Calcium and the Phosphoinositide Cycle in WRK-1 Cells

EFFECTS OF A23187 ON METABOLISM OF SPECIFIC PHOSPHATIDYLINOSITOL POOLS*

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WRK-1 cells possess a labile, hormone-sensitive pool of phosphatidylinositol which appears to be separate from the stable, hormone-insensitive phosphatidylinositol. It is the sensitive pool which turns over in response to treatment with vasopressin. Addition of the calcium ionophore A23187, on the other hand, selectively stimulates precursor incorporation into the hormone-insensitive pool of phosphatidylinositol, while causing nonspecific breakdown of both pools. The polyphosphoinositides are similarly affected. Ionophore-stimulated breakdown appears to be predominately phospholipase C-mediated, since there is a concomitant increase in inositol phosphates. These inositol phosphates are localized predominantly in the extracellular medium. Permeabilization of the cells may explain the extracellular location of the breakdown products. When added together with the hormone, A23187, at concentrations greater than $5 \times 10^{-8}$ M, inhibits both hormone-induced synthesis and breakdown of phosphatidylinositol. Omission of calcium from the medium abolishes the effects of the ionophore.

We have previously reported that vasopressin induces changes in phosphoinositide metabolism in WRK-1 rat mammary tumor cells (1-4); furthermore, these changes appear to involve a discrete pool of cellular phosphatidylinositol (2, 3). Stimulation of synthesis and breakdown by hormone involves a pool of lipid which accounts for approximately 17% of the total cellular phosphatidylinositol. A similar compartmentalization of the polyphosphoinositides is also seen (4).

Several investigators have reported effects of calcium ionophores on phosphoinositide metabolism, including increased de novo phosphatidylinositol synthesis (5-8) as well as increased phospholipase C and/or phospholipase A$_2$-mediated breakdown of the phosphoinositides (5, 6, 9-17), as well as inhibition of hormone-stimulated phosphoinositide turnover (18). We report here that the calcium ionophore A23187 can stimulate both synthesis and breakdown of the phosphoinositides in WRK-1 cells; however, these effects appear to be quite different from those of vasopressin. In addition, in the presence of high concentrations of ionophore, the effects of vasopressin are attenuated.

MATERIALS AND METHODS

Tissue culture media, sera, trypsin, and Dulbecco's phosphate-buffered saline, without Ca$^{2+}$ and Mg$^{2+}$ were supplied by Gibco.

* This investigation was supported by Grant PCM-8029437 from the National Science Foundation. The costs of publication of this article were deferred in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

Phosphate-free medium was from Flow Laboratories. Vasopressin was from Behring Diagnostics. $^{32}$P$_2$ (carrier free) was purchased from ICN, and $[3\text{H}]$myo-inositol (15 Ci/mmol) was from American Radiolabeled Chemicals. $[3\text{H}]$Choline and $[3\text{H}]$thymidine were from New England Nuclear. Phosopholipid standards and A23187 were from Sigma. Thin layer plates (IB2-F) were from J. T. Baker Chemical Co., and organic solvents were purchased from Fisher. Aquasol II was from New England Nuclear.

Cell Culture—WRK-1 cells were established from a dimethylbenz[a]anthracene-induced rat mammary tumor as previously described (19, 20). Growth characteristics and responses to neurohypophyseal hormones have also been described (21). In brief, cells were grown in monolayer cultures in a minimal essential medium containing Earle's salts and supplemented with penicillin (100 units/ml), streptomycin (100 $\mu$g/ml), fungizone (0.25 $\mu$g/ml), calf serum (5%), and rat serum (2%). For experiments, cells were harvested with a solution of trypsin (0.05%) and EDTA (0.02%) in 0.9% NaCl and replicated into 22-mm plastic dishes. Details for each experiment appear in the figure legends. Vasopressin was routinely used at a concentration of $10^{-7}$ M. A23187 was dissolved in dimethyl sulfoxide, and equal amounts of dimethyl sulfoxide were added to control cultures.

