Biochemical Pharmacology, Vol. 34, No. 13, pp. 2297–2301, 1985.
Printed in Great Britain.

0006–2952/85 $3.00 + 0.00

RELATIONSHIP BETWEEN STIMULATED INOSITOL LIPID HYDROLYSIS AND CONTRACTILITY IN GUINEA-PIG VISCERAL LONGITUDINAL SMOOTH MUSCLE

LEONARD BEST,* KEITH J. BROOKS and THOMAS B. BOLTON

Department of Pharmacology, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K.

(*Received* 26 *October* 1985; *accepted* 28 *January* 1985)

Abstract—Carbamylcholine caused a marked, concentration-dependent stimulation of [^{3}H]Ins P, [^{3}H]InsP$_2$ and to a lesser extent [^{3}H]InsP$_3$ production in guinea-pig longitudinal smooth muscle prelabelled with *myo*-[^{3}H]inositol. Accumulation of these three inositol phosphates showed differential sensitivity to LiCl. Muscle contraction was apparent at lower concentrations of carbamylcholine. Both responses were mediated via muscarinic-type receptors. An association of inositol phosphate production and contractility was also observed in response to substance P, histamine and noradrenaline, the latter via an α-adrenergic mechanism. The Ca^{2+}-channel agonist CGP 28392 failed to stimulate inositol phosphate production despite inducing a contractile response. Carbamycholine-induced inositol phosphate production persisted in the presence of D600 or Mn^{2+} despite loss of contractile activity. However, both responses showed a similar, marked dependence on the presence of Ca^{2+} in the extracellular medium. Mn^{2+} could restore basal and stimulated inositol phosphate production in low Ca^{2+} solutions but could not substitute for Ca^{2+} in restoring contractility.

The results suggest that stimulated inositol lipid hydrolysis in longitudinal smooth muscle does not result from Ca^{2+} entry into the tissue, although the response does depend on the concentration of divalent cations in the extracellular medium. This dependency may be related to the maintenance of membrane potential and possibly phospholipid conformation.

Accelerated hyrolysis of inositol-containing phospholipids has been demonstrated in a wide variety of neuronal, secretory and contractile tissues in which stimulation results in a rise in the cytosolic concentration of free Ca^{2+} (for reviews, see [1–5]). In visceral smooth muscle, enhanced ^{32}P-labelling of phosphatidylinositol and phosphatidic acid has been demonstrated in response to stimulation by a number of agonists [6, 7] suggesting increased inositol lipid breakdown upon receptor occupation. In rabbit iris smooth muscle, Abdel-Latif and co-workers reported breakdown of PtdInsP$_2$† upon exposure to either acetylcholine [8] or noradrenaline [9] and proposed that hydrolysis of this polyphosphoinositide may be an early event in stimulation of smooth muscle. More recently, increased inositol phosphate production has been demonstrated in guinea-pig ileum in response to substance P [10, 11], again suggesting accelerated inositol lipid breakdown upon stimulation, although no assessment was made as to which phosphoinositide(s) were involved in this effect. Furthermore, the absence of measurements in parallel of muscle contraction in the majority of the above studies prevents any firm conclusions regarding the role of enhanced inositol lipid metabolism in smooth muscle function.

In 1975, Michell [1] proposed a general hypothesis that stimulated phosphoinositide breakdown may be a determinant of the regulation of cytosolic Ca^{2+} concentrations in stimulated tissues. The demonstration that Ca^{2+}-antagonistic drugs did not impair enhanced phosphatidylinositol metabolism in guinea-pig ileum [12] was consistent with this hypothesis. In contrast, the breakdown of PtdInsP$_2$ in rabbit iris muscle upon cholinergic stimulation was found to be reduced in the absence of Ca^{2+} [13] and potentiated by high concentrations of Ca^{2+} [14] suggesting that lipid hydrolysis occurred as a result of increased Ca^{2+} uptake into the tissue.

In the present study, we have examined the nature of stimulated inositol lipid breakdown in guinea-pig longitudinal intestinal smooth muscle in response to a variety of agonists, and monitored contractility under similar conditions in order to define a relationship between these two aspects of smooth muscle function. In addition, we have focused attention on the calcium-dependency of these processes and suggest a possible role for Ca^{2+} in modulating agonist-induced inositol lipid hydrolysis.

