Carbachol Induces a Rapid and Sustained Hydrolysis of Polyphosphoinositide in Bovine Tracheal Smooth Muscle Measurements of the Mass of Polyphosphoinositides, 1,2-Diacylglycerol, and Phosphatidic Acid

Yoh Takuwa, Noriko Takuwa, and Howard Rasmussen
From the Departments of Cell Biology, Physiology and Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

The effects of carbachol on polyphosphoinositides and 1,2-diacylglycerol metabolism were investigated in bovine tracheal smooth muscle by measuring both lipid mass and the turnover of [3H]inositol-labeled phosphoinositides. Carbachol induces a rapid reduction in the mass of phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-monophosphate and a rapid increase in the mass of 1,2-diacylglycerol and phosphatidic acid. These changes in lipid mass are sustained for at least 60 min. The level of phosphatidylinositol shows a delayed and progressive decrease during a 60-min period of carbachol stimulation. The addition of atropine reverses these responses completely. Carbachol stimulates a rapid loss in [3H]inositol radioactivity from phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-monophosphate associated with production of [3H]inositol trisphosphate. The carbachol-induced change in the mass of phosphoinositides and phosphatidic acid is not affected by removal of extracellular Ca2+ and does not appear to be secondary to an increase in intracellular Ca2+. These results indicate that carbachol causes phospholipase C-mediated polyphosphoinositide breakdown, resulting in the production of inositol 1,4,5-trisphosphate and a sustained increase in the actual content of 1,2-diacylglycerol. These results strongly suggest that carbachol-induced contraction is mediated by the hydrolysis of polyphosphoinositides with the resulting generation of two messengers: inositol 1,4,5-trisphosphate and 1,2-diacylglycerol.

The intracellular events involved in the regulation of smooth muscle contraction remain a matter of controversy. It has been proposed that an increase in intracellular free Ca2+ concentration activates myosin light chain kinase, a Ca2+-calmodulin-dependent enzyme, leading to the phosphorylation of myosin light chain, and that this molecular event induces contraction through an increased interaction of myosin with actin (1-7). This model assumes that during the sustained phase of contraction, the intracellular free Ca2+ concentration and the amount of phosphorylated myosin light chain remain elevated. However, recent works done by Morgan and Jackson (8, 9) show that, when aequorin is employed as an intracellular calcium indicator, addition of either phenylephrine or angiotensin to vascular smooth muscle leads to a transient, rather than sustained, increase in intracellular free Ca2+, but a sustained contractile response. Likewise, Silver and Stull (10), and Aksoy et al. (11) have shown that the amount of phosphorylated myosin light chain rapidly rises after agonist addition to either tracheal or vascular smooth muscles and then gradually returns toward the base-line value during the sustained phase of contraction. These studies indicate that the mechanisms by which Ca2+ acts may be more complex than previously thought. They have led to the postulate that a second calcium-dependent mechanism operates during the sustained phase of smooth muscle contraction (10-12, 58).

In recent years it has been shown that the interaction of Ca2+-mobilizing hormones with their receptors activates a specific phospholipase C which catalyzes the hydrolysis of PtdIns-4,5-P2 in a variety of tissues or cells (13-17). This results in production of Ins-P2 and 1,2-diacylglycerol. It is currently believed that Ins-P2 causes the release of Ca2+ from an intracellular pool (presumably endoplasmic reticulum) and produces the initial intracellular Ca2+ transient (14, 18), thereby activating Ca2+-calmodulin-dependent enzymes. On the other hand, an increase in the 1,2-diacylglycerol content of the plasma membrane is thought to activate the Ca2+-activated, phospholipid-dependent protein kinase (C-kinase) (17, 19). Based on results obtained from the use of agents which bypass receptor-mediated events and directly activate Ca2+-calmodulin-dependent kinases and the C-kinase in smooth muscle, we have proposed that in smooth muscle contraction as well as secretory responses in many tissues, the calmodulin branch of the Ca2+ messenger system is transiently activated by a transient rise in cytosolic free Ca2+ concentration and is largely responsible for initiating cellular response; the C-kinase branch which is activated by both the sustained increase in plasma membrane Ca2+ influx rate and the increase in the 1,2-diacylglycerol content of the plasma membrane is responsible for sustaining the response (20, 21, 23-27). In this model a sustained increase in intracellular free Ca2+ is not a prerequisite for the sustained phase of smooth muscle contraction.

