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Nitric Oxide Activates the Glucose-dependent Mobilization of Arachidonic Acid in a Macrophage-like Cell Line (RAW 264.7) That Is Largely Mediated by Calcium-independent Phospholipase A_2*

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Herein, we demonstrate that nitric oxide is a potent (>20% release) and highly selective inducer of [^{3}H]arachidonic acid mobilization in the macrophage-like cell line RAW 264.7. Treatment of RAW 264.7 cells with (*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2*H*-tetrahydropyran-2-one resulted in the inhibition of the large majority (86%) of nitric oxide-induced [^{3}H]arachidonic acid release into the medium (IC_{50} <0.5 μM) and the concomitant inhibition of *in vitro* measurable calcium-independent phospholipase A_2 activity (92% inhibition) without demonstrable effects on calcium-dependent phospholipase A_2 activity. Since nitric oxide is a potent stimulator of glycolysis (and therefore glycolytically derived ATP) and since cytosolic calcium-independent phospholipase A_2 exists as a catalytic complex comprised of ATP-modulated phosphofructokinase-like regulatory polypeptides and a catalytic subunit, we examined the role of glucose in facilitating nitric oxide-mediated arachidonic acid release. Nitric oxide-induced release of [^{3}H]arachidonic acid possessed an obligatory requirement for glucose, was highly correlated with the concentration of glucose in the medium, and was dependent on the metabolism of glucose. Thus, [^{3}H]arachidonic acid release is coupled to cellular glucose metabolism through alterations in the activity of calcium-independent phospholipase A_2. Collectively, these results identify a unifying metabolic paradigm in which the generation of lipid second messengers is coordinately linked to the signalstimulated acceleration of glycolytic flux, thereby facilitating integrated metabolic responses to cellular stimuli.

Recent studies have underscored the unanticipated ubiquity, potency, and diversity of nitric oxide-mediated cellular activation (*cf.* Refs. 1–3). Although many mechanisms have been proposed to explain the sequence of molecular transformations that mediate the biologic sequelae of nitric oxide stimulation and some have received substantial experimental support (*e.g.* cGMP-mediated vascular relaxation (4)), the detailed chemical mechanisms underlying many of the effects of this gaseous second messenger remain enigmatic (*e.g.* Refs. 5 and 6). For example, macrophages undergo a dramatic biochemical and functional metamorphosis after nitric oxide stimulation and yet the chemical mechanisms mediating the majority of nitric oxide-induced alterations in macrophage chemistry and biology remain unknown.

Macrophage activation induced by a variety of different ligands (*e.g.* lipopolysaccharide, zymosan, etc.) results in increased cellular glycolytic flux, accelerated phospholipid catabolism, and stimulation of many macrophage physiologic functions (*e.g.* phagocytosis and chemotaxis) (7, 8). Macrophages contain both calcium-dependent (9, 10) and calcium-independent phospholipase A_2 (11) activities that are catalyzed by separate and distinct classes of polypeptides (*e.g.* Refs. 12 and 13). We have previously demonstrated that native calcium-independent phospholipase A_2 in several cell types exists as a high molecular weight catalytic complex (400 kDa) comprised of regulatory polypeptides highly homologous with, or identical to, phosphofructokinase and a 40-kDa catalytic entity (*e.g.* Refs. 14 and 16). Calcium-independent phospholipase A_2 activity is modulated by physiologic alterations in ATP concentration through the interaction of ATP with phosphofructokinase-like regulatory polypeptides, which are tightly associated with, and functionally coupled to, the 40-kDa catalytic subunit (16). These findings led us to suggest that glycolysis and phospholipolysis are interwoven metabolic pathways coupled through the interaction of the calcium-independent phospholipase A_2 catalytic complex with glycolytically derived ATP (16). Since nitric oxide is a potent stimulator of macrophage glucose uptake and glycolytic flux (7, 17), we hypothesized that some of the effects of nitric oxide on macrophage phospholipid catabolism and physiologic function could result from the calcium-independent phospholipase A_2-mediated mobilization of arachidonic acid in response to the nitric oxide-induced accelerated production of glycolytically derived ATP. We now report that: 1) nitric oxide is a potent activator of arachidonic acid mobilization in macrophages; 2) the highly selective mobilization of arachidonic acid by nitric oxide is predominantly mediated by calcium-independent phospholipase A_2; and 3) nitric oxide-mediated arachidonic acid mobilization by calcium-independent phospholipase A_2 possesses an obligatory dependence on glucose uptake and metabolism.

