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Early production of 1,4,5-Inositol Trisphosphate and 1,3,4,5-Inositol Tetrakisphosphate by Histamine and Carbachol in Ileal Smooth Muscle

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SUMMARY

We have examined the time course of the formation of inositol mono-, bis-, tris, and tetrakisphosphates ($InsP_1$, $InsP_2$, $InsP_3$, and $InsP_4$, respectively) in slices of the longitudinal muscle of guinea pig small intestine that had been prelabeled with *myo*-^{3}H-inositol. The agonists employed were histamine and carbachol. $InsP_3$ increases immediately with a time course which is similar to that of the increase in contractile force and remains elevated for the rest of the incubation period. High performance liquid chromatography analysis revealed that $InsP_3$ is composed of two isomers, the 1,4,5- and 1,3,4-isomers. The release of 1,4,5-inositol trisphosphate [Ins(1,4,5)P_3] was followed by the rapid accumulation of $InsP_4$ and later on by the formation of 1,3,4-inositol trisphosphate [Ins(1,3,4)P_3]. Ins(1,3,4)P_3 and $InsP_4$ were identified by co-chromatography with the Ins(1,3,4)P_3 and 1,3,4,5-inositol tetrakisphosphate prepared from ^{3}H-Ins(1,4,5)P_3 using a kinase from rat brain. The time course of accumulation of these compounds is consistent with a second messenger role of Ins(1,4,5)P_3 in initiation of smooth muscle contraction.

Interaction of ligands with Ca^{2+}-mobilizing receptors is thought to result in the generation of two second messengers, both of which are derived from phosphatidylinositol 4,5-bisphosphate. These are Ins(1,4,5)P_3 and diacylglycerol (1, 2). Diacylglycerol is believed to remain in the membrane where, together with Ca^{2+}, it can activate protein kinase C (3). The other messenger, Ins(1,4,5)P_3, apparently exerts its action by causing the release of Ca^{2+} from an intracellular site (4). This calcium store is located in a compartment of the endoplasmic reticulum and is released following activation of a specific Ins(1,4,5)P_3 receptor which has recently been detected by direct studies of the binding of radiolabeled Ins(1,4,5)P_3 to a microsomal fraction of rat liver (5).

Studies of Ins(1,4,5)P_3 in stimulated tissues have been complicated by the recent finding that another inositol trisphosphate, Ins(1,3,4)P_3, is formed on stimulation with the Ca^{2+}-mobilizing agonist carbachol (6, 7). In the light of the finding that a large proportion of the total $InsP_3$ is not the Ins(1,4,5)P_3 isomer but, rather, Ins(1,3,4)P_3, the relevance of ^{3}H-$InsP_3$ measurement could be questioned. However, the two isomers have entirely different kinetics of metabolism; Ins(1,4,5)P_3 is generated almost instantaneously, whereas production of Ins(1,3,4)P_3 is delayed, as is its decline on removal of the stimulus (8). One possible source of Ins(1,3,4)P_3 is dephosphorylation of 1,3,4,5-IP_4, which is formed as rapidly as Ins(1,4,5)P_3 in brain tissue and cultured GH_4 cells (9–11). The exact roles of Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4 are not known and it may be that both compounds play major roles as intracellular messengers (9, 11).

The sequence of events leading from receptor occupation to the initiation of smooth muscle contraction is unclear. However, it is known that an increase in cytoplasmic free Ca^{2+} is necessary for contraction, especially during early periods of stimulation (12). Recent studies in cultured vascular smooth muscle cells loaded with the calcium probe Quin 2 revealed that histamine and norepinephrine, acting on the H_1 histamine receptor and the α_1-adrenoceptor, respectively, may initiate the contraction by a rapid release of Ca^{2+} from cellular storage sites in the absence of extracellular Ca^{2+} (13, 14). Indirect studies of ^{32}P-labeled phosphate incorporation into phosphatidylinositol in longitudinal muscle of guinea pig ileum, rabbit iris, and dog trachea, and recent studies of accumulation of ^{3}H-inositol phos-

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ABBREVIATIONS: Ins(1,4,5)P_3, 1,4,5-inositol trisphosphate; Ins(1,3,4)P_3, 1,3,4-inositol trisphosphate; $InsP_3$, inositol trisphosphate; 1,3,4,5-IP_4, D-inositol 1,3,4,5-tetrakisphosphate; Ins(1,3,4,5)P_4, 1,3,4,5-inositol tetrakisphosphate; $InsP_4$, inositol tetrakisphosphate; TCA, trichloroacetic acid; IP_1, IP_2, IP_3 and IP_4, inositol mono-, bis-, tris-, and tetrakisphosphates, respectively; HPLC, high performance liquid chromatography; $InsP_2$, inositol bisphosphate; $InsP_1$, inositol monophosphate; Quin 2, 2-[[2-bis-(carboxymethyl)amino-5-methylphenoxy]-methyl]-6-methoxy-8-bis-(carboxymethyl)amino-quinoline tetrakis-acetoxymethyl)ester.

phates in these tissues, have indicated that each of the stimuli which provoked contraction also elicited, through the same receptor system, an increase in turnover of inositol lipids (15–20). The finding that Ins(1,4,5)P_3 induces the release of Ca^{2+} in saponin-permeabilized smooth muscle cells provides additional evidence that Ins(1,4,5)P_3 provides the link between receptor stimulation and internal Ca^{2+} release and, hence, activation of the contractile apparatus (20).