The present study was undertaken to determine whether in bovine tracheal smooth muscle an agonist, carbachol, causes polyphosphoinositide breakdown and generates the two messengers: inositol 1,4,5-trisphosphate and 1,2-diacylglycerol.
sengers, Ins-P₃ and 1,2-diacylglycerol. Our results show that
carbachol stimulation causes a rapid decrease in the mass of
the phosphoinositides and a small but significant increase in
the mass of 1,2-diacylglycerol and phosphatidic acid. Further-
more, these changes in the lipid mass are sustained during a
1-h period of hormone action.

MATERIALS AND METHODS

RESULTS

Effect of Carbachol on the Turnover of [³H]Inositol-labeled
Phosphoinositides—As shown in Fig. 1, the addition of car-
bachol to tracheal muscle strips prelabeled with [³H]inositol
causes a rapid decrease in radioactivity from both the PtdIns-
4,5-P₂ and PtdIns-4-P pools. A loss of 28% of [³H] radioactivity
from PtdIns-4,5-P₂ is detected at 30 s and is maximal at 2
min (a loss of 34%). The change in radioactivity of PtdIns-4-
P follows a similar time course to that of PtdIns-4,5-P₂. The
radioactivity in both the PtdIns-4,5-P₂ and PtdIns-4-P pool
remains reduced for the initial 10 min. In contrast, the radio-
activity of phosphatidylinositol shows no significant change
during this period.

Effect of Carbachol on Inositol Phosphate Production—Car-
bachol stimulates the production of Ins-P, Ins-P₃, and Ins-P₄
in [³H]inositol-prelabeled muscle strips (Fig. 2). Both Ins-P₂
and Ins-P₃ increase rapidly following carbachol addition and
reach peaks at 1 min (870% in Ins-P₂ and 800% in Ins-P₃ of
each control value). Then the values slightly fall but still
remain 6- to 7-fold higher than the control values during 10
min. Ins-P rises less rapidly and reaches a peak (190% of
control value) at 5 min and stays at that level. The rapid
decrease in radioactivity of PtdIns-4,5-P₂ and the concomi-
tant accumulation of Ins-P₃ is consistent with carbachol-
dependent activation of the phospholipase C-catalyzed cleav-
age of PtdIns-4,5-P₂.

Effect of Carbachol on the Absolute Mass of Phosphoinosi-
tides and Phosphatidic Acid—The time course of the changes
in the mass of phosphoinositides and phosphatidic acid is
shown in Fig. 3. The resting content of PtdIns-4,5-P₂ is about
0.4% of total phospholipid on a molar basis and also one-
tenth of the mass of phosphatidylinositol (about 4%). Upon
carbachol stimulation, the content of PtdIns-4,5-P₂ rapidly
decreases and reaches a nadir (50% of the resting value) at 1
min. Then the content of PtdIns-4,5-P₂ slightly recovers, but
still remains appreciably lower than the resting value for at
least 60 min. During a similar period of time, muscle strips
not treated with carbachol show no significant change in the
PtdIns-4,5-P₂ content. The content of PtdIns-4-P also de-
clines rapidly from the resting level of 0.4% and remains
during the carbachol stimulation of 60 min. Again, muscles
not stimulated with carbachol show no significant change
in the content of this phosphoinositide. These changes
in PtdIns-4,5-P₂ and PtdIns-4-P during the first 5 min are
apparently similar to the changes in radioactivity of poly-
phosphoinositide in [³H]inositol-labeled muscles (Fig. 1), but
the change in the mass of PtdIns-4,5-P₂ is quantitatively
larger than that in the labeling experiments (50% versus 34%
as a maximal decrease). The mass of phosphatidylinositol
shows a delayed decrease. The content of phosphatidylinositol
shows no significant change at 30 s and then declines slowly
and progressively for 60 min. The content of phosphatidyli-
nositol at 60 min is reduced to 44% of the resting content. In
contrast, the mass of phosphatidic acid rises rapidly and
progressively from a resting value of about 0.2% of total
phospholipid mass to reach a plateau of about 0.9% of this
mass at 30 min.