EXPERIMENTAL PROCEDURES

Materials—RAW 264.7 cells were obtained from American Type Tissue Culture Collection. 1-*O*-(*Z*)-Hexadec-1′-enyl-2-[9,10-^{3}H]octadec-9′-enoyl-*sn*-glycero-3-phosphocholine-(plasmenylcholine) and (*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2*H*-tetrahydropyran-2-one (BEL)[1] were synthesized and purified by established methods (18–21).

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[1] The abbreviations used are: BEL, (*E*)-6-(bromomethylene)-3-(1-naphthalenyl)-2*H*-tetrahydropyran-2-one; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LPS, lipopolysaccharide; SNP, sodium nitroprusside.

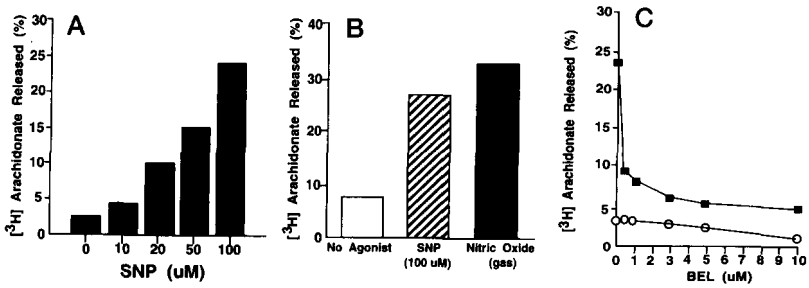


FIG. 1. **Nitric oxide induces the mobilization of [^{3}H]arachidonic acid from RAW 264.7 cell phospholipids, which is inhibited by the mechanism-based inhibitor BEL.** *A*, RAW 264.7 cells were prelabeled for 16 h with [^{3}H]arachidonic acid, unincorporated [^{3}H]arachidonic acid was removed and cells were incubated with the indicated concentrations of sodium nitroprusside for 60 min at 37 °C. Released fatty acids in the media (or phospholipids in cells) were purified by TLC and quantified by scintillation spectrometry as described under "Materials and Methods." Released [^{3}H]arachidonic acid is expressed as the percent of [^{3}H]arachidonic acid in the media divided by the total incorporated [^{3}H]arachidonate in RAW 264.7 cells. Results represent the mean of at least six independent determinations. *B*, RAW 264.7 cells prelabeled for 16 h with [^{3}H]arachidonic acid were incubated with buffer alone (control), buffer containing sodium nitroprusside (100 μM), or buffer previously bubbled for 10 min with nitric oxide gas (the pH was subsequently adjusted to pH 7.4 with 2 N NaOH) for 60 min at 37 °C. Fatty acids released into the media were quantified as described above. *C*, RAW 264.7 cells were prelabeled for 16 h with [^{3}H]arachidonic acid, preincubated with the indicated concentrations of BEL, and subsequently incubated for 60 min at 37 °C with either buffer alone (○) or with buffer previously bubbled with NO gas (■). [^{3}H]Arachidonate release into the media was quantified as described above.