Measurement of Precursor Incorporation into Lipids—Following incubation of cells with $^{32}$P$_2$, $[3\text{H}]$inositol, radioactive lipids were extracted and quantitated as previously described (2). The specific activity of $^{32}$P$_2$ was determined by quantitating the ratio of $^{32}$P to $^3$H in cells labeled acutely with $^{32}$P and chronically with $[3\text{H}]$inositol.

Measurement of $[3\text{H}]$inositol Phosphates—Cells were incubated for 3-5 days with $10 \mu$Ci/ml radioactive inositol. Cells were then harvested and suspended in glass test tubes in a final volume of 1 ml of minimal essential medium containing 10 mM lithium chloride. Following additions, cells were incubated at 37°C for 25 min. Cells were then collected by centrifugation, and the water-soluble products extracted and separated as previously described (22).

RESULTS

Effect of A23187 on Precursor Incorporation into Phosphatidylinositol—Fig. 1 illustrates that A23187, at concentrations ranging from $5 \times 10^{-8}$ M to $2 \times 10^{-5}$ M, stimulates incorporation of both $[3\text{H}]$inositol and $^{32}$P$_2$ into phosphatidylinositol. At the higher concentrations, the stimulation of $[3\text{H}]$inositol incorporation appears to diminish; however, when expressed as specific activity as shown for the $^{32}$P$_2$ incorporation data, the stimulation remains constant. It is probably the concurrent breakdown of phosphatidylinositol, as demonstrated below in Fig. 3, which accounts for the apparent decrease in incorporation.

To examine whether or not the effect of A23187 on incorporation of $^{32}$P$_2$ into phosphatidylinositol was related to that induced by vasopressin, that is, whether or not the increase represented changes in the hormone-sensitive pool, the following experiment was done: cells were preincubated with $^{32}$P$_2$ in the presence of A23187, and the ability of vasopressin to induce breakdown of this $^{32}$P$_2$phosphatidylinositol was measured. Fig. 2 illustrates the results. The increase in incorporation effected by A23187 does not involve the hormone-sensitive pool, since subsequent treatment with vasopressin does not
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Fig. 1. Effect of A23187 on incorporation of radioactive precursors into phosphatidylinositol (PI). Cells were plated in 22-mm plastic dishes and incubated for 2 h with $^{32}$P, or $^3$H]inositol in the presence of varying concentrations of A23187. Lipid extraction and quantitation was as described under "Materials and Methods." Results shown for $^3$H]inositol incorporation represents the total radioactivity per dish. In the case of $^{32}$P, incorporation, cells were chronically incubated with $^3$H]inositol and pulsed with 2 h with $^{32}$P. The value shown in the amount of $^{32}$P incorporated divided by the amount of $^3$H]inositol incorporated, i.e., a specific activity. Values represent the means of triplicate determinations ± 1 S.D.

Fig. 2. Effect of vasopressin (VP) on breakdown of A23187-induced phosphatidylinositol. Cells were preincubated for 2 h with $^{32}$P, in phosphate-free medium in the presence of either A23187 (2 × 10$^{-7}$ M) or vasopressin (1 × 10$^{-7}$ M). Following the preincubation, cells were washed with phosphate-buffered saline, and the incubation continued for an additional 4 h in phosphate-containing medium with or without vasopressin. Lipids were extracted and quantitated as described under "Materials and Methods." Values represent the means of triplicate determinations ± 1 S.D. For cells prelabeled in the absence of hormone or ionophore and subsequently treated with vasopressin, values were 686 ± 165 (C) and 889 ± 232 (VP).

not result in breakdown. In fact, one sees a slight increase in labeling.