As shown in Fig. 4, the changes in the mass of PtdIns-4,5-
P₂ and phosphatidic acid are dose-dependent and become

---

2 Portions of this paper (including "Materials and Methods," and Figs. 1 and 6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-1555, cite the authors, and include a check or money order for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
greater with increasing doses of carbachol. The \( \text{ED}_{50} \) for the carbachol-induced changes in PtdIns-4,5-P\(_2\) and phosphatidic acid is 5 and 3 \( \mu \text{M} \), respectively. The values are 15- to 30-fold higher than the \( \text{ED}_{50} \) for the carbachol-induced tension development (28). One possible interpretation of these data is the existence of large numbers of spare receptors. A second is that there is an amplification step between messenger generation and physiological responses.

The changes in the mass of polyphosphoinositides and phosphatidic acid are reversed by atropine, an antagonist of muscarinic-type receptor (Fig. 5). The content of PtdIns-4,5-P\(_2\) and phosphatidic acid mass is slower and takes 25 min before complete recovery is seen.

To see if these changes in the mass of phospholipids are a specific response to the agonist carbachol, the effect of 50 mM K\(^+\) in the extracellular fluid on polyphosphoinositide metabolism was examined. Eighty mM K\(^+\), a concentration which causes a maximal contraction, did not elicit any changes in the mass of polyphosphoinositides or phosphatidic acid in tracheal muscle (data not shown). These results also indicate that the phospholipase C activation resulting from carbachol stimulation is not a consequence of an increase in an intracellular Ca\(^{2+}\) concentration.

To determine the dependency of carbachol-mediated breakdown of polyphosphoinositides on extracellular calcium, the effect of carbachol on polyphosphoinositide metabolism was compared in the presence or absence of extracellular Ca\(^{2+}\). The carbachol-stimulated breakdown of polyphosphoinositides and the production of phosphatidic acid were similar in the presence or absence of extracellular Ca\(^{2+}\) (data not shown). Thus, carbachol-stimulated breakdown of polyphosphoinositides is not affected by removal of extracellular Ca\(^{2+}\).

**Effect of Carbachol on 1,2-Diacylglycerol Production**—The results shown in Figs. 1–3 clearly indicate that carbachol stimulates phospholipase C-mediated hydrolysis of polyphosphoinositides. Therefore, the change in the level of 1,2-diacylglycerol, the other product of phospholipase C-mediated hydrolysis of polyphosphoinositides, was examined. Initially, the quenching method of adding ice-cold chloroform/methanol (1:2, v/v) to muscle strips was employed. In experiments employing this method, no significant increase in the 1,2-diacylglycerol level in terms of changes in the absolute amount, or of radioactivity in \([\text{H}]\)glycerol- or \([\text{H}]\)arachidonic acid-labeled 1,2-diacylglycerol were found (data not shown). In experiments in which \([\text{H}]\)arachidonic acid-labeling was employed, a decrease in PtdIns-4,5-P\(_2\) and an increase in phosphatidic acid were found (Fig. 6). However, when another method for terminating the reaction, freeze-clamping the muscle, was employed, changes in 1,2-diacylglycerol content were found. As shown in Fig. 7, the mass of 1,2-diacylglycerol in freeze-clamped muscle rises rapidly and reaches a peak at 2 min after the addition of 2 \( \mu \text{M} \) carbachol. It then falls slightly to remain for 60 min at a value significantly above the control value. The plateau value is about 130% of the resting value. An experiment in which a higher concentration of carbachol (0.1 mM) was employed gives a similar result (data not shown). Thus, these data indicate that the choice...
of quenching method is critically important for the measurement of 1,2-diacylglycerol content of this tissue.

**DISCUSSION**

The present results demonstrate that carbachol, a muscarinic agonist, causes a rapid reduction in the mass of PtdIns-4,5-P₂ in bovine tracheal smooth muscle (Fig. 3). Because the change is associated with concomitant increases in [³H]Ins-4,5-P₂ in the contents of 1,2-diacylglycerol (Fig. 7) and phosphatidic acid (Figs. 3 and 6), these data indicate that a carbachol-induced decrease in the mass of PtdIns-4,5-P₂ is caused by a stimulation of the hydrolysis of PtdIns-4,5-P₂ catalyzed by phospholipase C. The present work is the first to report the measurement of polyphosphoinositide breakdown based upon measurement of lipid mass as well as radioisotopic labeling in a smooth muscle experiment.