EXPERIMENTAL PROCEDURES

Materials. *myo*-[2-^{3}H]inositol (10 Ci/mmole) was purchased from the Radiochemical Centre (Amersham, U.K.) and purified by applying to a column containing 0.5 ml Dowex AG1×8 (200–400 mesh, formate form) and eluting with 5 vol. H_2O. Carbamylcholine, substance P, histamine dihydrochloride, noradrenaline and atropine sulphate

* Present address and correspondence: Department of Biochemistry, University of Bristol, BS8 1TD, U.K.

† Abbreviations used: PtdIns, phosphatidylinositol; PtdIns P, phosphatidylinositol monophosphate; PtdInsP$_2$, phosphatidylinositol bisphosphate; InsP, *myo*-inositol 1-phosphate; InsP$_2$, *myo*-inositol bisphosphate; SInsP$_3$, inositol – trisphosphate.

were obtained from the Sigma Chemical Co. (Surrey, U.K.). Phentolamine and propranolol were supplied by ICI (Macclesfield, U.K.), ionophore A23187 by Calbiochem–Behring (San Diego, U.S.A.), and D600 by Knoll, AG (Ludwigschafen, West Germany). The calcium channel agonist CGP 28392 was obtained from Ciba–Geigy, Horsham, U.K.

Methods. The small intestine was removed from 1–2 guinea-pigs of either sex, freed of fat and mesenteric tissue and the longitudinal muscle carefully stripped off and placed in Krebs–Ringer bicarbonate (KRB) medium [15] gassed with 95% O_2–5% CO_2. The muscle was then finely chopped with scissors, extensively washed with fresh KRB and then incubated for 3 hr at 37° in a volume of 1 ml KRB with 30 μCi *myo*-[^{3}]inositol in an atmosphere of 95% O_2–5%CO_2. At the end of this preincubation period, the slices were washed four times with 10 ml KRB containing no radioactivity and once with KRB containing 10^{-2} M LiCl and 10^{-3} M unlabelled *myo*-inositol. In experiments employing Mn^{2+}, Hepes-buffered medium was used to avoid precipitation of Mn^{2+}-salts. 100 μl aliquots of tissue slices (approx. 5 mg dry wt) were then transferred to glass tubes containing 800 μl KRB to which had been added 10^{-2} M LiCl and 10^{-3} M unlabelled *myo*-inositol. Incubations were started by the addition of 100 μl KRB containing test substances, and terminated by the addition of 3 ml chloroform:methanol (200:100:1). The tubes were then vortex-mixed for 30 sec and the two phases separated by centrifugation at 500 *g* for 5 min. The upper, aqueous phase was removed, 3 ml H_2O added and applied to a column containing 1 ml. Dowex AG1×8 (200–400 mesh, formate form). Free inositol and inositol phosphates were eluted in a stepwise manner as described by Berridge *et al.* [16, 17]. In certain experiments, all inositol phosphates were eluted together as an index of inositol phospholipid hydrolysis. Results are expressed in dpm per 100 μl tissue suspension. In order to make direct comparisons between separate experiments, all control values from a series of experiments were pooled, and test values expressed in terms of this pooled control value. Statistical analyses were performed using Student's *t*-test and were restricted to comparisons within each individual experiment.

For studies of smooth muscle contractility, strips of longitudinal muscle from guinea-pig ileum were prepared as described previously [18] and placed in 0.1 ml organ baths [19] and perfused at a rate of 1.6 ml/min with gassed KRB heated to 37°. Tension was recorded using a UTC 2 isometric transducer coupled to an SC1105 bridge amplifier (Gould Inc., Oxnard, California, U.S.A.). Contractile responses were expressed in grams.

RESULTS

In the presence of 10^{-2} M LiCl, carbamylcholine induced a concentration-dependent stimulation of [^{3}H]inositol phosphate production in [^{3}H]inositol—prelabelled longitudinal smooth muscle slices (Fig. 1). This increase was most apparent in the [^{3}H] Ins P and [^{3}H]InsP_2; a relatively minor effect was observed on levels of [^{3}H]InsP_3. Carbamylcholine

