Biochemical Pharmacology, Vol. 36, No. 3, pp. 307–310, 1987.
Printed in Great Britain.

0006-2952/87/$3.00 + 0.00

RAPID COMMUNICATIONS

POLYPHOSPHOINOSITIDE METABOLISM IN CANINE TRACHEAL SMOOTH MUSCLE (CTSM) IN RESPONSE TO A CHOLINERGIC STIMULUS*

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(Received 7 October 1986; accepted 10 November 1986)

The contractile response of airway smooth muscle via pharmacomechanical coupling is a membrane potential independent process (1). The sequential series of events involves the binding of a drug to its receptor, increased intracellular calcium, the binding of calcium to calmodulin, activation of myosin light chain kinase, and increased interaction of actin and myosin filaments within smooth muscle cells (2). Michell (3) postulated that occupancy of certain receptors results in an increased turnover of phosphatidylinositol which, in turn, is a signal for calcium mobilization. It has been subsequently recognized in many nonmuscle and some smooth muscle preparations that receptor occupancy is more closely correlated with polyphosphoinositide hydrolysis (4,5). The products of a phospholipase C mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate are inositol 1,4,5-trisphosphate and diacylglycerol. The former substance is thought to act as a second messenger for intracellular calcium mobilization, whereas the latter acts to stimulate protein kinase C. Recent evidence indicates that both of these molecules participate in smooth muscle contraction.

Somlyo et al. (6) demonstrated that inositol trisphosphate can cause the release of calcium from internal stores and induce a contractile response in saponin permeabilized vascular smooth muscle strips. Yu (7) has isolated and characterized protein kinase C in various smooth muscle preparations and demonstrated its activation by diacylglycerol. Park and Rasmussen (8) have published data showing a synergistic contractile response to calcium ionophores and protein kinase C activators (i.e. phorbol esters) in airway smooth muscle. These and other reports (9-13) are consistent with the concept that inositol 1,4,5-trisphosphate and diacylglycerol may be involved in the initiation and maintenance of the contractile state in smooth muscle.

To date two reports have been published regarding the characterization of phosphatidylinositol metabolism in airway smooth muscle. Inositol phosphate formation and a functional response (i.e. development of contractile tension) in the same smooth muscle strip are seldom measured simultaneously. The only report of such a study was by Baron et al. (14). These authors were the first to find decreases in phosphatidylinositol and increases in phosphatidic acid after a minute of cholinergic stimulation in canine airway smooth muscle. The second and the most recent report (15) demonstrated that inositol phosphate formation is associated with muscarinic subtype 2 receptor occupancy

*This article is part of a dissertation to be submitted by R. Duncan for a Doctor of Philosophy in Pharmacology at the University of South Florida College of Medicine.

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in bovine tracheal smooth muscle. Both studies demonstrated inositol phosphate formation in airway smooth muscle. The first study assayed for changes beginning at 1 min of stimulation, whereas the second began assaying at 15 min of stimulation with a cholinergic agonist. Consistency with the hypothesis that inositol 1,4,5-trisphosphate and diacylglycerol act as second messengers demands that their formation precedes the contractile response. Initial studies in our laboratory revealed very short half-times (20 sec) to maximal tension development during cholinergic stimulation of canine airway smooth muscle. The purpose of this study was to determine the kinetic relationship between inositol phosphate formation and the contractile response at very short time intervals (i.e. 1 sec) during cholinergic stimulation in airway smooth muscle.

Adult dogs (18-30 kg) of random sex were anesthetized with intravenous pentobarbital (30 mg/kg). The trachea was excised to the carina and bathed in a Krebs-Ringer solution (NaCl, 117.0 mM; KCl, 4.0 mM; $NaHCO_3$, 25.0 mM; $MgSO_4$, 2.4 mM; NaH_2PO_4, 1.2 mM; $CaCl_2$, 2.5 mM; and dextrose, 11.0 mM). Smooth muscle strips (approximately 2-3 mm by 15-20 mm weighing 40 mg) were dissected from the cartilage and tied tightly at both ends with 4-0 braided surgical silk. Strips were mounted in a constant temperature incubator maintained at 37° and were superperfused at a flow rate of 2 ml/min with an aerated (95% O_2, 5% CO_2) Krebs buffer maintained at 37°. A basal tension of 1-2 g (0.85 g/cm^2) was applied to each strip and is consistent with the resting tension at L_{max} reported by Stephens (16) for canine tracheal smooth muscle. Contractile tension was monitored with a Grass FT03C force displacement transducer and a Grass model 7C polygraph. Strips were allowed to equilibrate for 30 min and responses were initiated by stimulation for approximately 180 sec with 10 μM acetylcholine. The time course of contraction was monitored. The drug was washed off, the tissue was allowed to equilibrate for 10 min, and the strips were labeled for 2 hr with [^{3}H]inositol (10 μCi/ml). After labeling, the strips were washed for 10 min with nonradioactive buffer, stimulated with 10 μM acetylcholine, and freeze clamped at various times with aluminum clamps precooled in liquid nitrogen. The tissues were quickly removed and stored under liquid nitrogen until further processing. Stimulated tissues were compared to control strips freeze clamped after the washout period.