Several investigators have shown that various agonists cause polyphosphoinositide breakdown in smooth muscle tissues employing radioactive tracer methods (13, 42-47, 60). These previous studies demonstrated agonist-stimulated changes in radioactivity of phosphoinositides and/or production of [³H]inositol phosphates in smooth muscle prelabeled with ³²P or [³H]inositol. When a freshly isolated tissue is employed for labeling experiments, it is unlikely that a true isotopic equilibrium is reached because of short periods of labeling. Therefore, it is not possible to accurately estimate a change in the lipid mass based upon experiments of this type. In addition, it is not easy to know from such labeling experiments whether or not polyphosphoinositide breakdown is sustained in response to agonists. The direct measurement of lipid mass gives one a means of overcoming these difficulties.

Recently a number of hormones and neurotransmitters have been shown to cause polyphosphoinositide breakdown in their target tissues. The resulting products, Ins-P₃, and 1,2-diacylglycerol, have been shown to function as intracellular messengers in the action of the particular agonist (14-17). One of the unanswered questions concerning polyphosphoinositide breakdown is whether polyphosphoinositide breakdown is sustained during the sustained phase of the hormonal response. The present study shows that the mass of PtdIns-4,5-P₂ and PtdIns-4-P remain lower than their resting values even after 60 min of continuous exposure to carbachol, and that the level of phosphatidylinositol continues to fall progressively during this period. In contrast, the mass of phosphatidic acid and 1,2-diacylglycerol remain higher than their resting values. These results clearly indicate that polyphosphoinositide breakdown is sustained and continues to generate messengers during the sustained response to carbachol. These results give support to the notion that polyphosphoinositide breakdown plays a messenger role during the tonic as well as the acute phase of carbachol-induced contraction in bovine tracheal smooth muscle. It is our postulate that the major intracellular pathway involved in the sustained phase of carbachol-induced contraction is the C-kinase pathway and that the C-kinase is maintained in its Ca²⁺-sensitive state during the sustained phase (20, 21, 23, 27, 28). Since 1,2-diacylglycerol in the plasma membrane is believed to be a physiological factor which activates the C-kinase in situ (17, 19), our data showing a sustained increase in the 1,2-diacylglycerol content of muscle strips strongly suggest that the C-kinase is actually maintained in its Ca²⁺-sensitive state during the sustained phase of carbachol-induced contraction.

To our knowledge, the only previous report in which long term effects of agonists on the mass change in phosphoinositides are reported is from the work of Farese et al. (48) in rat adrenal subcapsular cells. These workers showed that angiotensin II, a typical agonist causing phosphoinositide breakdown in this tissue (39, 61), induces increases in the mass of PtdIns-4,5-P₃, PtdIns-4-P, phosphatidylinositol, and phosphatidic acid at 60 min. Since agonists causing phosphoinositide breakdown stimulate both breakdown and resynthesis of phosphoinositides, it is possible that if the resynthesis is greater than the breakdown of phosphoinositides, one might see net increases in the mass of these lipids. In fact, a rapid increase in the mass of PtdIns-4,5-P₂ after the initial decrease has been reported to occur at an early time point (30 s) after thrombin addition in human platelets (49, 50). Thus, a balance between breakdown and resynthesis of phosphoinositides following agonist stimulation may determine the net change in the mass of phosphoinositides in a given tissue.

The decrease in the mass of PtdIns-4,5-P₃, seen in carbachol-stimulated tracheal muscle, is associated with an equally rapid loss of mass in the PtdIns-4-P pool (Fig. 3). The time course of Ins-P₃ production is also similarly as rapid as that of Ins-P₃ (Fig. 2). However, the reversal of PtdIns-4-P level toward the resting value after atropine addition takes a longer time than that of PtdIns-4,5-P₂ (Fig. 5). Therefore, a loss of the mass of PtdIns-4-P after carbachol addition may not be explained solely by phospholipase C-mediated breakdown of PtdIns-4-P. Accelerated conversion of PtdIns-4-P to PtdIns-4,5-P₂ is likely to contribute to a reduction in the mass of PtdIns-4-P. Similarly, the time course of a delayed decrease in the mass of phosphatidylinositol and the relatively small increase in InPs compared to the much larger decrease in the mass of phosphatidylinositol (Figs. 2 and 3) suggest that the bulk of the decrease in the mass of phosphatidylinositol occurs via phosphorylation to PtdIns-4-P and PtdIns-4,5-P₂.