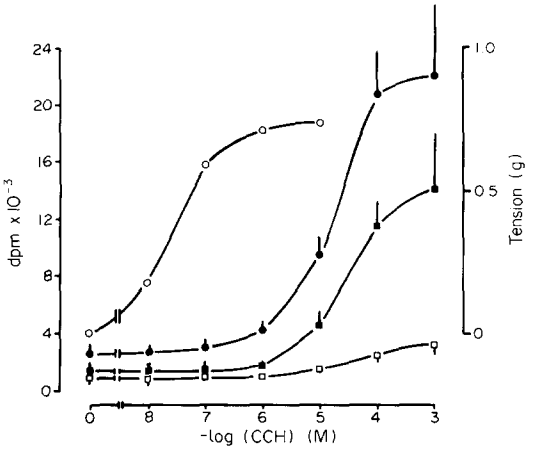

Fig. 1. Concentration–response curves of carbamylcholine-induced [^{3}H]InsP (●), [^{3}H]InsP_2 (■) and [^{3}H]InsP_3 (□) production in longitudinal smooth muscle slices in the presence of 10^{-2} M LiCl, and of contractility (○) in smooth muscle strips. Incubations were for 20 min. Each point represents the mean ± S.E.M. of three separate incubations. Typical responses are shown taken from at least three experiments on contractility.

also caused a concentration-dependent increase in smooth muscle tension (Fig. 1). Maximal contractile responses were observed at approx. 10^{-6} M carbamylcholine, a value two orders of magnitude smaller than the concentration of the drug necessary to induce maximal stimulation of [^{3}H]inositol phosphate production. The latter was found to be a linear process, at least during 20 min of stimulation with 10^{-4} M carbamylcholine (Fig. 2), a significant rise in the levels of [^{3}H]Ins P being detectable 1 min following addition of the drug; no lag being apparent.

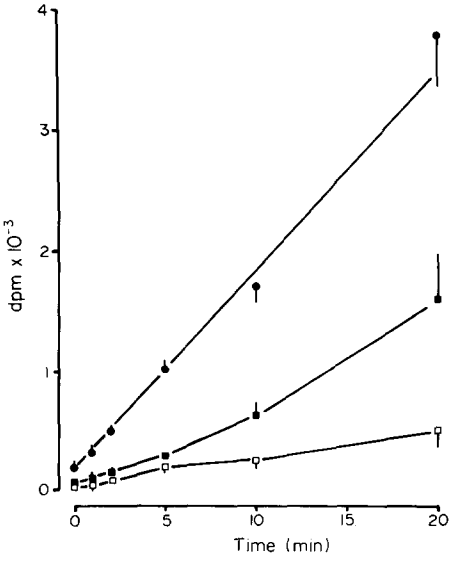

Fig. 2. Time courses of [^{3}H]InsP (●), [^{3}H]InsP_2 (■) and [^{3}H]InsP_3 (□) production in longitudinal smooth muscle slices in response to 10^{-4} M carbamylcholine. 10^{-2} M LiCl was present throughout. Each point represents the mean ± S.E.M. of three separate incubations.

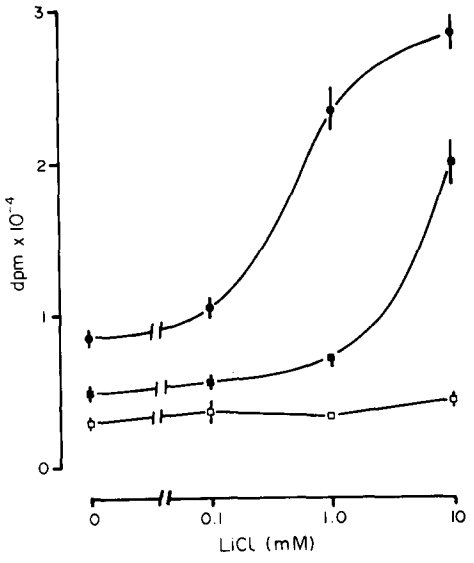

Fig. 3. [^{3}]InsP (●), [^{3}H]InsP_2 (■) and [^{3}H]InsP_3 (□) production in response to 10^{-4} M carbamylcholine effect of LiCl concentration. Incubations were for 20 min. Each point represents the mean ± S.E.M. of three separate incubations.

