Arachidonic Acid Release from Aortic Smooth Muscle Cells Induced by [Arg⁹]Vasopressin Is Largely Mediated by Calcium-independent Phospholipase A₂*

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To identify the phospholipase mediating the majority of [Arg⁹]vasopressin (AVP)-induced release of arachidonic acid in A-10 smooth muscle cells, we exploited the specificity inherent in the mechanism-based inhibitor, (E)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (HELSS), which possesses a 1,000-fold selectivity for inhibition of calcium-independent versus calcium-dependent phospholipases A₂. Utilizing [³H]arachidonic acid-labeled A-10 smooth muscle cells, one-half of AVP-inducible [³H]arachidonic acid release was inhibited by pretreatment with only 1 μM HELSS and two-thirds of AVP-stimulated [³H]arachidonic acid release was inhibited by 5 μM HELSS. The inhibition of [³H]arachidonic acid release by HELSS was saturable (i.e. no additional inhibition of [³H]arachidonic acid release was present at 10 μM HELSS), specific (i.e. the activities of six intracellular enzymes, as well as the rate of glucose oxidation, were not altered by HELSS treatment), and nontoxic (i.e. HELSS-treated cells excluded trypan blue dye and did not leak intracellular enzymes into the medium). Collectively, these results demonstrate that HELSS blocks AVP-induced arachidonic acid release by specific and irreversible inhibition of calcium-independent phospholipase A₂ and underscore the importance of calcium-independent phospholipase A₂ in agonist-induced arachidonic acid release in at least some cell types.

The generation of eicosanoid metabolites in the vascular bed is coordinately regulated by the sequential actions of a multiplicity of enzymes which amplify and propagate the flow of biologic information (cf. Ref. 1). Since the rate-determining step in the generation of eicosanoid metabolites in most cell types is the rate of calcium-independent phospholipase A₂ and underscores the importance of phospholipases A₂ have been characterized which represent the predominant phospholipase A₂ activity present in several agonist-responsive cell types including canine vascular smooth muscle (14–17). Traditionally, the importance of individual enzymes, receptors, or regulatory proteins has been elucidated through the utilization of inhibitors that possess substantial specificity toward the polypeptides under consideration. However, prior attempts to identify the importance of specific types of intracellular phospholipases A₂ have been confounded by the inability of traditional phospholipase inhibitors to distinguish among different phospholipases A₂ present in mammalian cells. Thus, the identification of (E)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (HELSS)¹ as a potent, irreversible, mechanism-based inhibitor that possessed over a 1000-fold selectivity for inhibition of calcium-independent versus calcium-dependent phospholipases A₂ (18) was met with enthusiasm, since it was an agent capable of elucidating the biologic role of calcium-independent phospholipase A₂ in receptor-mediated arachidonic acid release. The rat thoracic aortic A-10 smooth muscle cell line expresses vasopressin receptors of the V₁ subtype whose stimulation results in the selective release of arachidonic acid from membrane phospholipids (19, 20). Through specific mechanism-based inhibition of calcium-independent phospholipase A₂ by HELSS, we now report that the majority of arachidonic acid release induced by vasopressin stimulation of A-10 cells is due to the activation of calcium-independent phospholipase A₂.

MATERIALS AND METHODS

Cell Culture, Radiolabeling, Quantification of Lipids, and Phospholipase A₂ Assays—Rat thoracic aortic smooth muscle cells (A-10) were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium (DMEM; containing 20% fetal calf serum (FCS)). Cells were radio-labeled by incubation of 20 μCi of [³H]arachidonic acid (Amesham Corp.) with the cells at 37 °C for 24 h. The cells were subsequently extensively washed with fresh DMEM/FCS medium (DMEM plus 20% FCS) and were incubated for 15 min at 37 °C with medium (heat-inactivated) containing either vehicle alone (0.1% EtOH final concentration) or the indicated concentration of HELSS dissolved in vehicle. Next, the cells were incubated for 5 min at 37 °C in fresh medium containing either vehicle alone (0.1% NaCl) or AVP. The incubation of lipid storage pools (see, e.g., Refs. 1 and 2), the identification and characterization of the intracellular phospholipases responsible for the release of arachidonic acid is a prominent issue in vascular biology.