In the present study, some differences in the data on phosphoinositide breakdown are noted between the [³H]inositol-labeling experiment (Fig. 1) and the measurement of the lipid mass (Fig. 3). In the labeling experiment the radioactivity in phosphatidylinositol shows no significant change for at least 10 min (Fig. 1), while the mass of phosphatidylinositol shows a decrease of 11% at 1 min and 27% at 5 min (Fig. 3). Moreover, the extent of decrease in PtdIns-4,5-P₂ after carbachol addition is larger in absolute amount than that estimated by radioactivity measurements. Because muscles were labeled for 3 h with [³H]inositol and presumably true isotopic equilibrium was not reached in the present experiment (51, 52), stimulated incorporation of [³H]inositol into phosphoinositides associated with enhanced resynthesis of these lipids may account for the smaller relative changes observed in the labeling experiment. It is also possible that [³H]inositol is incorporated into only a particular pool of each phosphoinositide (53) and changes in radioactivity do not represent overall changes in the mass of these lipids.

The changes in the mass of phosphoinositides and phosphatidic acid are carbachol-specific events because the application of 80 mM K⁺ leads to no significant change in the mass of these lipids. These data also indicate that simply raising intracellular Ca²⁺ concentration with high extracellular K⁺ is not sufficient to activate the phospholipase C-mediated breakdown of the phosphoinositides. Furthermore, carbachol-mediated breakdown of phosphoinositides is not dependent on extracellular Ca²⁺. These data are similar to previous reports in a variety of cells or tissues (14, 15, 52).

In the present study, the quenching method of adding ice-cold chloroform/methanol (1:2, v/v) to the incubation medium was initially employed for the measurement of 1,2-diacylglycerol mass. However, the experiments using this quenching method were unsuccessful in detecting a significant increase in 1,2-diacylglycerol content. Likewise, a significant change
in [³H]arachidonic acid- or [³H]glycerol-labeled 1,2-diacylglycerol was not observed using this approach. In contrast, in freeze-clamped muscle an increase in the mass of 1,2-diacylglycerol is seen (Fig. 7). The base-line value of the 1,2-diacylglycerol content in the freeze-clamped muscle is one-seventh of that determined using the quenching method. These results suggest that the use of such a method as freeze clamping, which allows biochemical reactions to be terminated immediately, is critically important for the determination of the 1,2-diacylglycerol content of solid tissues like tracheal smooth muscle. Otherwise a small change in 1,2-diacylglycerol mass might be masked by an artificial increase in the mass of this lipid derived from the degradation of phospholipids or triacylglycerol during the solvent-based quenching procedure.

The increase in the actual 1,2-diacylglycerol content appears to be small compared to the larger mass changes of phosphatidic acid and polyphosphoinositides (Fig. 3) (57). Using the present methods we could show a substantial increase (2-fold) in the mass of 1,2-diacylglycerol in Swiss 3T3 fibroblasts upon bombesin stimulation. Also, the densitometer, and Nancy Canetti and Ann Levine for their excellent editorial and secretarial assistance.

C-kinase activity is an especially interesting avenue to be explored. Because arachidonic acid is known to be mobilized from phosphatidyl-