The levels of [^{3}H]inositol phosphates achieved upon stimulation with 10^{-4} M carbamylcholine were also dependent on the concentration of LiCl in the incubation medium (Fig. 3). 10^{-3} M Li^+, which caused a near-maximal accumulation of [^{3}H]Ins P, produced only a small increment in [^{3}H]InsP_2 levels. A higher concentration of Li^+ (10^{-2} M) resulted in a pronounced increase in the concentration of [^{3}H]InsP_2, although the levels of [^{3}H]InsP_3 were not significantly affected by the presence of Li^+. 10^{-2} M Li^+ had no apparent effect on longitudinal muscle contractility in response to carbamylcholine (results not shown).

Stimulation of [^{3}H]inositol prosphate production and of contractile activity in longitudinal smooth muscle was also observed upon exposure to substance P, histamine and noradrenalin (Table 1). Maximal concentrations of these agonists were less effective than carbamylcholine in stimulating [^{3}H] inositol phosphate production despite maximal contractile activity. The stimulation of [^{3}H]inositol phosphate formation by carbamylcholine (10^{-4} M) and noradrenalin (10^{-4} M) were inhibited by atropine (10^{-6} M) and phentolamine (10^{-5} M) respectively (Table 1). Propranolol (10^{-5} M) had no significant effect on noradrenaline-induced [^{3}H]inositol phosphate production. Similarly atropine (10^{-6} M) and phentolamine (10^{-5} M) inhibited, respectively, carbamylcholine (10^{-4} M) and noradrenaline (10^{-4} M)-induced contractile activity, whilst the latter was unaffected by propranolol (10^{-5} M).

Carbamylcholine-stimulated [^{3}H] inositol phosphate production was found to persist in the presence of the calcium-channel blocker D600 (Table 1), which inhibited the contractile response to carbamylcholine. However, the effects of carbamylcholine (10^{-4} M) on [^{3}H]inositol phosphate production showed a similar dependence to contraction on the presence of Ca^{2+} in the extracellular medium (Fig. 4). Both responses were markedly inhibited by reducing the Ca^{2+} concentration of the medium from 10^{-3} M to 10^{-5} M. At 10^{-6} M Ca^{2+}, contractility was completely suppressed, whilst [^{3}H]inositol phosphate formation persisted, though at a very much reduced level. The impaired production of [^{3}H]inositol phosphates in response to carbamylcholine in low Ca^{2+} medium was restored by 10^{-3} M Mn^{2+}, despite

Table 1. Effects of agonists and antagonists on total [^{3}H]inositol phosphate ([^{3}H]Ins P) production and contractility in guinea-pig longitudinal smooth muscle

Conditions	[^{3}H]Ins P DPM	P		Contractility (g)
1. Control	6717 ± 648(24)			0
2. 10^{-5} M carbamylcholine	28090 ± 4997(3)	$P < 0.05$	vs line 1	0.73 ± 0.01(8)
3. 10^{-5} M carbamylcholine +10^{-6} M atrophine	9660 ± 817(3)	$P < 0.05$	vs line 2	0
4. 10^{-6} M carbamylcholine +2 × 10^{-6} M D600	22543 ± 3197(3)	P < N.S.	vs line 2	0
5. 10^{-4} M carbamylcholine	60884 ± 8277(9)	$P < 0.001$	vs line 1	0.76 ± 0.03(3)
6. 10^{-4} M histamine	29700 ± 1864(3)	$P < 0.01$	vs line 1	0.61 ± 0.08(3)
7. 10^{-4} M carbamylcholine +10^{-4} M histamine	74524 ± 2906(3)	$P < 0.05$	vs line 5	0.66 ± 0.09(3)
8. 10^{-4} M noradrenalin	20284 ± 2646(6)	$P < 0.05$	vs line 1	0.71 ± 0.09(7)
9. 10^{-4} M noradrenalin +10^{-5} M phentolamine	12278 ± 3568(3)	$P < 0.05$	vs line 8	0.50 ± 0.08(5)
10. 10^{-4} M noradrenalin +10^{-5} M propranolol	18093 ± 1455(3)	N.S.	vs line 8	0.94 ± 0.09(3)
11. 10^{-8} M substance P	14508 ± 393(3)	$P < 0.005$	vs line 1	0.37 ± 0.03(3)
12. 10^{-7} M substance P	20911 ± 2524(3)	$P < 0.05$	vs line 1	0.60 ± 0.03(3)
13. 10^{-6} M substance P	34230 ± 2591(3)	$P < 0.01$	vs line 1	0.73 ± 0.01(3)
14. 10^{-5} M CGP 28392	9567 ± 1273(3)	N.S.	vs line 1	0.46 ± 0.06(6)
15. 10^{-5} M A23187	10610 ± 438(3)	N.S.	vs line 1	0.25 ± 0.05(3)

All incubations for measurement of [^{3}H]inositol phosphates were for 20 min. The figures in parentheses represent the number of determinations. P values refer to [^{3}H]Ins P measurements and were determined by Student's *t*-test.