The detailed examination of the stoichiometry and specific activities of eicosanoid precursor pools and metabolites has collectively demonstrated the importance of phospholipase A₂ as the major mediator of agonist-induced arachidonic acid release in most cell types (see, e.g., Refs. 2–4). During the last decade, several novel families of intracellular phospholipases A₂ have been identified and characterized (5–9). Although it was previously assumed that calcium was the predominant activator of intracellular phospholipase A₂ during cellular stimulation, recent studies have demonstrated that calcium is neither necessary nor sufficient for activation of several recently described intracellular phospholipases A₂ (see, e.g., Refs. 10–13). Indeed, a novel class of calcium-independent phospholipases A₂ have been characterized which represent the predominant phospholipase A₂ activity present in several agonist-responsive cell types including canine vascular smooth muscle (14–17). Traditionally, the importance of individual enzymes, receptors, or regulatory proteins has been elucidated through the utilization of inhibitors that possess substantial specificity toward the polypeptides under consideration. However, prior attempts to identify the importance of specific types of intracellular phospholipases A₂ have been confounded by the inability of traditional phospholipase inhibitors to distinguish among different phospholipases A₂ present in mammalian cells. Thus, the identification of (E)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (HELSS)¹ as a potent, irreversible, mechanism-based inhibitor that possessed over a 1000-fold selectivity for inhibition of calcium-independent versus calcium-dependent phospholipases A₂ (18) was met with enthusiasm, since it was an agent capable of elucidating the biologic role of calcium-independent phospholipase A₂ in receptor-mediated arachidonic acid release. The rat thoracic aortic A-10 smooth muscle cell line expresses vasopressin receptors of the V₁ subtype whose stimulation results in the selective release of arachidonic acid from membrane phospholipids (19, 20). Through specific mechanism-based inhibition of calcium-independent phospholipase A₂ by HELSS, we now report that the majority of arachidonic acid release induced by vasopressin stimulation of A-10 cells is due to the activation of calcium-independent phospholipase A₂.

¹ The abbreviations used are: HELSS, haloenol lactone suicide substrate (E)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one; AVP, [Arg⁹]vasopressin; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; KRB, Krebs-Ringer bicarbonate buffer.

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media or cells were extracted by the Bligh and Dyer method, and fatty acid was quantified after TLC by scintillation spectrometry. Phospholipase A2 assays were performed utilizing either cytosolic or microsomal fractions from control, AVP-treated, or AVP + inhibitor-treated A-10 cells employing either radiolabeled plasmalogen substrate (1-O-
(2Z)-10,12-eicosapentaenoic-9-enzymo-glycerol-3-phosphocholine) for calcium-independent phospholipase A2 or phosphatidylcholine substrate (1-hexadecanoyl-2-[5,6,8,9,11,12,14,15-3H]eicoso-5,8,11,
14,17-tetraenyl-an-glycerol-3-phosphocholine) for calcium-dependent phospholipase A2. Briefly, the indicated protein fraction was added to media or cells were extracted by the Bligh and Dyer method, and fatty acid species were separated by anion exchange chromatography and were quantified by scintillation spectrometry as described previously (21). Chromatography of Inositol Phosphates—Cells were labeled with 90 pCi of myo-[2-3H]inositol in each 35-mm culture dish and incubated for 24 h. Inositol metabolites in control, stimulated, and inhibitor-treated cells were extracted and radiolabeled mono-, bis-, and tri-, and tetrakisphosphates were separated by anion exchange chromatography and were quantified by scintillation spectrometry as described previously (21). Determination of Intracellular Calcium Concentration, Glucose Oxidation Rates, and Nonrelated Smooth Muscle Cell Enzymatic Activities—A-10 cells were plated onto Cell Tak-coated glass coverslips and incubated overnight at 37 °C. The attached cells were pretreated with either vehicle alone or with 10 μM HELSS for 10 min at 37 °C. Intra-
cellular calcium in Fura-2-loaded cells was quantified as described previously (21). Glucose oxidation in A-10 cells was assessed in cells grown for 3 days on Cytodex 3 beads (Pharmacia LKB Biotechnology Inc.) after treatment with vehicle alone or treatment with inhibitor (10 μM HELSS) by trapping released [14C]CO₂ from L-[1-14C]glucose onto filter paper and subsequent scintillation spectrometry as previously described (21–23). The integrity of intracellular enzymes in A-10 cells treated for 15 min with 10 μM HELSS was assessed after two freeze-
thaw cycles by measuring alkaline phosphatase, creatine kinase, lact
tate dehydrogenase, aspartate aminotransferase, alanine aminotrans
erase, and γ-glutamyl transpeptidase employing previously described techniques (22, 25–27).