Radiolabeling of RAW 264.7 Cells and Measurement of Phospholipase A_2 Activity in the Cytosolic and Microsomal Fractions—RAW 264.7 cells were maintained in culture at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Cells were seeded and incubated for 6 h, and 0.5 ml of fresh DMEM containing 10% FBS and 0.25 μCi of either [^{3}H]arachidonic acid (100 Ci/mmol) or [^{3}H]oleic acid (10 Ci/mmol) was added to each well. The media contained 6 μM arachidonic acid and 7 μM oleic acid (as endogenous constituents in FBS) rendering the final specific activity of these fatty acids to values of 183,330 and 157,140 dpm/nmol, respectively. After 16 h, media were removed, and cells were washed (3 times) with phosphate-buffered saline and incubated with either vehicle alone (EtOH, final concentration, <0.1% v/v) or vehicle containing BEL (0.5–10 μM as indicated) for 15 min. After washing with phosphate-buffered saline containing 0.25% bovine serum albumin (3 times), cells were subsequently treated with either media containing albumin (0.25 mg/ml), media containing the indicated concentration of SNP and albumin, or media containing albumin previously bubbled with nitric oxide (the final pH was adjusted with 1 N NaOH) for 60 min at 37 °C. Fatty acids and phospholipids were extracted, separated, and quantified as described previously (21). Phospholipase A_2 activity in the cytosolic or microsomal fractions of control or BEL-treated RAW 264.7 cells was measured by quantifying the release of radiolabeled fatty acid from synthetically prepared plasmenylcholine or purchased phosphatidylcholine(1-octadecanoyl-2-[5,6,8,9,11,12,14,15-^{3}H]eicosa-5,8,11,14-tetraenoyl-*sn*-glycero-3-phosphocholine) as described previously (21).

RESULTS

Nitric Oxide-induced Mobilization of [^{3}H]Arachidonic Acid from Macrophage-derived RAW 264.7 Cellular Phospholipids—Incubation of [^{3}H]arachidonic acid-prelabeled RAW 264.7 cells with sodium nitroprusside resulted in the dose-dependent mobilization of [^{3}H]arachidonic acid from endogenous cellular phospholipid storage depots (Fig. 1*A*). The mobilization of [^{3}H]arachidonic acid from RAW 264.7 cells induced by SNP-generated nitric oxide was indistinguishable from that induced by bubbling nitric oxide gas through the media (Fig. 1*B*). The identity of the storage species of nitric oxide in plasma as a thionitroso adduct of cysteine residues in proteins has been well documented (22–25). The presence and persistence of the thionitroso adducts in the media after nitric oxide (gas) bubbling was demonstrated by the appearance of new uv peaks (λ_{max} = 335 and 545 nm), which are stable for hours under the conditions of the experiments (23). Since BEL possesses over a 1,000-fold selectivity for inhibition of calcium-independent phospholipase A_2 compared with calcium-dependent phospholipase A_2 (20, 26), we exploited the specificity inherent in mechanism-based inhibition to identify the type of phospholipase A_2 activity mediating the nitric oxide-induced release of arachidonic acid. The overwhelming majority of arachidonic acid mobilization induced by nitric oxide was inhibited by BEL with over 75% inhibition manifest even at the lowest concentration of inhibitor employed (*i.e.* 0.5 μM BEL) (Fig. 1*C*).

Nitric Oxide Selectively Releases Arachidonic Acid from Endogenous RAW 264.7 Cell Phospholipid Storage Pools—The nitric oxide-induced mobilization of fatty acids from endogenous RAW 264.7 phospholipid storage depots mediated by SNP was highly selective for arachidonic acid in comparison with oleic acid (Fig. 2). Furthermore, SNP-mediated mobilization of [^{3}H]arachidonic acid was greater (>20% release) than that manifested by saturating concentrations of LPS or zymosan (Fig. 2). Over 95% of radiolabeled products released by SNP, LPS, or zymosan in RAW 264.7 cells were identified as bona fide arachidonic acid and not oxygenated eicosanoid metabolites (Fig. 2). The large majority of LPS and zymosan-induced [^{3}H]arachidonic acid mobilization in macrophages was inhibited by BEL demonstrating that the release of [^{3}H]arachidonic acid after stimulation by these effectors largely results from hydrolysis mediated by calcium-independent phospholipase A_2.