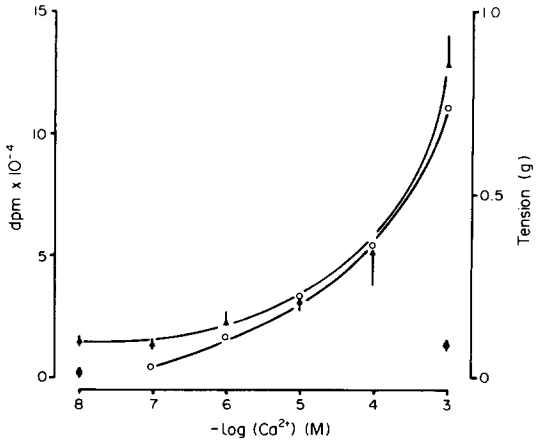

Fig. 4. Total [^{3}H]inositol phosphate (▲) production and contractility (○) in longitudinal smooth muscle in response to 10^{-4} M carbamylcholine: effect of $CaCl_2$ concentration. Incubations were for 20 min. Each point represents the mean ± S.E.M. of three separate incubations. Typical responses are shown taken from at least three experiments on contractility. ? represents basal production of inositol phosphates at 10^{-8} and 10^{-3} M Ca^{2+}.

complete inhibition of smooth muscle contraction (Table 2). The calcium channel agonist CGP 28392 produced a marked contractile response but had no significant influence on [^{3}H]inositol phosphate production in smooth muscle (Table 1). Similarly, the calcium ionophore A23187 failed to significantly affect [^{3}H]inositol phosphate production.

DISCUSSION

The present investigation has employed the measurement of [^{3}H]inositol phosphates, the water-soluble products of inositol phospholipid hydrolysis [16, 17], as an index of the latter in stimulated guinea-pig longitudinal smooth muscle. A similar approach was recently used to demonstrate hydrolysis of inositol lipids in guinea-pig ileum and hypothalamus [10] and rat small intestine [11] in response to substance P. We have further shown that several neurotransmitter substances which cause contraction of longitudinal smooth muscle, namely cholinergic, α-adrenergic and histaminergic stimuli, also provoke inositol lipid hydrolysis. The most potent agonist employed was carbamylcholine which induced both contraction and phosphoinositide breakdown by an interaction with a muscarinic type cholinergic receptor. A similar mechanism has been demonstrated in guinea-pig ileum resulting in enhanced ^{32}P-labelling of phosphatidylinositol [6], which presumably results from an initial stimulation of inositol lipid breakdown. Part of the present study was carried out to assess which of the phosphoinositides might be involved in such lipid breakdown. The detection of $InsP_2$ and to a lesser extent $InsP_3$ suggests strongly that an initial hydrolysis of Ptd Ins P_2 and possibly PtdIns P may occur in longitudinal smooth muscle during cholinergic stimulation. This effect may correspond to polyphosphoinositide breakdown during cholinergic stimulation of rabbit iris muscle [13]. The simultaneous formation of InsP is likely to result from the enzymatic degradation of Ins P_3 and Ins P_2 [17], although we cannot exclude the possibility that some hydrolysis of Ptd Ins may also occur [6].

It has recently been shown that $InsP_3$ occurs in rat parotid gland as two anomers; the biologically active Ins(1,4,5)P_3 (derived from PtdIns(4,5)P_2) and an inactive form Ins(1,3,4)P_3 (presumably derived from PtdIns(3,4)P_2 hydrolysis) [20]. Furthermore, prolonged stimulation of parotid with carbamylcholine resulted in a proportionally increased formation of Ins(1,3,4)P_3. The chromatography system used in the present study for the separation of inositol phosphates would not distinguish between the two anomeric forms of $InsP_3$, and so it should be borne in mind that these anomers may make a variable contribution to the detectable IP_3, depending on the period of stimulation with carbamylcholine.