RESULTS AND DISCUSSION

The major measurable phospholipase A2 activity in smooth muscle A-10 cells grown in culture was calcium-independent (specific activities in the microsomal and cytosolic fractions were 250 and 120 pmol/mg min, respectively), selective for plasmalogen substrate, and exquisitely sensitive to inhibition by the mechanism-based inhibitor (E)-6-chromomethylene/ter
rubydro-3-(1-naphthalenyl)-2H-pyran-2-one (>95% inhibition at 5 μM inhibitor). These characteristics are similar to those of the major phospholipase activity present in adult rat thoracic aortic smooth muscle which had been previously characterized (16).

Incubation of [3H]arachidonic acid-labeled A-10 cells with 1 μM AVP for 5 min at 37 °C resulted in the release of ~320,000 dpm of [3H]arachidonic acid (equivalent to ~4% of the [3H]arachidonic acid incorporated into cellular phospholipids) into the culture medium (Fig. 2). The only metabolite released was arachidonic acid, without demonstrable amounts of lipox
genase or cyclooxygenase products present (Fig. 1, lanes 2 and 3). Incubation of prelabeled rat aortic A-10 smooth muscle cells with 10 μM HELSS for 15 min immediately prior to AVP stimulation resulted in the ablation of the majority of arachidonic acid release into the medium (Fig. 1, compare lanes 2 and 3 with lanes 4 and 5). One-half of [3H]arachidonic acid release into the medium was blocked by preincubation with only 1 μM HELSS, and two-thirds of agonist-induced [3H]arachidonic acid release was inhibited by 5 μM HELSS (Fig. 2). Importantly, pretreatment with 10 μM HELSS did not result in further inhibition of arachidonic acid release, demonstrating saturation of inhibition through HELSS-sensitive pathways at the 70% of release level. The remaining 30% of [3H]arachidonic acid release is likely mediated by other phospholipases including a 30-kDa dimeric calcium-responsive phospholipase A2 (6), a 85-
kDa calcium-responsive phospholipase A2 (7, 8), the low molecular weight phospholipases A2 (28), and/or phospholipases C (5, 29–32) and D (33). Measurements of phospholipase A2 activities from control and AVP-treated A-10 cells demonstrated that calcium-independent phospholipase A2 activity in the microsomal fraction increased modestly after AVP stimulation (118% of control value), which was accompanied by a parallel decrease in calcium-dependent phospholipase A2 activity in the cytosolic fraction (84% of control activity). As anticipated, treatment of A-10 cells with HELSS (10 μM) inhibited over 95% of calcium-independent phospholipase A2 activity in both cyto
sic and microsomal subcellular fractions in both control and AVP-stimulated cells. Calcium-dependent phospholipase A2 activity represented <10% of total measurable phospholipase A2 activity employing either plasmalogenyl or phosphatidylcholine substrate, was predominantly present in the microsomal fraction in both control and AVP-treated cells, decreased modestly after AVP-treatment, and was not inhibited by HELSS. Collectively, these results demonstrate that diminu
tive concentrations of HELSS are capable of inhibiting the majority of agonist-induced arachidonic acid release in a concen
tration-dependent and saturable manner and identify calcium-
dependent phospholipase A2 as the enzymic mediator responsible for the majority (~70%) of [3H]arachidonic acid release in this system.