Measurement of RAW 264.7 Cell Phospholipase A_2 Activities and [^{3}H]Arachidonic Acid Reacylation Rates—Measurement of RAW 264.7 cell phospholipase A_2 activity *in vitro* demonstrated the presence of both calcium-dependent and -independent phospholipase A_2 activities (Fig. 3*A*). Calcium-dependent phospholipase A_2 activity was predominant in the cytosolic fraction, while calcium-independent phospholipase A_2 predominated in the microsomal fraction. Stimulation of RAW 264.7 cells with SNP did not result in substantial alterations of *in vitro* measurable calcium-dependent or -independent phospholipase A_2 activities in either the cytosolic or microsomal fractions utilizing plasmenylcholine substrate (plasmenylcholine is not susceptible to hydrolysis by phospholipase A_1) (Fig. 3*A*) or phosphatidylcholine substrate containing [^{3}H]arachidonic acid at the *sn*-2 position (data not shown). Treatment of RAW 264.7 cells with BEL (10 μM) resulted in the inhibition of 92% of calcium-independent phospholipase A_2 activity without inhibition of calcium-dependent phospholipase A_2 activity. Collectively, these results demonstrate that: 1) nitric oxide-mediated stimulation of RAW 264.7 cells does not result in stimulation of measurable phospholipase A_2 activity in *in vitro* homogenates; 2) BEL specifically inhibits calcium-independent phospholipase A_2 (and not calcium-dependent phospholipase A_2) when administered to intact RAW 264.7 cells; and 3) BEL ablates the mobilization of [^{3}H]arachidonic acid in RAW 264.7 cells under conditions in which calcium-independent phospholipase A_2 activity is inhibited and calcium-dependent phospholipase A_2

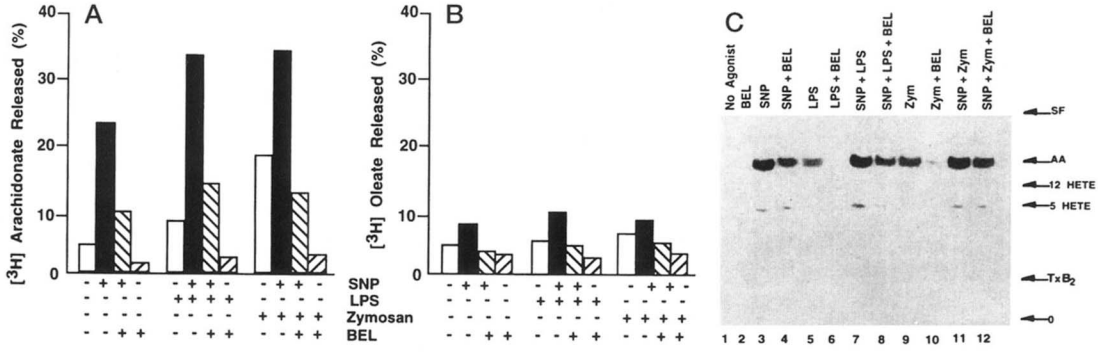
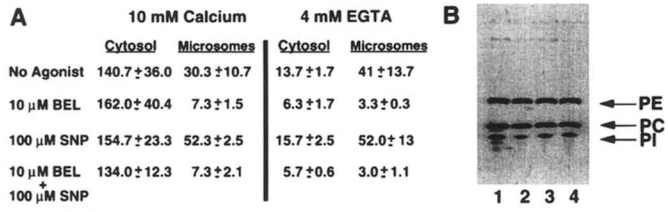