In longitudinal smooth muscle of guinea pig small intestine, which is one of the most important preparations for the study of smooth muscle function, inositol phosphates also seem to be involved (21, 22), but the situation is more complex. Changes in accumulation of total inositol phosphates have been observed, but incubation times of minutes have been employed, whereas the onset of the contraction occurs within a few seconds. In addition, whereas the changes in inositol phosphate accumulation elicited by carbachol are blocked in the expected fashion by atropine, the responses to histamine are less dramatic, observed only at high concentrations of the agonist, and they do not appear to be blocked by the H_1 antagonist mepyramine (21). These observations have led Donaldson and Hill (21) to suggest that a novel type of histamine receptor may be involved. The recent reports of the rapid formation of Ins(1,3,4,5)P_4 and the metabolism of $InsP_3$ mentioned above prompted us to investigate the levels of $InsP_3$ and $InsP_4$ at time intervals similar to those over which contraction is observed. These findings are reported here.

Materials and Methods

Separation of ^{3}H-inositol phosphates. Hartley guinea pigs (weight 200–300 g) were used for all experiments. Slices (350 × 350 μm) of ileal longitudinal muscle were obtained with a McIlwain tissue chopper and were then incubated at 37° in 3 ml of oxygenated Krebs-Henseleit buffer. After 60 min the Krebs buffer was replaced by fresh buffer containing *myo*[1,2-^{3}H]inositol (~1.5 μM) and the incubation was continued for a further 2 hr at 37° in a shaking incubator. The prelabeled slices were then washed and resuspended in fresh Krebs medium. Aliquots of gravity-packed slices (about 1 mg of protein) were transferred to vials containing 200 μl of Krebs medium containing LiCl (10 mM). When antagonists were used, they were added 15 min before the addition of the agonist. Incubation was terminated by addition of 300 μl of 1 M TCA. Samples were thoroughly mixed and allowed to stand for at least 20 min at room temperature and then centrifuged at 3000 × *g* for 15 min. Aliquots of 500 μl of supernatant were washed five times with 2 volumes of water-saturated diethyl ether, and the pH was adjusted to between 7 and 8 by addition of 5 mM $NaHCO_3$. The extract was then subjected to anion exchange chromatography on Dowex-1 columns (8% cross-linked, mesh size 200–400, formate form). *myo*-Inositol, IP_1, and IP_2 were eluted by the stepwise addition of solutions containing increasing levels of formate as described by Berridge *et al.* (23). IP_3 and 1,3,4,5-IP_4 were successively eluted with 10 ml of 0.1 M formic acid/0.8 M ammonium formate and 10 ml of 0.1 M formic acid/1.0 M ammonium formate, respectively (11).

The effectiveness of this separation method was confirmed by using authentic samples of ^{3}H-*myo*-Ins(1,4,5)P_3 (Amersham) and ^{3}H-$InsP_4$ that was prepared from rat brain as described below. The recovery from parallel runs with ^{3}H-*myo*-Ins(1,34,5)P_3 in the $InsP_3$ fraction isolated by Dowex-1 anion exchange chromatography was 96 ± 8%.

Formation of Ins(1,4,5)P_3, Ins(1,3,4)P_3, and $InsP_4$ in the homogenates of rat brain and guinea pig longitudinal muscle. Ten pmol of ^{3}H-*myo*-Ins(1,4,5)P_3 were incubated for various times at pH 7.5 in 0.05 M Tris-maleate buffer containing 0.05 ml of a homogenate of rat brain or guinea pig longitudinal muscle in 0.15 M sucrose (24). The final volume was 0.2 ml and the solution contained 10 mM ATP and 20 mM $MgCl_2$. The reaction was terminated by addition of 0.5 ml of 20% ice-cold TCA and 0.05 ml of 5% bovine serum albumin. The TCA was removed by four diethyl ether washes and the mixture was neutralized with ammonium hydroxide. The mixtures were then subjected to anion exchange chromatography on Dowex-1 anion exchange columns, and the inositol phosphates were separated as described above. The $InsP_3$ and $InsP_4$ fractions were freeze dried and then used for HPLC analysis.