To confirm that the reduction in arachidonic acid release was due to specific inhibition of calcium-dependent phospholipase A2 and not the result of unforeseen effects on other enzymes or overall cellular viability, a multiplicity of independent criteria were evaluated. First, cellular ultrastructure was unchanged in control or HELSS-pretreated A-10 cells. Second, cellular permeability was unaltered after HELSS treatment as assessed by trypan blue exclusion. Similarly, HELSS treatment did not result in the release of lactate dehydrogenase into the medium,
Mediation of AVP-induced Arachidonic Acid Release

**Fig. 2.** Dose-response profile of the inhibition of AVP-induced [3H]arachidonic acid release from A-10 cells by HELSS. Nearly confluent A-10 cells were prelabeled with [3H]arachidonic acid and stimulated for 5 min with 1 μM AVP immediately following a 15-min incubation with either vehicle alone or 1, 5, or 10 μM HELSS. Released [3H]arachidonic acid was extracted from the media, separated by TLC, and quantified by scintillation spectrometry as described under “Materials and Methods.” Each data point represents the mean (± S.E.M.) of at least six different culture plates. AVP-induced arachidonic acid release from HELSS-pretreated cells was significantly different from control (*, p < 0.01; **, p < 0.001) utilizing a non-directional Student’s t test. **Fig. 3.** [3H]vasopressin-induced alterations in intracellular calcium and their modulation by HELSS. Vascular smooth muscle A-10 cells were plated on 25-mm coverslips and cultured overnight at 37°C under an atmosphere of 5% CO2. Nearly confluent A-10 cells were then washed free of growth media and loaded with 10 μM Fura-2 AM (in KRB/bovine serum albumin) for 30 min at room temperature. The cells were subsequently incubated for 10 min at 37°C in the presence of either HELSS (10 μM) or vehicle (0.1% EtOH) and perfused with appropriate media in a thermostatically regulated (37°C) chamber for the microfluorimetry experiments. Dual-wavelength excitation was performed at 340 nm (Ca2+-bound Fura-2) and 380 nm (unbound Fura-2) and emission was monitored at 505 nm. The ratio of 340/380 nm was used as an index of cytosolic Ca2+ and single cell recordings of 340/380 nm fluorescence ratios are presented. Panels A and B, cells were perfused for 10 min with KRB media containing 1 mM EGTA and no added Ca2+ prior to exposure to 1 μM AVP (as indicated by the arrows). Panel A, control cells; panel B, HELSS-pretreated cells. Panels C and D, cells were perfused in KRB media containing 2.5 mM Ca2+ and 1 μM AVP, washed, and cytosolic Ca2+ quantitated as described above. Panel C, control cells; panel D, HELSS-pretreated cells.

under-scoring the sustained functional integrity of the A-10 cell plasma membrane after exposure to HELSS. Third, the activities of multiple enzymes (unrelated to phospholipase A2) present in A-10 cells (including alkaline phosphatase, creatine kinase, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, and γ-glutamyl transpeptidase) were unaltered by HELSS treatment. Fourth, the rates of glucose oxidation in control and HELSS-pretreated cells were nearly identical, demonstrating the metabolic integrity of a multiplicity of enzymes participating in the sequential oxidation of glucose. Fifth, control and HELSS-treated cells manifested similar amounts of agonist-induced phospholipase C-mediated hydrolysis of inositol phospholipids (i.e., no significant differences in the amounts of [3H]inositol phosphate or [3H]inositol triphosphate released after AVP stimulation of [3H]inositol prelabeled cells). Collectively, these results demonstrate the morphologic, ultrastructural, functional and metabolic integrity of A-10 cells following inhibitor treatment and underscore the selectivity of the mechanism-based inhibitor HELSS.