The measurement of inositol phosphates resulting from agonist-induced phosphoinositide hydrolysis is dependent on the presence of Li^+ which inhibits the hydrolysis of Ins P to free inositol [21]. Our results also demonstrate that high concentrations ($>10^{-2}$ M) of Li^+ also potentiate the accumulation of $InsP_2$, whilst $InsP_3$ levels appeared to be relatively insensitive to Li^+. These findings suggest that in longitudinal smooth muscle, the three phosphatases responsible for the hydrolysis of the three above

Table 2. Total [^{3}H]inositol phosphate ([^{3}H]Ins P) production and contracility in guinea-pig longitudinal muscle in response to carbamylcholine (CCH; 10^{-4} M). Effect of substituting Ca^{2-} with Mn^{2+}

Conditions	[^{3}H]Ins P DPM	P		Contractility
10^{-3} M Ca^{2+}				
1. Control	10665 ± 801(3)			0
2. CCH	95819 ± 1856(3)	$P < 0.001$	vs line 1	0.58 ± 0.09(5)
10^{-5} M Ca^{2+}				
3. Control	5312 ± 479(3)	$P < 0.01$	vs line 1	0
4. CCH	46255 ± 2067(3)	$P < 0.001$	vs line 2	0.33 ± 0.06(4)
10^{-5} M Ca^{2+}, 10^{-3} M Mn^{2+}.				
5. Control	7731 ± 568(3)	N.S.	vs line 1	0
6. CCH	92768 ± 13254(3)	N.S.	vs line 2	0.10 ± 0.03(3)

All incubations for measurements of [^{3}H]inositol phosphates were for 20 min. The figures in parentheses represent the number of determinations. P values refer to [^{3}H] Ins P measurements and were determined by Student's *t*-test.

inositol phosphates are distinct enzymes which show differential sensitivity to Li^+. The short-term exposure of smooth muscle strips to Li^+ did not affect their contractile responses, confirming the results of Watson and Downes [10] and implying that neither Li^+ nor inositol phosphates had acute effects on stimulus-contraction coupling.

In general, a close correlation was apparent between smooth muscle contractility in response to neurotransmitter stimuli and inositol lipid hydrolysis. The relative concentration–response curves for these phenomena indicate that maximal contractile activity is elicited by concentrations of carbamylcholine which induce the breakdown of only a small proportion of available inositol lipids, suggesting the presence of 'spare receptors' [22]. Furthermore, it was found that maximal concentrations of substance P, histamine and noradrenaline produced contractile responses comparable to those obtained with maximal concentrations of carbamylcholine, despite the fact that the latter drug resulted in higher levels of inositol phosphates. This observation also suggests that the contractile apparatus can be fully activated when inositol lipid breakdown is submaximal. It is of additional interest that noradrenaline potentiated inositol lipid breakdown in response to a maximal concentration of carbamylcholine, suggesting that occupation of all cholinergic receptors was insufficient to cause the breakdown of all available inositol phospholipids. Thus there appears to be, at least in longitudinal smooth muscle, a reserve not only of receptors but also of the tissue potential for hydrolysing phosphoinositides.