Separation of inositol phosphates by HPLC. The samples were reconstituted in 1 ml of water containing ATP (30 μg/ml) as a marker since, on our HPLC system, this compound co-chromatographed with the 1,3,4-isomer (6). The isomers of $InsP_3$ and $InsP_4$ were separated by a modification of the methods of Irvine *et al.* (6) and Binder *et al.* (25). The chromatography column was a 0.46 × 25 cm Hypersil APS-1 high performance anion exchange column with 5 μm of packing material packed by Chromatography Sciences Company (St. Laurent, Quebec). The column was fitted with a 0.2 × 2 cm guard column filled with Whatman Pell PAC (Cyano-amino groups bonded to 35-μm beads). A 300–500-μl portion of the sample was injected into a Rheodyne 7125 valve fitted with a 1-ml sample loop and eluted with water at a flow rate of 1.2 ml/min for the first 5 min and then a 25-min gradient increasing from water to 1.7 M ammonium formate adjusted to pH 3.7 with phosphoric acid, using equipment from Waters (Mississauga, Ontario). Ultraviolet absorbance was monitored at 254 nm in order to detect ATP and 1-min fractions were collected. This procedure is referred to as procedure A. For the samples incubated with *myo*-[1,2-^{3}H]inositol which had not been subjected to anion exchange chromatography, a modified stepwise gradient was performed. Solvent A was water and solvent B was 1.7 M ammonium formate buffered to pH 3.7 with phosphoric acid; the flow rate was 1.0 ml/min. The solvent program was water for 5 min, a linear increase over 30 min to 60% solvent B; 60% solvent B for 6 min, followed by a linear gradient over 10 min to 100% solvent B. Volumes of the collected fractions were 1 ml (0–18 min), 0.5 ml (18–28 min), and 1 ml (28–70 min). Volumes of the collected fractions after injection of ^{3}H-$InsP_4$ separated from rat brain homogenate were 1 ml (10–24 min), 0.25 ml (24–40 min), and 1 ml (40–70 min). This procedure is referred to as procedure B. Under these HPLC conditions, standard ^{3}H-*myo*-Ins(1,4,5)P_3 was eluted at 23–24 min and the standards AMP, ADP, and ATP were eluted at 15, 18, and 22 min, respectively. Radioactivity was determined by liquid scintillation counting in a Beckman LS 6800 scintillation counter using commercial scintillant (United Technologies Packard). Radioactivity was calculated as dpm/mg of protein in each sample. Protein was determined by a modification of the Lowry procedure (26).

Measurement of contractile response in longitudinal smooth muscle. Longitudinal muscle strips from guinea pig ileum prepared as described by Rang (27) were suspended under a resting tension of 0.5 g in organ baths of 10 ml working volume containing Krebs-Henseleit solution, gassed with O_2/CO_2 (95:5), and maintained at 37°. Isometric contractions were recorded by means of Grass force displacement transducers (model FT03) connected to a Grass 7D polygraph. The strips were allowed to equilibrate for 1 hr before drugs were applied. Contractions in response to a single dose of histamine or carbachol (10^{-4} M) were recorded for 45 min.

Drugs. *myo*-[1,2-^{3}H]Inositol (47 Ci/mmol) was purchased from New England Nuclear. D-*myo*-[2-^{3}H]Inositol 1,4,5-triphosphate (1 Ci/mmol) was obtained from Amersham. In a previous study we found that *myo*-[1,2-^{3}H]inositol breaks down spontaneously to yield radioactive decomposition products which have been found in all fractions corresponding to inositol phosphates (28). Therefore, immediately before its addition to the incubation medium, the tracer was purified by passing through a column of Dowex resin in formate form. All other chemicals and drugs were purchased from Sigma Chemical Co. or Fisher Scientific.

Statistics. Statistical comparisons were conducted by an analysis of variance. The significance level chosen was 0.05 throughout.

Results

Formation of inositol phosphates. Data for the time course of the production of $InsP_3$, $InsP_4$, $InsP_2$ and $InsP_1$ are shown in Fig. 1 for carbachol and Fig. 2 for histamine. The values are expressed as percentage of a nonstimulated control. Within the first minute of stimulation by carbachol (0.1 mM) there was no significant elevation of the levels of $InsP_1$. Incubation for 10 min provided a significant elevation of $InsP_1$, while after 30 min there was a 9-fold increase over control levels corresponding to a radioactivity of 41,633 ± 6,434 dpm/mg of protein. Similar data were obtained when $InsP_2$ was measured. There was a dramatic increase in $InsP_2$ at 10 and 30 min to 17,693 ± 2,199 and 25,620 ± 3,584 dpm/mg of protein, respectively (907 and 1,314%), whereas over the first minute of stimulation $InsP_2$ levels did not change significantly. When $InsP_3$ and $InsP_4$ were measured after carbachol stimulation, a different picture emerged. There was an elevation of $InsP_3$ at 1, 5, and 10 sec to 842 ± 92, 1,058 ± 98, and 1,036 ± 108 dpm/mg of protein, representing 137, 172, and 169% of control values, respectively. After 1 min, the levels did not differ significantly from control values, but after 10 and 30 min of stimulation there was, again, a highly significant increase. It should be pointed out that the anion exchange chromatographic technique used to separate the various inositol polyphosphates does not enable us to separate isomers of $InsP_3$, a point which is relevant to the interpretation of these findings and which is discussed later. Finally, the $InsP_4$ was elevated above control