Since alterations in intracellular calcium concentration constitute an important element in the initiation and propagation of cellular activation which has been temporally and chemically related to the activation of at least some intracellular phospholipases, additional experiments were performed to determine the effects of HELSS treatment on the alterations in cytosolic Ca2+ induced by AVP stimulation of A-10 cells. First, treatment of A-10 smooth muscle cells with 1 μM AVP in calcium-free buffer resulted in a transient spike in calcium ion (as ascertained by the 340/380 nm fluorescence ratio of Fura-2) and the intensity and duration of the spike was unchanged after treatment with HELSS (Fig. 3, panels A and B). Thus, the intracellular machinery involved in the release and reuptake of intracellular Ca2+ was not altered by HELSS treatment. Furthermore, treatment of smooth muscle A-10 cells with AVP in buffer containing 2.5 mM Ca2+ resulted in a calcium transient that was similar to that present in the absence of extracellular Ca2+ ion but that was substantially prolonged due to calcium influx from the extracellular medium (Fig. 3, panel C). In prior studies Thibonnier (34) demonstrated that treatment of A-10 smooth muscle cells with arachidonic acid prior to AVP stimulation shortens the time necessary for Ca2+ levels to return to base line. Accordingly, we anticipated, and found, that inhibitor-treated cells demonstrated an extended prolongation of the elevation in intracellular calcium ion after exposure to agonist in comparison to control cells (compare panels C and D in Fig. 3; see Fig. 4). Since HELSS treatment attenuates the AVP-induced release of arachidonic acid, these results clearly show the presence of one functional sequela (i.e., a change in temporal longevity of the calcium signal) resulting from HELSS-mediated attenuation of arachidonic acid release and underscore the functional importance of calcium-independent phospholipase A2 in regulating the temporal duration of the calcium signal in agonist-stimulated A-10 cells.

Collectively, these results demonstrate the importance of calcium-independent phospholipase A2 in mediating the majority of agonist-induced arachidonic acid release in vascular smooth muscle A-10 cells and identify one functional sequela of the inhibition of arachidonic acid release after receptor stimulation. Since arachidonic acid accelerates phosphorylation of the myosin light chain and attenuates the rate of its dephosphorylation (35), it seems likely that calcium-independent phospholipase A2 is an important modulator of the increase in contractile force of smooth muscle after AVP stimulation. The bio-
logic role of calcium-independent phospholipase A<sub>2</sub> in mediating the release of arachidonic acid in other cell types, or in modulating the kinetics of other agonist-stimulated cellular events, will hopefully be facilitated by future studies with HELSS (or other membrane-permeable specific inhibitors) to assess the biochemical and functional roles of each of the different types of intracellular phospholipase A<sub>2</sub>.