Effect of A23187 on Phosphoinositide Breakdown—Fig. 3 illustrates that A23187 stimulates breakdown of prelabeled phosphoinositides in a dose-dependent manner. Approximately 25% of the radioactivity disappears from the lipid fraction. The loss of radioactivity from the lipid fraction is accompanied by a concomitant increase in water-soluble radioactivity present predominantly in the extracellular medium. At the lower concentrations of ionophore there is also a slight increase in the cellular radioactivity. A detailed characterization of the water-soluble inositol compounds is shown in Table I. Although all the water-soluble compounds increase, the greatest increase appears to be in the inositol diphasphate fraction. In a similar experiment, breakdown of the polyphosphoinositides was specifically monitored, as well as production of lysophosphatidylinositol. Results are shown in Fig. 4. Breakdown of phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-bisphosphate is even greater than that of PI and there is no appreciable accumulation of lysophosphatidylinositol.

The abbreviation used is: PI, phosphatidylinositol.

To determine whether breakdown was occurring nonspecifically, or involved a specific pool of phosphatidylinositol, cells were prelabeled with $^{32}$P, under conditions which specifically labeled the hormone-sensitive or -insensitive pool; that is, cells were incubated for 2 h with vasopressin to label the sensitive pool and without vasopressin to label the insensitive pool. Fig. 5 illustrates that both pools are subject to ionophore-induced breakdown.

Effect of A23187 on Hormone-induced Synthesis and Breakdown of Phosphatidylinositol—When vasopressin and ionophore are added simultaneously, the effects of the two on synthesis appear to be additive at low concentrations of ionophore (less than 5 × 10$^{-4}$ M), whereas at higher concentrations, the effect of the hormone to increase $^{32}$P incorporation into phosphatidylinositol is inhibited (Fig. 6). When breakdown products of hormone-induced phosphoinositide turnover are examined, one observes that high concentrations of A23187 inhibit the accumulation of cellular inositol phosphates induced by vasopressin (Table II). A full dose-response curve for the inhibitory effect of A23187 on vasopressin-induced inositol phosphate accumulation is shown in Fig. 7. The inhibitory effect of the ionophore is not due to increased secretion of these metabolites, since there is no concomitant increase in extracellular radioactivity above that seen with ionophore alone (data not shown).

Dependence of the Ionophore's Effect on Extracellular Calcium—Table III illustrates that the effect of A23187 is secondary to alterations in calcium concentrations. In the absence of extracellular calcium, there is no ionophore-induced accumulation of extracellular inositol compounds, and there is no inhibition of vasopressin-stimulated inositol phosphate production.

Effect of A23187 on Cell Permeability and Viability—The presence of polar inositol compounds in the medium in the presence of A23187 raises the question of whether A23187 is stimulating secretion of these compounds or merely compromising the integrity of the cell membrane, allowing polar compounds to escape. Two types of experiments were performed to assess the effect of A23187 on the cells. The release of other polar compounds in the presence of A23187 was evaluated. Table IV illustrates that the level of both $^3$H]choline-labeled compounds as well as $^3$H]thymidine-labeled...
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TABLE I
Effect of A23187 on the accumulation of water-soluble inositol compounds in media and cells

Cells were prelabeled for 3 days with [3H]inositol (10 μCi/ml). Cells were then harvested and resuspended in serum-free medium containing 10 mM lithium chloride with or without A23187 for 25 min at 37 °C. Radioactivity was characterized as described under “Materials and Methods.” Results shown represent the total radioactivity per tube and are expressed as the means of triplicate determinations ± 1 S.D. Abbreviations: I, inositol; GPI, glycerophosphorylinositol; IP, inositol monophosphate; IP2, inositol diphosphate; IP3, inositol triphosphate.