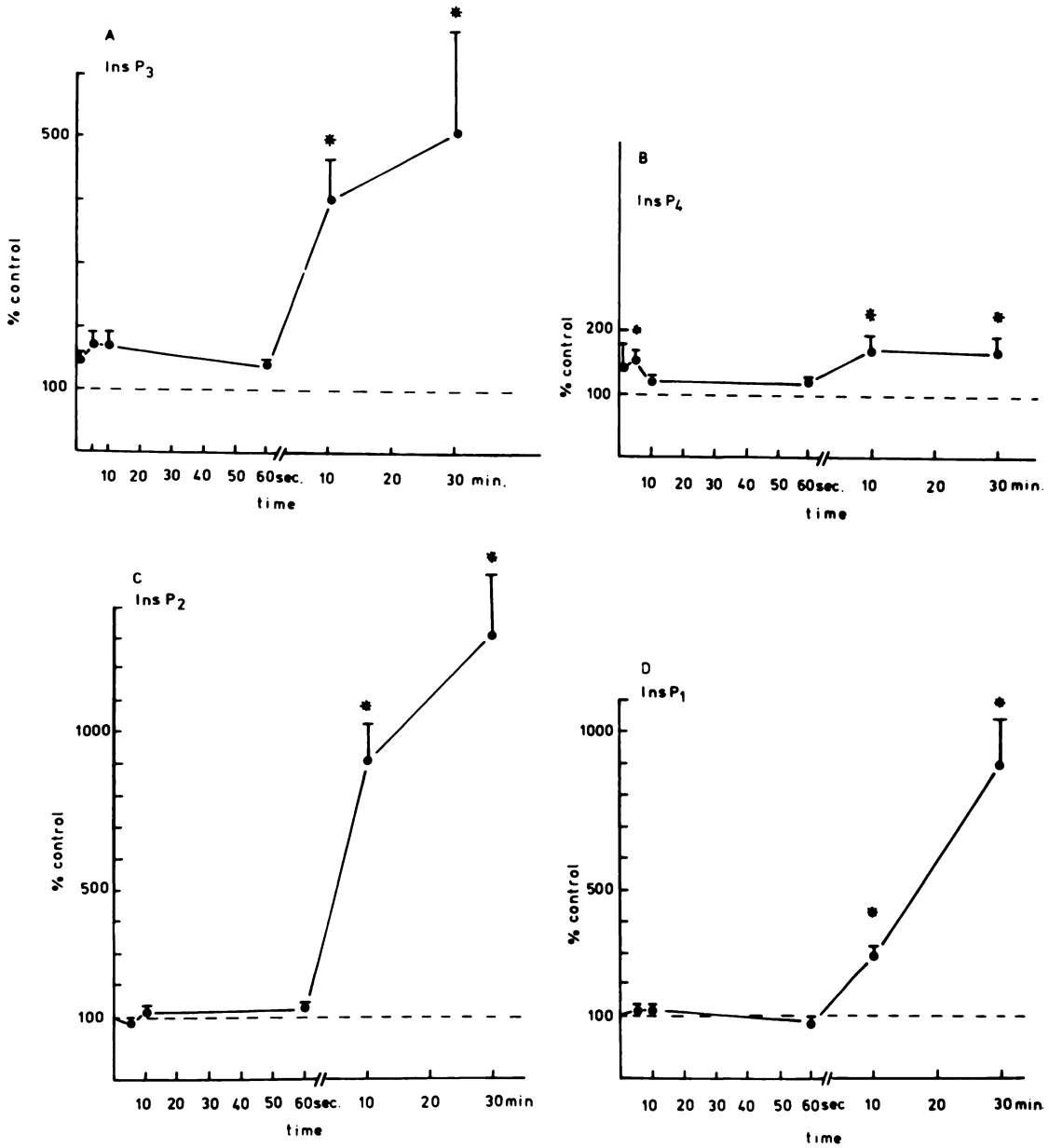

Fig. 1. Time course of formation of $InsP_1$, $InsP_2$, $InsP_3$, and $InsP_4$ in ileal slices stimulated with carbachol (0.1 mM). Results are expressed as percentages of corresponding controls (means ± SE; $n = 14$, except for $InsP_4$ when $n = 20$ and except at 1 sec when $n = 3$). *, significantly different from control ($p < 0.05$). A, ^{3}H-$InsP_3$; B, ^{3}H-$InsP_4$; C, ^{3}H-$InsP_2$; D, ^{3}H-$InsP_1$. Basal ^{3}H-$InsP_3$ accumulation in the absence of carbachol was 615 ± 33 dpm/mg of protein (mean ± SE, $n = 26$); basal ^{3}H-$InsP_4$ = 486 ± 22 dpm/mg of protein ($n = 34$); basal ^{3}H-$InsP_2$ = 1950 ± 412 dpm/mg of protein ($n = 21$); basal ^{3}H-$InsP_1$ = 4666 ± 415 dpm/mg of protein ($n = 15$).