REFERENCES

1. Needleman, P., Turk, J., Jakusch, B. A., Morrison, A. R., and Lefkowitz, J. R. (1986) Annu. Rev. Biochem. 55, 69–102
2. Thomas, J. M. F., Hulin, F., Chap, H., and Douste-Blazy, L. (1984) Thromb. Res. 34, 117–123
3. Meade, C. J., Turner, G. A., and Bateman, P. E. (1986) Biochim. J. 236, 425–438
4. Garcia-Gil, M., and Siragianian, R. P. (1986) J. Immunol. 136, 3825–3828
5. Wolf, R. A., and Gross, R. W. (1985) J. Biol. Chem. 260, 7285–7303
6. Leeb, L. A., and Gross, R. W. (1986) J. Biol. Chem. 261, 10467–10479
7. Leslie, C. C., Volcker, D. R., Channon, J. Y., Wall, M. M., and Zabary, P. T. (1988) Biochim. Biophys. Acta 963, 476–492
8. Kramer, R. M., Roberts, R. F., Manetta, J., and Putnam, J. E. (1991) J. Biol. Chem. 266, 5289–5272
9. Clark, J. D., Lin, L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A. Lin, A. Y., Tschopp, N., and Knoop, J. L. (1991) Cell 65, 1043–1051
10. Hazen, S. L., Stuppy, R. J., and Gross, R. W. (1990) J. Biol. Chem. 265, 10622–10630
11. Zupan, L. A., Kruza, K. K., and Gross, R. W. (1991) FERS Lett. 284, 27–30
12. Hazen, S. L., Ford, D. A., and Gross, R. W. (1991) J. Biol. Chem. 266, 5629–5633
13. Wijkander, J., Sundler, F. (1992) Biochim. Biophys. Res. Commun. 184, 118–124
14. Hazen, S. L., and Gross, R. W. (1991) J. Biol. Chem. 266, 14526–14534
15. Hazen, S. L., and Gross, R. W. (1991) Biochim. J. 280, 581–587
16. Miyake, R., and Gross, R. W. (1992) Biochim. Biophys. Acta 1165, 167–176
17. Gross, R. W., Ramanadham, S., Kruza, K. K., Hao, X., and Turk, J. (1993) Biochemistry 32, 327–336
18. Hazen, S. L., Zupan, L. A., Weiss, R. H., Getman, D. P., and Gross, R. W. (1991) J. Biol. Chem. 266, 7227–7232
19. Stassen, F. L., Beckman, G., Schmidt, D., Aiyar, N., Nambi, P., and Crooke, S. T. (1987) Mol. Pharmacol. 31, 259–269
20. Grillo, L. S., Clark, M. A., Geoffrey, R. W., Stassen, F., and Crooke, S. T. (1988) J. Biol. Chem. 263, 2655–2663
21. Hazen, S. L., Leeb, L. A., and Gross, R. W. (1991) Methods Enzymol. 197, 400–411
22. Ramanadham, S., Gross, R. W., Han, X., and Turk, J. (1993) Biochemistry 32, 337–345
23. McDaniel, M. L., King, S., Anderson, S., Fink, J., and Lacy, P. E. (1974) Diabetologia 10, 393–398
24. Hughes, J. H., Eason, R. A., Wolf, B. A., Turk, J., and McDaniel, M. L. (1989) Diabetes 38, 1251–1257
25. Wacker, W. E. C., Ulmer, D. D., and Yallee, B. L. (1956) J. Biol. Chem. 260, 581–587
26. Gay, R. J., McComb, R. B., and Bewers, G. N., Jr. (1968) Clin. Chem. 14, 215–2163
27. Bergmeyer, H. U., Scheibe, P., and Wohlfeld, A. W. (1978) Clin. Chem. 24, 58–73
28. Davidson, F. F., Dennis, F. A. (1990) J. Biol. Chem. 265, 228–238
29. Kretzschmar-Simmons, S., (1979) J. Clin. Invest. 63, 565–577
30. Hofman, S. L., and Majerus, P. W. (1982) J. Biol. Chem. 257, 6461–6469
31. Shub, P. G., Ryu, S. H., Moon, K. H., Shub, H. W., and Rhue, S. G. (1986) Cell 54, 161–169
32. Stahl, M. L., Ferens, C. R., Kelleher, K. L., Kriz, R. W., and Knoop, J. (1988) Nature 332, 269–272
33. Agwa, D. E., McPhail, L. C., Chabot, M. C., Daniel, L. W., Wykle, R. L., and McCullough, C. E. (1989) J. Biol. Chem. 264, 1405–1413
34. Tribouillard, M., Bayer, A., L. Simonse, M. S., and Rester, M. (1991) Endocrinology 129, 3845–3956
35. Gong, M. C., Fuglaas, A., Alessi, D., Kobayashi, S., Cohen, P., Sembly, A. V., and Sembly, A. P. (1992) J. Biol. Chem. 267, 21492–21496