REFERENCES

1. Bolton, T. B. (1979) Physiol. Rev. 59, 606–718
2. Adelstein, R. S., and Eisenberg, E. (1980) Annu. Rev. Biochem. 49, 921–956
3. Van Breeman, C., and Seig, B. (1980) Circ. Res. 46, 426–429
4. Stull, J. T. (1980) Adv. Cyclic Nucleotide Res. 15, 39–93
5. Small, J. V., and Sobieszek, A. (1977) Eur. J. Biochem. 76, 521–530
6. Hartshorne, D. J., and Siemankowski, R. F. (1980) Annu. Rev. Physiol. 43, 519–530
7. Kerrick, W. G. L., Hoar, P. E., and Cassidy, P. S. (1980) Fed. Proc. 39, 1558–1563
8. Morgan, J. P., and Morgan, K. G. (1982) Pflügers Arch. 396, 75–97
9. Morgan, J. P., and Morgan, K. G. (1984) J. Physiol. 351, 155–167
10. Silver, P. J., and Stull, J. T. (1984) Mol. Pharmacol. 25, 267–274
11. Aksoy, M. O., Mraa, S., Kamm, K. E., and Murphy, R. A. (1980) Am. J. Physiol. 245, C255–C270
12. Murphy, R. A., Aksoy, M. O., Dillon, P. F., Gerthoffer, W. T., and Kamm, K. E. (1983) Fed. Proc. 42, 51–56
13. Abdel-Latif, A. A., Akhtar, R. A., and Hawthorne, J. N. (1977) Biochem. J. 162, 61–75
14. Berridge, M. J. (1984) Biochem. J. 220, 395–360
15. Michell, R. H. (1982) Cell Calcium 3, 285–294
16. Hokin, L. E. (1985) Annu. Rev. Biochem. 54, 205–235
17. Nishizuka, Y. (1984) Nature 308, 699–698
18. Streib, H., Irvine, R. F., Berridge, M. J., and Schulz, I. (1983) Nature 306, 67–69
19. Nishizuka, Y. (1984) Science 225, 1365–1370
20. Rasmussen, H., Forder, J., Kojima, I., Scriabine, A. (1984) Biochem. Biophys. Res. Commun. 122, 776–784
21. Forder, J., Scriabine, A., and Rasmussen, H. (1985) J. Pharmacol. Exp. Ther. 235, 267–273
22. Shakh, N. A., and Palmer, F. B. S. C. (1977) J. Neurochem. 28, 355–402
23. Park, S., and Rasmussen, H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 8835–8839
24. Kojima, I., Lipps, H., Kojima, K., and Rasmussen, H. (1983) Biochem. Biophys. Res. Commun. 116, 555–562
25. Zawalich, W., Brown, C., and Rasmussen, H. (1983) Biochem. Biophys. Res. Commun. 117, 448–455
26. Delbeke, D., Kojima, I., Dannels, P. S., and Rasmussen, H. (1984) Biochem. Biophys. Res. Commun. 123, 735–741
27. Rasmussen, H., and Barrett, P. Q. (1984) Physiol. Rev. 64, 938–984
28. Silver, P. J., and Stull, J. T. (1984) Mol. Pharmacol. 25, 267–274
29. Kojima, I., Kojima, K., Kreutzer, D., and Rasmussen, H. (1984) J. Biol. Chem. 259, 14448–14457
30. Carney, D. H., Scott, D., Gordon, E. A., and Labelle, E. F. (1986) Cell 22, 479–488
31. Bansbach, M. W., Geison, R. L., and Kojima-Neaverson, M. (1981) Biochim. Biophys. Acta 663, 34–45
32. Bockino, S. B., Blackmore, P. F., and Exton, J. H. (1985) J. Biol. Chem. 260, 14201–14207
33. Bansbach, M. W., Geison, R. L., and O’Brien, J. F. (1974) Anal. Biochem. 59, 617–627
34. Berridge, M. J., Dawson, R. M. C., Downes, L. P., Heslop, J. P., and Irvine, R. F. (1983) Biochem. J. 212, 473–483
35. Hanks, H. H., and Derr, J. E. (1975) Anal. Biochem. 53, 607–613
36. van Dongen, C. J., Zwiers, H., and Gispen, W. H. (1985) Anal. Biochem. 144, 104–109
37. Baron, C. B., Cunningham, M., Strauss, J. F., III, and Coburn, R. F. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 6899–6903
38. Baron, C. B., and Coburn, R. F. (1984) J. Liq. Chromatogr. 7, 2793–2801
39. Bitman, J., and Wood, D. D. (1982) J. Liq. Chromatogr. 5, 1155–1162
40. Touchstone, J. C., Levin, S. S., Dobbins, M. F., and Beers, P. C. (1983) J. Liq. Chromatogr. 6, 179–192
41. Macala, L. J., Yu, R. K., and Anda, S. (1983) J. Lipid Res. 24, 1243–1250
42. Akhtar, R. A., and Abdel-Latif, A. A. (1984) Biochem. J. 224, 291–300
43. Smith, J. B., Smith, L., Brown, E. R., Barnes, D., Sabir, M. A., Davis, J. S., and Farese, R. V. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 7812–7816
44. Hashimoto, T., Hirata, M., and Ito, Y. (1985) Br. J. Pharmacol. 86, 191–199
45. Brock, T. A., Rittenhouse, S. E., Powers, C. W., Ekstein, L. S., Giromone, M. A., Jr., and Alexander, R. W. (1985) J. Biol. Chem. 260, 14155–14162
46. Campbell, M. D., Deth, R. C., Payne, R. A., and Honeyman, T. W. (1985) Eur. J. Pharmacol. 116, 129–136
47. Derian, C. K., and Moskowitz, M. A. (1986) J. Biol. Chem. 261, 3831–3837
48. Farese, R. V., Sabir, M. A., and Larson, R. E. (1980) J. Clin. Invest. 66, 1428–1431
49. Perret, B. P., Plantavid, M., Chap, H., and Douste-Blazy, L. (1983) Biochem. Biophys. Res. Commun. 110, 660–667
Polypolposinoid Breakdown and Smooth Muscle Contraction