A key question regarding the possible role of stimulated phosphoinositide hydrolysis in tissues has been the relationship with calcium mobilization [1, 3–5]. In rabbit iris muscle, $PtdInsP_2$ breakdown in response to acetylcholine was impaired in the absence of Ca^{2+} from the external medium [14], suggesting that this process may be Ca^{2+}-dependent. In contrast, enhanced ^{32}P-labelling of PtdIns in guinea-pig longitudinal muscle during carbamylcholine stimulation was unaffected by a number of Ca^{2+}-antagonistic drugs [12], implying that accelerated turnover of this lipid was not a secondary response to increased Ca^{2+} uptake. In the present study, carbamylcholine-stimulated inositol lipid hydrolysis persisted in the presence of either D600 or Mn^{2+}, two calcium channel blockers which completely inhibited contractile responses. Furthermore, the calcium channel agonist CGP 28392, which was effective in causing smooth muscle contraction, failed to significantly influence phosphoinositide hydrolysis, again suggesting that Ca^{2+} entry *per se* was not sufficient to trigger lipid breakdown. However, reduction of the Ca^{2+} concentration of the external medium from 10^{-3} M to 10^{-6} M progressively suppressed both contractility and phosphoinositide breakdown, suggesting that the presence of Ca^{2+}, if not its entry into the tissue, was required for maximal inositol lipid hydrolysis. The possibility that Ca^{2+} plays a permissive rather than an active role in this process is supported by the observation that the reduced formation of inositol phosphates in low Ca^{2+} conditions could be restored by Mn^{2+} despite continued suppression of smooth muscle contractility. Thus, the use of D600 or Mn^{2+} reveals a dissociation between contractility and phosphoinositide breakdown, and further suggests that the latter is not itself triggered by increased Ca^{2+} entry, but is nevertheless dependent on the concentration of divalent cations (normally Ca^{2+}) in the extracellular medium. It is possible that Mn^{2+} can substitute for Ca^{2+} in supporting inositol lipid hydrolysis but not smooth muscle contraction. Alternatively, the relative concentrations of Ca^{2+} on either side of the plasma membrane may be important in modulating inositol lipid metabolism, possibly through the maintenance of membrane electrical potential [23, 24]. It is interesting in this respect that Mn^{2+} causes a repolarisation of the plasma membrane and recovery of resistance following exposure of visceral smooth muscle to a Ca^{2+}-free solution [23]. The maintenance of electrical potential by ionic gradients may be an important determinant of membrane phospholipid conformation and hence susceptibility to enzymatic hydrolysis.

Acknowledgements—This work was supported by the Medical Research Council.

REFERENCES

1. R. H. Michell, *Biochim. biophys. Acta.* **415**, 81 (1975).
2. J. N. Hawthorne and M. R. Pickard, *J. Neurochem.* **32**, 5 (1975).
3. M. J. Berridge, *Molec. Cell. Endocrinol.* **24**, 141 (1981).
4. R. H. Michell, *Cell Calcium* **3**, 285 (1982).
5. J. N. Fain, in *Hormone Receptors* (Ed. L. D. Kohn), pp. 237–276. John Wiley & Sons, New York (1982).
6. S. S. Jafferji and R. H. Michell, *Biochem. J.* **154**, 653 (1976).
7. O. Canessa de Scarnati and E. G. Lapetina, *Biochim. biophys. Acta.* **360**, 298 (1974).
8. A. A. Abdel-Latif, R. A. Akhtar and J. N. Hawthorne, *Biochem. J.* **162**, 61 (1977).
9. A. A. Abdel-Latif, K. Green and J. P. Smith, *J. Neurochem.* **32**, 225 (1979).
10. S. P. Watson and C. P. Downes, *Eur. J. Pharmac.* **93**, 245 (1983).
11. S. P. Watson, *Biochem. Pharmac.* **33**, 3733 (1984).
12. S. S. Jafferji and R. H. Michell, *Biochem. J.* **160**, 163 (1976).
13. R. A. Akhtar and A. A. Abdel-Latif, *Biochem. J.* **192**, 783 (1980).
14. R. A. Akhtar and A. A. Abdel-Latif, *J. Pharmac. exp. Ther.* **204**, 655 (1978).
15. H. A. Krebs and K. Henseleit, *Hoppe-Zeyler's Z. physiol. Chem.* **210**, 33 (1932).
16. M. J. Berridge, C. P. Downes and M. R. Hanley, *Biochem. J.* **206**, 578 (1982)
17. M. J. Berridge, R. M. C. Dawson, C. P. Downes, J. P. Heslop and R. F. Irvine, *Biochem. J.* **212**, 473 (1983).
18. T. B. Bolton, *J. Physiol.* **220**, 647 (1972).
19. T. B. Bolton and J. P. Clark, *Br. J. Pharmac.* **72**, 319 (1981).
20. R. F. Irvine, A. J. Letcher, D. J. Lander and C. P. Downes, *Biochem. J.* **223**, 237 (1984).
21. J. H. Allison and M. E. Blisner, *Biochem. Biophys. Res. Commun.* **68**, 1332 (1976).
22. R. H. Michell, S. S. Jafferji and L. M. Jones, *FEBS Lett.* **69**, (1976).
23. E. Bülbring and T. Tomita, *Proc. Roy. Soc. Lond. B.* **172**, 121 (1969).
24. E. Bülbring and T. Tomita, *Proc. Roy. Soc. Lond. B.* **197**, 271 (1977).