| Medium | I (CPM × 10^-3) | GPI (CPM × 10^-3) | IP (CPM × 10^-3) | IP2 (CPM × 10^-3) | IP3 (CPM × 10^-3) |
|--------|-----------------|-------------------|------------------|------------------|------------------|
| No addition | 4,853 ± 919 | 2,568 ± 714 | 976 ± 62 | 610 ± 200 | 50 ± 21 |
| A23187 (5 × 10^-7 M) | 7,178 ± 2,132 | 6,303 ± 361 | 3,283 ± 373 | 3,734 ± 1,319 | 258 ± 17 |
| A23187 (2 × 10^-6 M) | 16,579 ± 1,806 | 9,762 ± 1,138 | 5,784 ± 303 | 7,083 ± 2,949 | 297 ± 62 |

Cells

| No addition | 7,677 ± 261 | 1,028 ± 29 | 3,082 ± 817 | 588 ± 175 | 187 ± 11 |
| A23187 (5 × 10^-7 M) | 5,966 ± 243 | 777 ± 20 | 6,542 ± 204 | 977 ± 200 | 257 ± 33 |
| A23187 (2 × 10^-6 M) | 5,076 ± 358 | 721 ± 34 | 2,964 ± 44 | 533 ± 50 | 156 ± 25 |

compounds is increased in the extracellular medium when the ionophore is present. In addition, Table V indicates that uptake of the cell-impermeable dye erythrosin B is enhanced as a result of ionophore treatment. In Experiment A, 44% of control cells have become permeabilized. However, this is a direct result of detaching cells from the dish. If the identical experiment is carried out with cells remaining attached to the dish (Experiment B), very few of the control cells become permeabilized.

FIG. 4. Effect of A23187 on breakdown of the polyphosphoinositides and accumulation of lysophosphatidylinositol. Methods were as described for Fig. 3. The concentration of A23187 was 2 × 10^-5 M. Values represent the means of triplicate determinations ± 1 S.D. LPI, lysophosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol 4,5-bisphosphate.

FIG. 5. Effect of A23187 on breakdown of [32P]phosphatidylinositol. Cells were incubated with 32Pi for 3 days with [3H]inositol (10 μCi/ml). Radioactivity ([32P]PI) was then monitored for 3 h at 37 °C. Turnover of the phosphoinositides mimics that induced by hormones and neurotransmitters; however, important differences have been noted, such as distribution of breakdown products: lysophosphatidylinositol and/or inositol diphosphate predominate, depending on the system under investigation.

FIG. 6. Effect of A23187 and vasopressin on the incorporation of [32P] into phosphatidylinositol. Cells were incubated with [32P]Pi for 2 h in serum-free phosphate-free medium containing varying concentrations of A23187 plus (○—○) or minus (■—■) vasopressin (10^-7 M). PI was extracted and quantitated as described under “Materials and Methods.” Values represent the means of triplicate determinations ± 1 S.D.

DISCUSSION
Numerous investigators have demonstrated various effects of calcium and calcium ionophores on phosphoinositide metabolism. Removal of calcium or addition of manganese to the extracellular medium has been shown to increase incorporation of [3H]inositol into phospholipids (12, 23). Addition of the calcium ionophore A23187 has also been demonstrated to stimulate precursor incorporation into phosphoinositides, probably through the de novo pathway (5–8). At the same time, inhibition of phosphatidylinerine synthesis is observed (24). The ionophore is also capable of inducing breakdown of the phosphoinositides at higher concentrations (generally >10^-6 M). Analysis of the breakdown products has suggested a phospholipase C and/or a phospholipase A2-mediated mechanism (5, 6, 9–17). In many ways, the ionophore-induced turnover of the phosphoinositides mimics that induced by hormones and neurotransmitters; however, important differences have been noted, such as distribution of breakdown products: lysophosphatidylinositol and/or inositol diphosphate predominate, depending on the system under investigation.