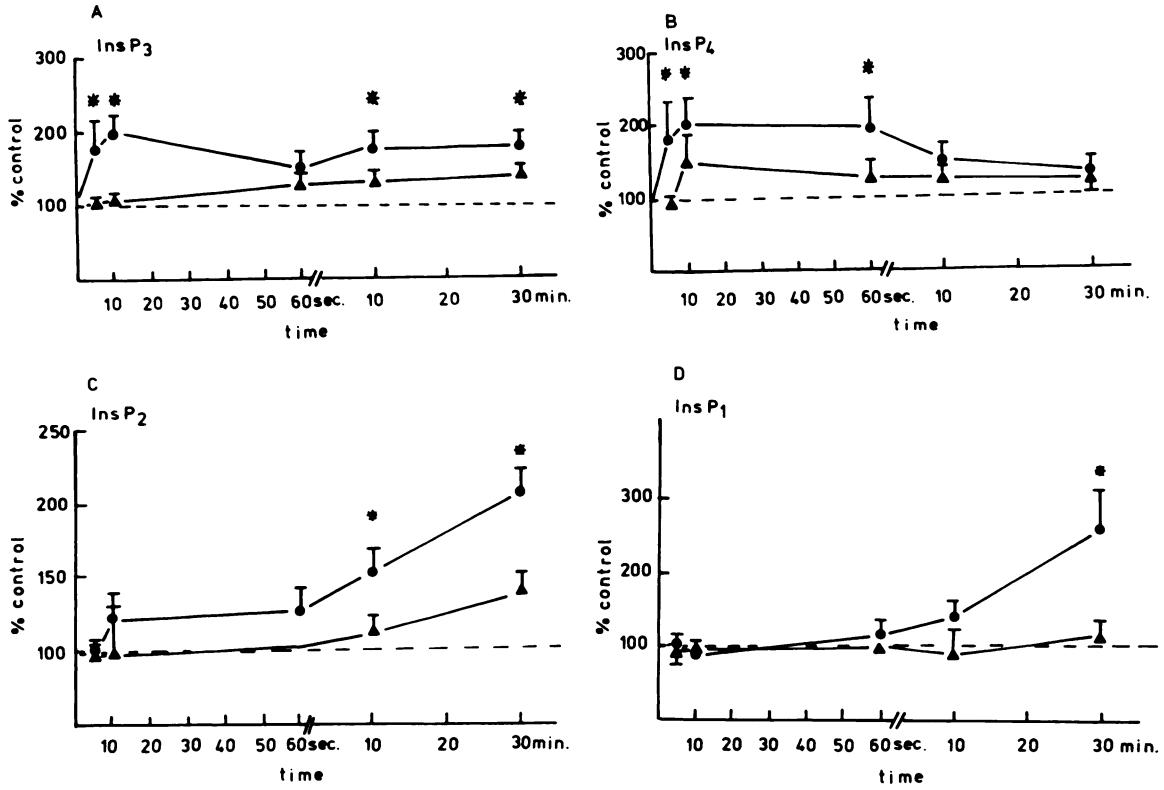

Fig. 2. Time course of formation of $InsP_1$, $InsP_2$, $InsP_3$, and $InsP_4$ in ileal slices stimulated with histamine (0.1 mM) in the absence (n = 12) and presence of mepyramine (▲, 10^{-7} M, n = 7). Results are expressed as percentages of corresponding controls (mean ± SE). *, significantly different from control ($p < 0.05$). A, ^{3}H-$InsP_3$; B, ^{3}H-$InsP_4$; C, ^{3}H-$InsP_2$; D, ^{3}H-$InsP_1$. Basal ^{3}H-$InsP_3$ accumulation in the absence of histamine was 646 ± 31 dpm/mg of protein (mean ± SE, n = 16); basal ^{3}H-$InsP_4$ = 501 ± 40 dpm/mg of protein (n = 20); basal ^{3}H-$InsP_2$ = 2533 ± 214 dpm/mg of protein (n = 20); basal ^{3}H-$InsP_1$ = 5852 ± 773 dpm/mg of protein (n = 24).

levels from the earliest time period (144 and 157% at 1 and 5 sec, respectively), and then declined at 10 and 60 sec and was somewhat elevated at 10 and 30 min. The second accumulation of radioactivity in the $InsP_4$ fraction on stimulation with carbachol might represent $InsP_5$ and $InsP_6$, reported recently by Heslop *et al.* (9) in GH_4 cells. However, using our present HPLC conditions, we could not detect any accumulation of radioactivity corresponding to these higher inositol phosphates, and the origin of second peak of radioactivity detected by anion exchange chromatography remains to be established. Studies of the response to histamine showed a somewhat similar picture. There seemed to be a progressive increase in $InsP_1$ as the incubation of the tissue proceeded (Fig. 2D), but this did not reach statistical significance until the incubation had proceeded for 30 min. This increase, which presents 15,425 ± 3,247 dpm/mg of protein, was reduced by mepyramine (0.1 μM). Similar results were obtained when $InsP_2$ was measured (Fig. 2C). $InsP_3$ (Fig. 2A) reached a peak level at 10 sec of incubation, 1,270 ± 180 dpm/mg of protein, 197%, then declined. $InsP_4$ (Fig. 2B) was increased after 5 and 10 sec of stimulation and then returned to control levels at 10 and 30 min. The responses at 5, 10, and 60 sec were mepyramine sensitive.

Fig. 3 shows the log dose-response curve for histamine stimulation of $InsP_3$ formation at 10 sec compared with the dose-response curve for the induced contraction of longitudinal muscle strips from guinea pig small intestine. The dose-response curve for contraction lies well to the left of the dose-response curve for the $InsP_3$ accumulation (EC_{50} 6 × 10^{-6} M).

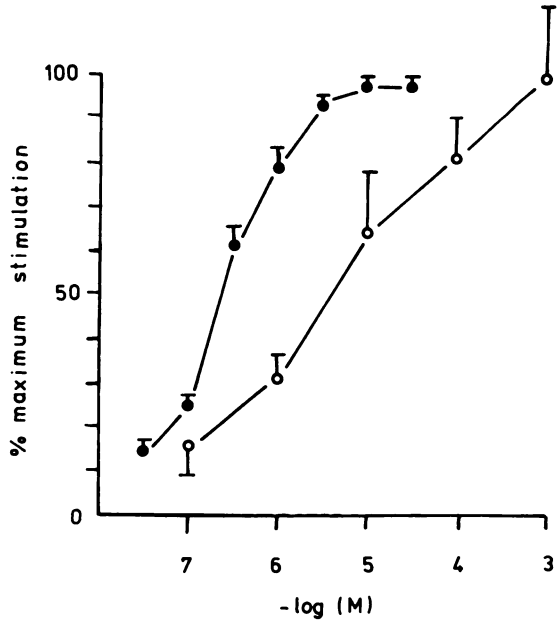

Fig. 3. Comparison of dose-response curves for histamine-mediated responses of the guinea pig longitudinal muscle. Contraction (●, n = 12) and ^{3}H-$InsP_3$ formation at 10 sec of stimulation (○, n = 10). The results are expressed as the percentage of the maximum stimulation (mean ± SE).

