent process. The results are consistent with inhibition by BPB of Ca^{2+} influx.

[3] S. Pocotte, R. W. Holz, and T. Ueda, unpublished observations.

TABLE 1

Inhibition by BPB of nicotinic agonist-induced phosphorylation of tyrosine hydroxylase

The results are expressed as the area (square centimeters) of the tyrosine hydroxylase peak from densitometer tracings of an autoradiogram of sodium dodecyl sulfate/polyacrylamide slab gel after electrophoresis of ^{32}P-labeled proteins. There were four samples per group. See text for experimental details.

	Phosphorylation of tyrosine hydroxylase
Control	0.55 ± 0.02
10 μM DMPP	2.59 ± 0.30
100 μM BPB	0.40 ± 0.07
10 μM DMPP + 100 μM BPB	0.36 ± 0.11

Ca^{2+} influx induced by elevated K^+ probably occurs via the voltage-sensitive Ca^{2+} channel (3, 4); Ba^{2+} entry may also occur through this channel. Ca^{2+} influx stimulated by DMPP probably occurs at least partially through the ionic channel associated with the nicotinic receptor (4, 31). The phospholipase A_2 inhibitors may block Ca^{2+} influx through both of these channels either directly or indirectly, possibly by inhibiting phospholipase A_2.

In some systems phospholipase A_2 inhibitors block secretion induced by the Ca^{2+} ionophore A23187 (9–13, 15), which indicates that phospholipase A_2 inhibitors can block the secretory process at a step distal to Ca^{2+} entry. Because we have been unable to stimulate catecholamine secretion with A23187 or another Ca^{2+} ionophore, ionomycin, without causing cell toxicity, we have not been able to investigate the effects of the phospholipase A_2 inhibitors on events following Ca^{2+} influx that are important in exocytosis. We are currently investigating chromaffin cell phospholipase A_2 activation and its relationship to Ca^{2+} influx and catecholamine secretion.

In summary, our results indicate that commonly used phospholipase A_2 inhibitors inhibit Ca^{2+} influx and indicate that these agents can have varied actions. Hence, experiments utilizing these phospholipase A_2 inhibitors must be interpreted with caution.

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