Tissues were homogenized in 10% trichloroacetic acid, an aliquot was removed for protein determination (17), and samples were centrifuged at 1000 g for 10 min, after which the upper aqueous phase was collected. Inositol phosphate accumulation was determined by the method of Berridge et al. (18). Data are expressed as cpm/mg protein and significance was determined by Student's T-test.

Figure 1(a) shows a typical time course for the contractile response to 10 μM acetylcholine for 60 sec at which time the strip was frozen. The half-time to maximum contraction was 20.6 sec with a range of 18-22 sec. The initiation of contractile tension begins at 2-3 sec after cholinergic stimulation. Figure 1 (b-d) shows the mean changes in inositol trisphosphate (IP_3), inositol bisphosphate (IP_2), and inositol monophosphate (IP) accumulation for each time point after stimulation with 10 μM acetylcholine. Table 1 gives means and standard errors for each time point. Inositol trisphosphate was increased significantly (497%) over basal values ($P < 0.05$) after 1 sec of stimulation. The response was biphasic in that all inositol phosphates began increasing a second time at 120 sec (Table 1). The increases after 2 min are consistent with a previous report (15) of inositol phosphate accumulation in tracheal airway smooth muscle stimulated with acetylcholine. In a single experiment (not shown), the time course for the contractile response was monitored in the presence of 10 mM LiCl. Lithium caused no change in the time course but caused an accumulation of inositol 1-phosphate.

Table 1. Time course for CTSM inositol phosphate formation during stimulation with 10 μM acetylcholine

Time (sec)	N	Inositol Phosphate Formation (cpm/mg protein) IP_3	IP_2	IP
0	4	55.9 ± 13.8	75.3 ± 15.7	361.3 ± 155.5
1	4	277.6 ± 93.7*	181.7 ± 75.3	543.5 ± 183.9
5	4	140.5 ± 71.8	70.7 ± 22.1	307.5 ± 112.2
10	4	78.5 ± 10.7	78.8 ± 31.4	252.0 ± 126.9
30	4	71.7 ± 9.1	103.3 ± 50.9	219.3 ± 109.5
60	3	71.6 ± 4.7	55.3 ± 38.3	267.0 ± 112.9
90	3	36.0 ± 5.8	90.6 ± 54.9	505.3 ± 246.7
120	3	333.0 ± 238.6	205.3 ± 176.3	976.0 ± 713.8

Values are means ± S.E. * $P < 0.05$.

The data presented indicate that the formation of inositol trisphosphate preceded the contractile event in tracheal smooth muscle stimulated with acetylcholine. This is the first report demonstrating polyphosphoinositide hydrolysis at very short time intervals in an isolated contracting smooth muscle preparation. The time course for inositol trisphosphate formation is consistent with its role as a second messenger in muscarinic mediated contractile responses in airway smooth muscle.

The biphasic nature of the response is of interest since this pattern has been reported recently in muscarinic receptor stimulation of rat cerebral cortical slices and rat parotid glands (19-21). This second rise in inositol phosphates has been attributed to the formation of inositol 1,3,4,5-tetrakisphosphate and inositol 1,3,4-trisphosphate from continued phosphatidylinositol 4,5-bisphosphate hydrolysis and calcium-dependent phosphatidylinositol hydrolysis (22,23). Preliminary data from our laboratory indicate changes in the HPLC separation profile of the inositol tris- and tetrakisphosphates at 1 and 120 sec of stimulation with 10 μM acetylcholine. This work is being pursued at present.

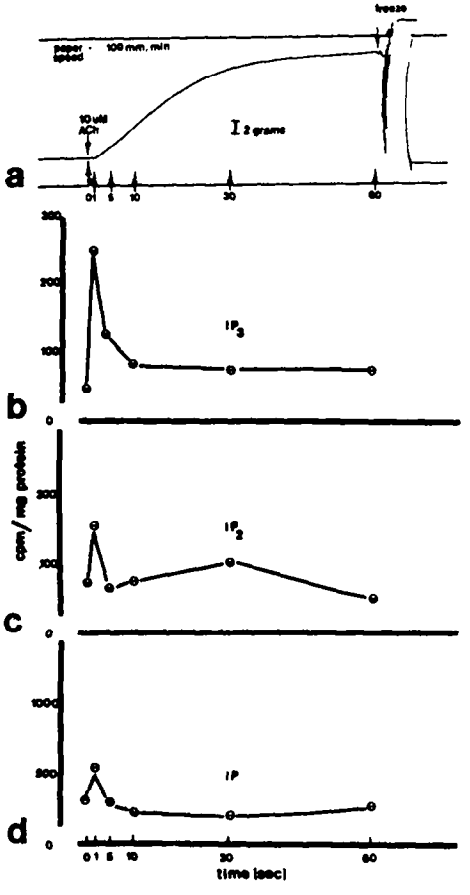

Fig. 1. (a) Time course for the contractile response of CTSM to 10 μM acetylcholine. (b-d) Time course for inositol tris-, bis-, and monophosphate (IP_3, IP_2, IP) formation up to 60 sec of stimulation with 10 μM acetylcholine.