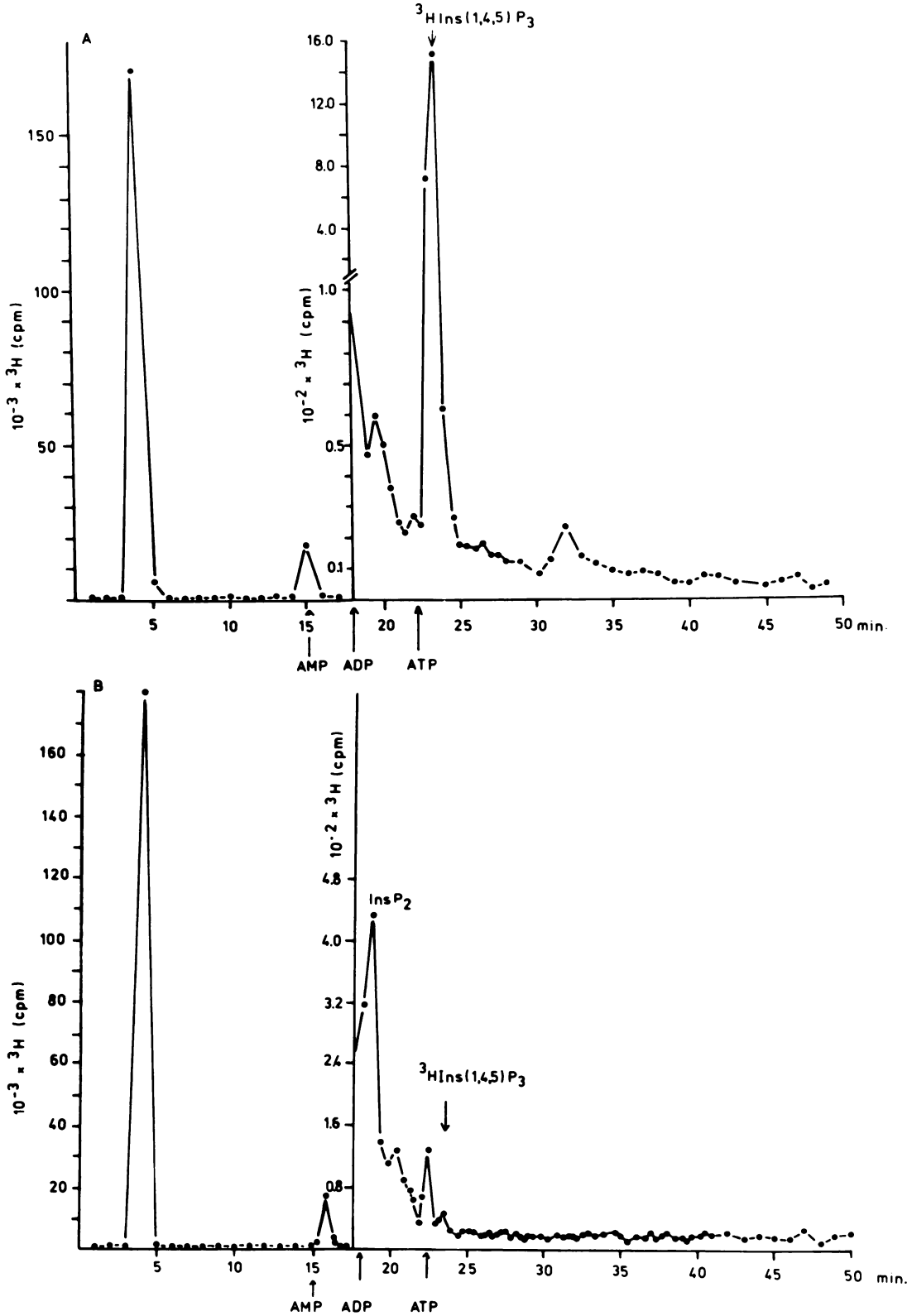

Fig. 4. HPLC analysis of metabolites of ^{3}H-*myo*-inositol from slices of longitudinal muscle of guinea pig ileum using elution procedure B. The tissue slices were prelabeled with ^{3}H-*myo*-inositol and incubated for 10 sec (A) and 10 min (B) with 0.1 mM carbachol, then, the radiolabeled products were recovered and analyzed by HPLC as described in Materials and Methods. The position of ^{3}H-Ins(1,4,5)P_3 standard is indicated by the *arrows*. ^{3}H-Ins(1,3,4)P_3 is considered to correspond to ATP.