50. Broekman, M. J. (1984) Biochem. Biophys. Res. Commun. 120, 226-231
51. Michell, R. H. (1975) Biochem. Biophys. Acta 415, 81-147
52. Michell, R. H. (1985) in Molecular Mechanism of Transmembrane Signaling (Cohen, P., and Houslay, M. D., eds) pp. 3-16, Elsevier Scientific Publishing Co., Amsterdam
53. Hansson, A., Serhan, C. N., Haggstrom, J., Ingelman-Sundberg, M., and Samuelsson, B. (1986) Biochem. Biophys. Res. Commun. 134, 1215-1222
54. Larue, J., Dorian, B., Demond-Henri, J., and Brucal, H. (1981)

Biochem. Biophys. Res. Commun. 101, 868-867
55. Coughlin, S. R., Moskowitz, M. A., Antoniadis, H. N., and Levine, L. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 7134-7138
56. Siess, W., Drey, F., Seillan, C., and Ody, C. (1981) Biochem. Biophys. Res. Commun. 99, 608-616
57. Hasse, E. P., Patu, M. D., and Kerriek, W. G. L. (1985) J. Biol. Chem. 260, 8790-8794
58. Schacht, J. (1981) Methods Enzymol. 72, 626-631
59. Hashimoto, T., Hirata, M., Itoh, T., Kannura, Y., and Kuriyama, H. (1985) J. Physiol. (Lond.) 370, 605-618
60. Enyedi, P., Buki, B., Muesi, L., and Spat, A. (1985) Mol. Cell. Endocrinol. 41, 105-112

Preparation and Inoculation of Tracheal Smooth Muscle

Tracheal smooth muscle were obtained from a local supplier and transported at 4°C in a modified Krebs-Ringer solution (in mM): NaCl 118.2, KCl 4.7, CaCl₂ 1.8, MgCl₂ 1.0, glucose 0.1 HEPES-KOH 2.2, and d-CaCl₂ 1.0. Tracheal smooth muscle were then trimmed using a scalpel and small trephine rings (7 mm in cross-sectional width with 4 mm length) were prepared (except for the case of 1.2-diacylglycerol measurement) in forceps-loaded manner, which was done in a single large ring. All chemicals were used. All procedures were done at room temperature in Tyrode buffer saturated with 95% O₂/5% CO₂.

51. Michell, R. H. (1985) in Molecular Mechanisms of Transmembrane Signaling (Cohen, P., and Houslay, M. D., eds) pp. 3-16, Elsevier Scientific Publishing Co., Amsterdam
52. Michell, R. H. (1985) in Molecular Mechanisms of Transmembrane Signaling (Cohen, P., and Houslay, M. D., eds) pp. 3-16, Elsevier Scientific Publishing Co., Amsterdam
53. Michell, R. H. (1985) in Molecular Mechanisms of Transmembrane Signaling (Cohen, P., and Houslay, M. D., eds) pp. 3-16, Elsevier Scientific Publishing Co., Amsterdam
54. Michell, R. H. (1985) in Molecular Mechanisms of Transmembrane Signaling (Cohen, P., and Houslay, M. D., eds) pp. 3-16, Elsevier Scientific Publishing Co., Amsterdam
55. Michell, R. H. (1985) in Molecular Mechanisms of Transmembrane Signaling (Cohen, P., and Houslay, M. D., eds) pp. 3-16, Elsevier Scientific Publishing Co., Amsterdam
56. Michell, R. H. (1985) in Molecular Mechanisms of Transmembrane Signaling (Cohen, P., and Houslay, M. D., eds) pp. 3-16, Elsevier Scientific Publishing Co., Amsterdam
57. Michell, R. H. (1985) in Molecular Mechanisms of Transmembrane Signaling (Cohen, P., and Houslay, M. D., eds) pp. 3-16, Elsevier Scientific Publishing Co., Amsterdam
58. Michell, R. H. (1985) in Molecular Mechanisms of Transmembrane Signaling (Cohen, P., and Houslay, M. D., eds) pp. 3-16, Elsevier Scientific Publishing Co., Amsterdam
59. Michell, R. H. (1985) in Molecular Mechanisms of Transmembrane Signaling (Cohen, P., and Houslay, M. D., eds) pp. 3-16, Elsevier Scientific Publishing Co., Amsterdam
60. Michell, R. H. (1985) in Molecular Mechanisms of Transmembrane Signaling (Cohen, P., and Houslay, M. D., eds) pp. 3-16, Elsevier Scientific Publishing Co., Amsterdam