FIG. 2. **Fatty acyl selectivity of nitric oxide-induced phospholipid hydrolysis and comparisons with lipopolysaccharide and zymosan-mediated RAW 264.7 cell stimulation.** *A* and *B*, RAW 264.7 cells were prelabeled for 16 h with either [^{3}H]arachidonic acid (*A*) or [^{3}H]oleic acid (*B*). Cells were either pretreated with buffer alone (control) or with BEL (10 μM) for 15 min. Subsequently, cells were incubated with either buffer alone (control), sodium nitroprusside (100 μM), lipopolysaccharide (5 μg/ml) or zymosan (100 μg/ml) alone, or the indicated combinations of agonists. Fatty acid released into the media was quantified as described in Fig. 1. *C*, RAW 264.7 cells were labeled for 16 h with [^{3}H]arachidonic acid (*AA*) and incubated for 15 min with either buffer alone or with BEL (10 μM). Subsequently, cells were stimulated for 60 min with either buffer alone (control), sodium nitroprusside (100 μM), lipopolysaccharide (5 μg/ml), or zymosan (*Zym*, 100 μg/ml), either alone or in the indicated combinations. The media were extracted by the Bligh and Dyer technique and applied to a Whatman Silica Gel 60A TLC plate, and metabolites were resolved utilizing a mobile phase comprised of chloroform/methanol/acetic acid/H_2O (90:8:1:0.8, v/v).



A

	10 mM Calcium		4 mM EGTA	
	Cytosol	Microsomes	Cytosol	Microsomes
No Agonist	140.7±36.0	30.3±10.7	13.7±1.7	41±13.7
10 μM BEL	162.0±40.4	7.3±1.5	6.3±1.7	3.3±0.3
100 μM SNP	154.7±23.3	52.3±2.5	15.7±2.5	52.0±13
10 μM BEL + 100 μM SNP	134.0±12.3	7.3±2.1	5.7±0.6	3.0±1.1

FIG. 3. **Measurement of phospholipase A_2 activities in RAW 264.7 cells after nitric oxide stimulation or inhibitor treatment and examination of deacylation-reacylation cycling rates.** *A*, phospholipase A_2 activity in the cytosolic or microsomal fractions was quantified by determining the initial rate of fatty acid release from 1-*O*-(*Z*)-hexadec-1′-enyl-2-[9,10-^{3}H]-octadec-9′-enoyl-*sn*-glycero-3-phosphocholine substrate as described under "Materials and Methods." Phospholipase A_2 activity was assessed in *in vitro* homogenates after incubation of cells with either buffer alone (control) or sodium nitroprusside (100 μM) following a 15-min preincubation period with either buffer (control) or BEL (10 μM). The data are presented in pmol/mg·min. *B*, RAW 264.7 cells were incubated with [^{3}H]arachidonic acid and either buffer (*lane 1*), sodium nitroprusside (100 μM) (*lane 2*), sodium nitroprusside (100 μM) and BEL (*lane 3*), or BEL alone (10 μM) (*lane 4*) for 60 min at 37 °C. Cells were drenched in MeOH/H_2O (1:1, v/v), and lipids were extracted by the Bligh and Dyer procedure, resolved by TLC (chloroform/methanol/ammonium hydroxide (65:25:5, v/v)) and visualized by autoradiography. *PE*, phosphatidylethanolamine; *PC*, phosphatidylcholine; *PI*, phosphatidylinositol.

activity is fully active.

Accumulation of [^{3}H]arachidonic acid in the culture medium could reflect either increased release of [^{3}H]arachidonic acid from endogenous phospholipid storage depots or decreased reincorporation of released [^{3}H]arachidonic acid into cellular phospholipids. Concomitant incubation of cells with [^{3}H]arachidonic acid and either SNP or BEL (or the combination of SNP and BEL) did not substantially alter the rate of incorporation of [^{3}H]arachidonic acid into cellular phosphatidylethanolamine, phosphatidylcholine, or phosphatidylinositol in comparison with control (Fig. 3). Thus, nitric oxide-induced accumulation of [^{3}H]arachidonic acid in the medium was the result of accelerated phospholipid catabolism and not decreased reacylation of released [^{3}H]arachidonic acid into endogenous phospholipid storage depots.