Fig. 4 illustrates the HPLC analysis of the inositol phosphates from longitudinal smooth muscle of guinea pig ileum that had been prelabeled with ^{3}H-*myo*-inositol and then stimulated with 0.1 mM carbachol for 10 sec (Fig. 4A) or 10 min (Fig. 4B) in the presence of 10 mM LiCl. The elution procedure was procedure B (see Materials and Methods). AMP corresponded to $InsP_1$. The radiolabeled compound which co-eluted with ADP was designated $InsP_2$. The compound eluted just after $InsP_2$ may represent an isomer of Ins(1,4)P_2 such as Ins(1,3)P_2. The elution profile of the $InsP_3$ shows two peaks. The ratio of the height of the two peaks varies with the duration of stimulation. The peak eluted just after ATP corresponds to standard ^{3}H-*myo*-Ins(1,4,5)P_3 and it predominates at 10 sec of stimulation. The radioactivity which co-eluted with ATP appears to correspond to the Ins(1,3,4)P_3 and it predominates at 10 min of stimulation. To verify that the peak with the longer retention time observed at 10 sec of stimulation was in fact Ins(1,3,4,5)P_4, radioactive material was isolated from rat brain homogenate and separated by Dowex ion exchange chromatography. The $InsP_4$ fraction was reanalyzed by HPLC. Fig. 5 shows the HPLC elution pattern of this fraction using procedure B. There is only one major peak at 31 min which presumably corresponds to Ins(1,3,4,5)P_4. It implies that Ins(1,3,4,5)P_4 formation precedes Ins(1,3,4)P_3 accumulation, in agreement with recent work in rat tissues (11, 24). We carried out HPLC analysis of the inositol phosphates from homogenates of guinea pig longitudinal muscle incubated with ^{3}H-*myo*-Ins(1,4,5)P_3 under the conditions reported to be optimal for Ins(1,4,5)P_3-3-kinase in rat brain, liver, and pancreas (24). After isolation by Dowex anion exchange chromatography using elution procedure A, only one peak was observed in the $InsP_4$ fraction at 21 min, which corresponds to the retention time of Ins(1,3,4,5)P_4 separated from rat brain (Fig. 6). Further analysis of the samples incubated for 5 min with ^{3}H-Ins(1,4,5)P_3 and separated by Dowex anion exchange chromatography in the $InsP_3$ fraction showed two peaks, one at 16 and one at 18 min (Fig. 7); the first peak co-eluted with ATP and corresponds to Ins(1,3,4)P_3, and the second one co-eluted with ^{3}H-Ins(1,4,5)P_3. Reanalysis of the sample of the rat brain homogenate incubated with ^{3}H-Ins(1,4,5)P_3 for 10 min and separated as the $InsP_3$ fraction showed a single peak at 16 min, corresponding to Ins(1,3,4)P_3. Rapid conversion of ^{3}H-*myo*-Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 in homogenates of rat brain and guinea pig small intestine confirms the involvement of the inositol tris/tetrakisphosphate route in the rat brain and implies the existence of this novel pathway in smooth muscle.

Contractility studies. The equivalent contractility data are shown in Fig. 8. Both histamine and carbachol produced a rapid phasic response in this preparation with mean times to peak tension of 4.5 ± 0.4 sec for histamine and 3.45 ± 0.19 sec for carbachol and mean times to 50% maximum of 1.45 ± 0.9 sec for histamine and 1.58 ± 0.06 sec for carbachol. The response then declined somewhat, and then a sustained tonic response which represented about 50% of the maximum response became established. Under these conditions it has been shown that significant desensitization exists (29). Thus, the accumulation of $InsP_1$ and $InsP_2$ which these studies and others have demonstrated after prolonged incubation is a phenomenon which is not apparent early on, and thus does not parallel the initial contraction. There appears to be an early increase in $InsP_3$ with a time course which is similar to the mechanical response followed by a late accumulation of this compound, whereas $InsP_4$ peaks early but is no longer present in this form after prolonged incubation in the case of histamine, although there