Extraction of Lipids and Thin-Layer Chromatography

In the present lipid experiment, muscles were homogenized in 100 mM (pH 7.0) trichloroacetic acid. The homogenate was centrifuged at 1000 g for 10 min. The supernatant was used for lipid analysis and the pellet was analyzed for phospholipids. Phospholipids were extracted with chloroform/methanol (2:1, v/v). The organic phase was dried under a flow of nitrogen gas. Phospholipids were separated by thin-layer chromatography (TLC) using silica gel G plates. Phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA) were visible under ultraviolet light, and were identified by comparison with authentic standards. This is the column system; the degrading of PA was measured by using silica gel 60 plates with a solution of chloroform/methanol/acetone (5:4:1, v/v/v). The bands of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidic acid in the separated phospholipids were scraped off for phosphorus assay as described below.

For thin-layer chromatography, muscles were homogenized in quenching solution; chloroform/methanol (1:1, v/v) 99% ethanol was added and then homogenate was taken. The upper phase was separated using 1/3 volumes of chloroform and was collected. The organic phase was washed with 1/3 volumes of chloroform and water. The upper phase was separated using 1/3 volumes of chloroform/methanol (99% ethanol) and was collected. The bands of phospholipids were scraped off for phosphorus assay as described below.

Analysis of Water-Soluble Lecithin Phosphates

The preparation of a muscle homogenate was neutralized with 60% and applied to an exchange resin column. The column was washed with distilled water. The column was then washed with a mixture of distilled water and 0.1 M sodium chloride, followed by 0.5 M sodium chloride. The column was washed with 0.5 M sodium chloride, followed by 0.5 M sodium chloride, followed by 0.5 M sodium chloride, followed by 0.5 M sodium chloride, followed by 0.5 M sodium chloride, followed by 0.5 M sodium chloride.

Measurement of phospholipid phosphorylation

Each 50 of the large chromatography plates was scraped onto a 12.5 cm glass tube and digested with 200 ml of 60% perchloric acid and 5% HClO₃. The digested solution was dried at 100°C for 4 h. The residue was dissolved in 10 ml of distilled water. The solution was applied to a thin-layer chromatography plate (silica gel 60F254, Merck). The bands of phospholipids were scraped off for phosphorus assay as described below.

Chemical procedure for 1.2-diacylglycerol measurement

For quantification of 1.2-diacylglycerol, an ion-exchange chromatography technique was employed, according to the method of Munson et al. (31). Briefly, after development of this layer chromatography, the plates were stained with 100% EtOH in 100% EtOH. The bands of 1.2-diacylglycerol were scanned, and the absorbance was used for calculation. The absorbance was scanned at 510 nm with a Shimadzu CS-901 scanning densitometer, and the integrated areas were computed by a Shimadzu CS-901 data processor (Shimadzu Scientific Instruments, Inc., Columbia, MD). The standard curve was measured using 1.2-diacylglycerol standard and the lipid extract of muscle was calculated as 1.2-diacylglycerol.

Figure 1. The time course of corticotropin-stimulated changes in Eiclonelabeled phospholipids.

Biocytin (technique smooth muscle strips were labeled with [3H]leucine with 0.1 M sodium perchloric acid for 4 h). The bands of phospholipids were scraped off for phosphorus assay as described below.

Figure 2. The time course of corticotropin-stimulated changes in Eiclonelabeled phospholipids.

Biocytin (technique smooth muscle strips were labeled with [3H]leucine with 0.1 M sodium perchloric acid for 4 h). The bands of phospholipids were scraped off for phosphorus assay as described below.

Figure 3. The time course of corticotropin-stimulated changes in Eiclonelabeled phospholipids.