The Coupling of Nitric oxide-induced Mobilization of [^{3}H]Arachidonic Acid with Cellular Glucose Metabolism—Nitric oxide-induced mobilization of [^{3}H]arachidonic acid required the presence of glucose in the media, and the magnitude of nitric oxide-induced [^{3}H]arachidonic acid release was highly correlated with the concentration of glucose present in the media (Fig. 4*A*). As anticipated from prior studies (7), the magnitude of nitric oxide-stimulated glucose oxidation was also highly correlated with the concentration of glucose in the media (data not shown). Furthermore, nitric oxide-induced [^{3}H]arachidonic acid mobilization required the metabolism of glucose since neither 2-deoxyglucose nor 3-*O*-methylglucose (nonmetabolizable glucose analogs) could facilitate the nitric oxide-induced mobilization of [^{3}H]arachidonic acid (data not shown). Importantly, preincubation of cells with from 1 to 25 mM glucose for 2 h followed by subsequent incubation with 25 mM glucose prior to nitric oxide stimulation resulted in similar amounts of [^{3}H]arachidonic acid release in comparison with cells kept at 25 mM glucose during the entire experimental interval (Fig. 4*B*). Thus, attenuation of nitric oxide-induced [^{3}H]arachidonic acid mobilization by low glucose concentration was entirely reversible after restoration of glucose to normal culture levels (*i.e.* 25 mM glucose). Cells treated with 1 mM glucose for 2 h remained attached to the cell culture surface, excluded trypan blue, grew normally for at least 48 h after the experimental interval, and did not release LDH into the medium. Furthermore, cells incubated with 1 or 3 mM glucose in the presence of 10% FBS grew at rates greater than 60% of the growth rate manifest at 25 mM glucose for extended intervals (*i.e.* 16 h) and incorporated [^{3}H]thymidine over the 3-h experimental interval at nearly identical rates (within 10%) at either 1, 3, 8, or 25 mM glucose.

DISCUSSION

The present study demonstrates that: 1) nitric oxide is a potent activator of arachidonic acid mobilization in mammalian cells; 2) nitric oxide-induced arachidonic acid mobilization is predominantly mediated by a calcium-independent phospholipase A_2; 3) the activation of calcium-independent phospholipase A_2 by nitric oxide results in the highly selective release of arachidonic acid; 4) the dose-response profile of SNP-mediated arachidonic acid mobilization is similar to the dose-response profile of other SNP-mediated responses in RAW 264.7 cells (27); and 5) the nitric oxide-induced release of arachidonic acid is dependent on glucose uptake and metabolism. Since arachidonic acid and its oxygenated eicosanoid metabolites have specific effects on distinct cell types, these results demonstrate a novel mechanism through which nitric oxide can mediate a complex repertoire of biologic effects.

Recent studies have recognized the preponderance of calcium-independent phospholipase A_2 activity in multiple tissues (*e.g.* heart, smooth muscle, brain, and kidney (13, 15, 28–30))