We have previously demonstrated the existence of two discrete pools of phosphoinositides in WRK-1 cells: one is hormone-sensitive with respect to breakdown, whereas the other is hormone-insensitive (2–4). In the present study we have examined the effects of A23187 on the metabolism of the individual phosphoinositide pools. All concentrations of A23187 tested stimulated an increase in incorporation of labeled precursor in phosphoinositides. At the higher concentrations (>5 × 10^-6 M) incorporation sometimes appears to
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**TABLE II**

*Effects of A23187 and vasopressin on the accumulation of cellular water-soluble inositol products*

| Addition                  | I (cpm)       | IP (cpm)      | IP (cpm)      | IP (cpm)      |
|---------------------------|---------------|---------------|---------------|---------------|
| None                      | 5,047 ± 775   | 3,115 ± 50    | 2,785 ± 100   | 700 ± 57      |
| Vasopressin (1 × 10⁻⁷ M)  | 5,272 ± 400   | 11,402 ± 675  | 6,380 ± 315   | 1,225 ± 102   |
| A23187 (1 × 10⁻⁸ M)      | 4,025 ± 525   | 4,195 ± 22    | 2,845 ± 450   | 575 ± 40      |
| A23187 (2 × 10⁻⁸ M)      | 3,772 ± 592   | 4,272 ± 632   | 1,965 ± 645   | 592 ± 135     |
| Vasopressin + A23187 (1 × 10⁻⁸ M) | 4,597 ± 172   | 12,257 ± 1,145 | 5,805 ± 690  | 1,115 ± 250   |
| Vasopressin + A23187 (2 × 10⁻⁸ M) | 3,577 ± 202   | 4,722 ± 235   | 1,630 ± 70    | 522 ± 25      |

**TABLE III**

*Dependence of the effect of A23187 on extracellular calcium*

| Addition                  | Radioactivity (cpm) |
|---------------------------|---------------------|
|                           | Medium              | Cellular          |
| None                      | 12,821 ± 534        | 21,390 ± 1,272    |
| Calcium (1.5 mM)          | 11,290 ± 555        | 26,540 ± 402      |
| A23187 (2 × 10⁻⁸ M) + calcium (1.5 mM) | 18,766 ± 574 | 36,866 ± 1,650 |
| A23187 (2 × 10⁻⁸ M) + calcium (1.5 mM) | 70,188 ± 1,657 | 5,841 ± 1,658 |

**TABLE IV**

*Effect of A23187 on the presence of [³H]choline- and [³H]thymidine-labeled metabolites in the extracellular medium*

| Addition | [³H]Choline metabolites (cpm/dish) | [³H]Thymidine metabolites (cpm/dish) |
|----------|-----------------------------------|-------------------------------------|
| None     | 94,302 ± 3,481                    | 27,466 ± 937                        |
| A23187   | 2 × 10⁻⁷ M 34,275 ± 1,362          | 40,153 ± 1,418                      |
|          | 2 × 10⁻⁸ M 141,989 ± 8,467         | 164,722 ± 10,229                    |
|          | 5 × 10⁻⁹ M 82,109 ± 4,559          | 182,109 ± 4,559                     |
|          | 1 × 10⁻⁹ M 675 ± 690               | 315 ± 1,225                         |
|          | 2 × 10⁻⁹ M 98 ± 57                 | 70 ± 522                            |

**TABLE V**

*Effect of A23187 on cell permeability*

For Experiment A, cells were harvested with phosphate-buffered saline/EDTA and resuspended in serum-free medium containing 35 μg/ml inositol and 10 mM lithium chloride with or without A23187 for 25 min at 37 °C. Then erythrosin B (0.0625%) was added to each tube, and the number of positive cells counted. Values shown are the average of two separate determinations. For Experiment B, cells remained in the dish. Similar results were seen in several experiments.

| Experiment | % positive cells |
|------------|------------------|
| A          | No addition      |
|            | 44               |
| A23187     | 2 × 10⁻⁷ M 64    |
|            | 5 × 10⁻⁷ M 54    |
|            | 2 × 10⁻⁸ M 87    |
|            | 2 × 10⁻⁹ M 98    |
| B          | No addition      |
|            | 7                |
| A23187     | 1 × 10⁻⁸ M 68    |
|            | 1 × 10⁻⁹ M 99    |