A perfusion apparatus that allows nearly instant access to tissues has been useful for relating the rapid changes in inositol phospholipid metabolism to changes in contractile tension. Goldman et al. (24) incorporated a "caged IP_3" into skinned vascular smooth muscle and used photolysis to release an active inositol trisphosphate. These authors conclude that once released, IP_3 can act rapidly enough to qualify as a second

messenger. Our data confirm and extend the work of others (14,15) that inositol phospholipid hydrolysis occurs during muscarinic stimulation in airway smooth muscle. One of the relevent changes in inositol phospholipid metabolism was the formation of inositol trisphosphate at very short time intervals (1 sec). This report further supports a role for inositol trisphosphate and diacylglycerol as second messengers in the initiation and maintenance of the contractile state in smooth muscle.

Acknowledgements--The authors wish to thank Leigh West and Laura Weakland for their technical advice and Patricia Claytor for her secretarial assistance.

REFERENCES

1. A.V. Somlyo and A.P. Somlyo, J. Pharmac. exp. Ther. 159, 129 (1968).
2. K.E. Kamm and J.T. Stull, A. Rev. Pharmac. Toxic. 25, 593 (1985).
3. R.H. Michell, Biochim. biophys. Acta 415, 81 (1975).
4. M.J. Berridge, Biochem. J. 212, 849 (1983).
5. R.A. Akhtar and A.A. Abdel-Latif, Biochem. J. 224, 291 (1984).
6. A.V. Somlyo, M. Bond, A.P. Somylo and A. Scarpa, Proc. natn. Acad. Sci. U.S.A. 82, 5231 (1985).
7. B. Yu, Kobe J. med. Sci. 27, 225 (1981).
8. S. Park and H. Rasmussen, Proc. natn. Acad. Sci. U.S.A. 82, 8835 (1985).
9. R.W. Alexander, T.A. Brock, M.A. Gimbrone and S.E. Rittenhouse, Hypertension U.S.A. 7, 447 (1985).
10. J.W. Baraban, R.J. Gould, S.J. Peroutka and S.H. Snyder, Proc. natn. Acad. Sci. U.S.A. 82, 604 (1985).
11. M.J. Grimes, A.A. Abdel-Latif and G.O. Carrier, Biochem. Pharmac. 28, 3213 (1979).
12. M.C. Sekar and B.D. Roufogalis, Biochem. J. 223, 527 (1984).
13. J.B. Smith, L. Smith, E.R. Brown, D. Barnes, M.A. Sabir, J.S. Davis and R.V. Farese, Proc. natn. Acad. Sci. U.S.A. 81, 7812 (1984).
14. C.B. Baron, M. Cunningham, J.F. Strauss and R.F. Coburn, Proc. natn. Acad. Sci. U.S.A. 81, 6899 (1984).
15. B.M. Grandordy, F.M. Cuss, A.S. Sampson, J.B. Palmer and P.J. Barnes, J. Pharmac. exp. Ther. 238, 273 (1986).
16. N.L. Stephens, in Airway Dynamics: Physiology and Pharmacology (Ed. A. Bouhuys), pp. 191-208. Charles C. Thomas, Springfield (1970).
17. O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. biol. Chem. 193, 265 (1951).
18. M.J. Berridge, R.M.C. Dawson, C.P. Downes, J.P. Neslop and R.F. Irvine, Biochem. J. 212, 473 (1983).
19. I.R. Batty, S.R. Nahorski and R.F. Irvine, Biochem.J. 232, 211 (1985).
20. R.F. Irvine, A.J. Letcher, D.J. Lander and C.P. Downes, Biochem.J. 223, 237 (1984).
21. R.F. Irvine, E.E. Anggard, A.J. Letcher and C.P. Downes, Biochem.J. 229, 505 (1985).
22. C.P. Downes, P.T. Hawkins and R.F. Irvine, Biochem. J. 238, 501 (1986).
23. P.T. Hawkins, L. Stephens and C.P. Downes, Biochem.J. 238, 507 (1986).
24. Y.E. Goldman, G.P. Reid, A.P. Somlyo, A.V. Somlyo, D.R. Trentham and J.W. Walker, J. Physiol. 377, 100P (1986).