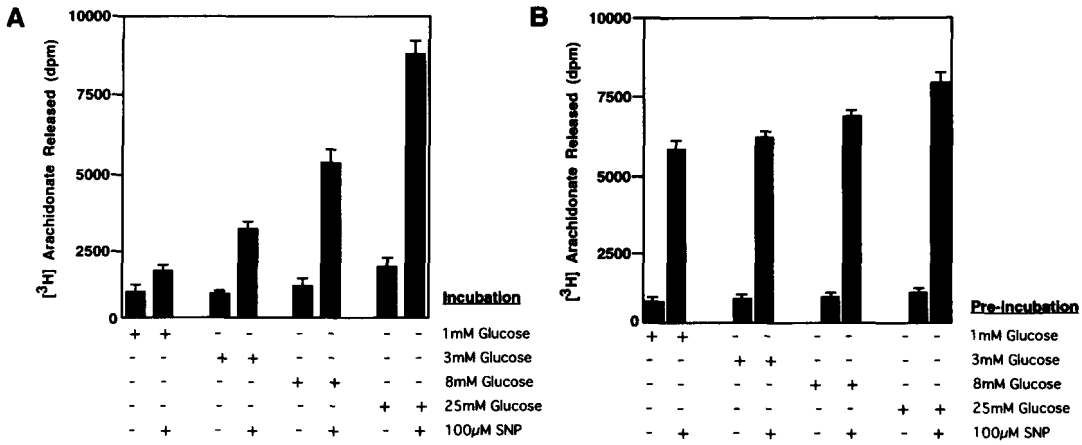


FIG. 4. **Nitric oxide-stimulated [^{3}H]arachidonic acid release from RAW 264.7 cell phospholipid storage depots is coupled to glucose utilization.** *A*, RAW 264.7 cells were prelabeled with [^{3}H]arachidonic acid for 16 h as described under "Materials and Methods." After exhaustive washing to remove unincorporated [^{3}H]arachidonic acid, cells were incubated with the indicated concentrations of glucose for 2 h prior to stimulation with sodium nitroprusside (100 μM) or incubation with buffer alone for 60 min (control). *B*, experiments performed in *B* were conducted similarly to those in *A*, except that the cells were first preincubated with the indicated concentrations of glucose for 2 h prior to a subsequent 2-h incubation with 25 mM glucose for re-equilibration before stimulation with sodium nitroprusside (100 μM) or incubation with buffer alone (control) for 60 min. [^{3}H]Arachidonate released into the media was quantified as described under "Materials and Methods." Results represent $x \pm$ S.E. of four determinations.

and have demonstrated that calcium-independent phospholipase A_2 is responsible for arachidonic acid mobilization after either ligand-receptor coupling or cellular stimulation in at least some cell types (26, 30). Calcium-independent phospholipase A_2 exists as a catalytic complex comprised of regulatory and catalytic constituents, whose activity is regulated by physiologic alterations in ATP concentration (16, 31). The prior demonstration that the polypeptides mediating ATP-induced activation of calcium-independent phospholipase A_2 catalytic activity were isoforms of phosphofructokinase suggested that ATP-induced allosteric regulation of phosphofructokinase could couple glycolytic metabolism to arachidonic acid mobilization (16). Based upon the known interaction of phosphofructokinase with calcium-independent phospholipase A_2 in purified systems, in conjunction with the inhibition of arachidonic acid mobilization by BEL in intact cells, the present results suggest that intracellular alterations in local ATP concentrations could represent a key regulatory element modulating phospholipid catabolism and the resultant generation of biologically active lipid second messengers (*e.g.* eicosanoids and lysolipids). Many cell types respond to a variety of different stimuli by concomitant increases in glycolytic flux and the release of lipid second messengers (*e.g.* Ref. 32). The translocation of glycolytic complexes that can catalyze substrate-based ATP synthesis to specific membrane compartments during cellular perturbation is well known (33). Accordingly, we propose that increased glycolytic flux and alteration of cytosolic ATP concentration in critical subcellular loci represent a master control switch, which couples glucose metabolism with the generation of lipid second messengers through the activation of Ca^{2+}-independent phospholipase A_2. Collectively, the present results provide a unifying hypothesis integrating alterations in glycolytic flux with the generation of lipid second messengers, thereby providing a fundamental mechanism through which cellular activation and intercellular communication can be mediated. The power inherent in the coordinated regulation of complex cellular metabolic networks (*e.g.* endothelium, smooth muscle, and macrophages) employing interwoven alterations in nitric oxide production, glycolytic flux, and eicosanoid generation through the activation of calcium-independent phospholipase A_2 is evident.

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