**FIG. 7. Effect of A23187 on vasopressin-induced accumulation of cellular inositol phosphates (IP).** Cells were grown for 3 days with [³H]inositol (10 μCi/ml). They were then harvested and resuspended in serum-free medium containing 10 mM lithium chloride with the additions indicated for 25 min at 37 °C. Radioactivity was characterized as described under "Materials and Methods." Results are the means of triplicate determinations ± 1 S.D. Abbreviations are as described for Table I.

**TABLE VI**

*Dependence of the effect of A23187 on extracellular calcium*

| Addition                  | Radioactivity (cpm) |
|---------------------------|---------------------|
|                           | Medium              | Cellular          |
| None                      | 12,821 ± 534        | 21,390 ± 1,272    |
| Calcium (1.5 mM)          | 11,290 ± 555        | 26,540 ± 402      |
| A23187 (2 × 10⁻⁸ M) + calcium (1.5 mM) | 18,766 ± 574 | 36,866 ± 1,650 |
| A23187 (2 × 10⁻⁸ M) + calcium (1.5 mM) | 70,188 ± 1,657 | 5,841 ± 1,658 |

**increase; however, when expressed in terms of specific activity, there is no decrease in incorporation, but rather an increase in the breakdown which is occurring simultaneously. Farese et al. (16) have reported similar findings. The stimulated incorporation does not involve the hormone-sensitive pool, since subsequent treatment with vasopressin does not result in loss of radioactivity incorporated in the presence of the ionophore.**

With respect to breakdown, no specificity is observed. A23187 induces a loss of radioactivity from both sensitive and insensitive pools of lipid. The mechanism appears to involve the action of phospholipase C, since the inositol phosphates comprise a large portion of the breakdown products. The increase in the radioactivity in the glycerophosphorylinositol fraction suggests that an increase in phospholipase A activity may also be involved. There is no significant increase in lysophosphatidylinositol. The water-soluble breakdown products resulting from A23187 treatment accumulate predominantly in the extracellular medium. This appears to be due to an increased permeabilization of the cells, as evidenced by 1) the presence of other polar compounds in the medium as well as 2) an increased uptake of erythrosin B by ionophore-treated cells. Such permeabilization has also been reported for 3T3...
cells (25). It should be noted that, in other studies of ionophore-induced turnover of phosphoinositides, the medium was generally not checked for the presence of metabolites.

In addition to stimulating breakdown, higher concentrations of A23187 also block vasopressin-induced turnover. Both the increase in $^{32}$P incorporation into phosphatidylinositol as well as the accumulation of inositol phosphates observed in the presence of vasopressin are inhibited at high concentrations of ionophore. Similar findings were reported for the effects of vasopressin on hepatocytes (18). At lower ionophore concentrations, the effects of the two compounds appear to be additive.

The results presented here further support the notion of the compartmentalization of the phosphoinositides within the cell. This concept was first suggested by Fain and Berridge (26) and has more recently been elaborated by this (2-4) and other laboratories (27–30). The ability of substances such as calcium ionophores and manganese to stimulate differentially proton incorporation into a hormone-insensitive pool of phosphoinositides leads to speculation regarding the origin and mechanics of synthesis of the sensitive pool. The data in WRK-1 cells indicate that there is no mixing of the ractoactivity incorporated into individual pools (3). Additional studies are underway to determine the location and mode of synthesis of the hormone-sensitive phosphoinositide pools.

In addition, it is evident from this and numerous other studies that data derived from experiments in which calcium ionophores are used to mimic physiological agonists must be interpreted with extreme caution, since there are apparently many important differences between ionophore-induced and hormone-induced metabolic changes.

Acknowledgment—I would like to thank Catherine Malichio for her excellent secretarial assistance.

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