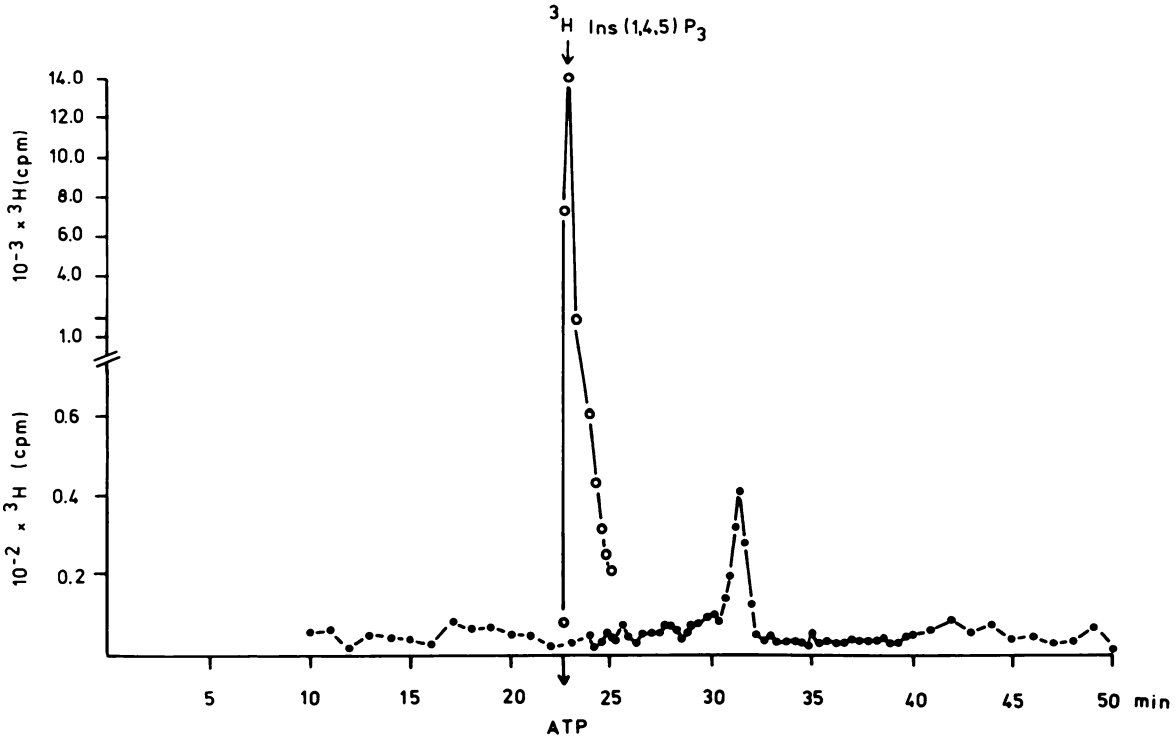

Fig. 5. Separation of inositol 1,3,4,5-tetrakisphosphate by HPLC using elution procedure B. ^{3}H-Ins(1,4,5)P_3 was incubated for 5 min with a homogenate of rat brain. The ^{3}H-$InsP_4$ fraction purified from Dowex columns was analyzed by HPLC as described in Materials and Methods. The ^{3}HIns(1,4,5)P_3 standard is indicated by an *arrow*.

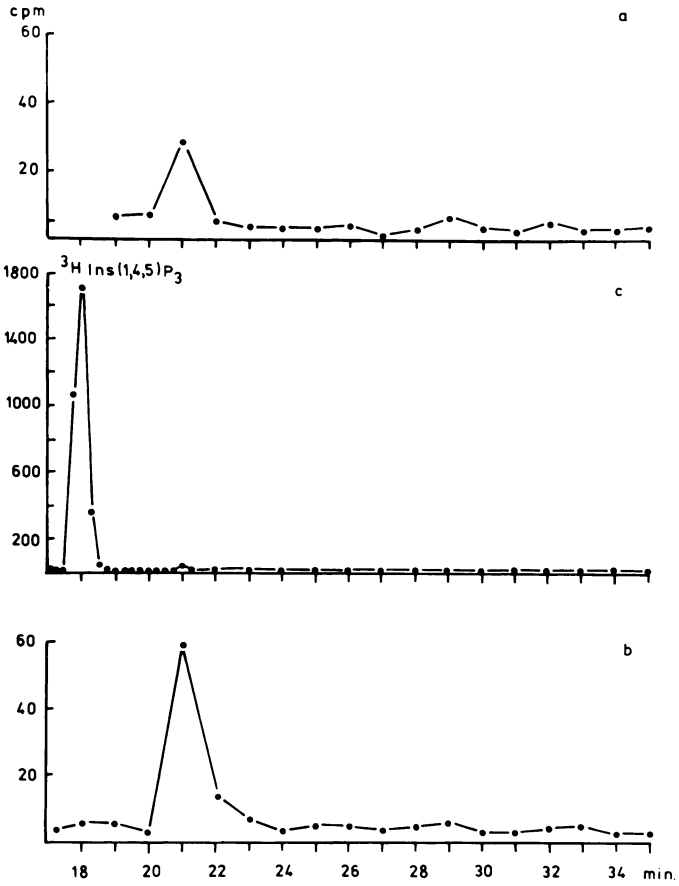

Fig. 6. Separation of inositol 1,3,4,5-tetrakisphosphate by HPLC using elution procedure A. a and b illustrate experiments where the ^{3}H-Ins(1,4,5)P_3 was incubated for 5 min with homogenate of guinea pig ileum and rat brain, respectively. The ^{3}H-InsP_4 fractions purified from Dowex columns were analyzed by HPLC as described in Materials and Methods. c illustrates the original ^{3}H-Ins(1,4,5)P_3 which was analyzed using the same technique.

seems to be a late elevation in IP_4 after stimulation with carbachol.

Discussion

The observations reported here confirm previous demonstrations that concentrations of histamine and carbachol that induce smooth muscle contraction also stimulate polyphosphoinositide hydrolysis and inositol phosphate accumulation in longitudinal muscle slices. The new information contributed by the present study is that the predominant inositol trisphosphate formed in smooth muscle during the first seconds of stimulation with carbachol or histamine is the Ins(1,4,5)P_3 isomer only. The identification of this compound as Ins(1,4,5)P_3 is supported by its chromatographic behavior relative to standard ^{3}H-*myo*-Ins(1,4,5)P_3, and by detection of Ins(1,3,4,5)P_4 as its metabolic product.

This last compound is probably a precursor of Ins(1,3,4)P_3 in muscle slices and in homogenates of smooth muscle. The observation of an early stimulation of the formation of Ins(1,4,5)P_3 by carbachol and histamine suggests that the following sequence of events may occur. First, there is rapid formation of Ins(1,4,5)P_3 followed by intracellular release of Ca^{2+} from sarcoplasmic reticulum. Second, the second messenger action of Ins(1,4,5)P_3 is terminated both by conversion to

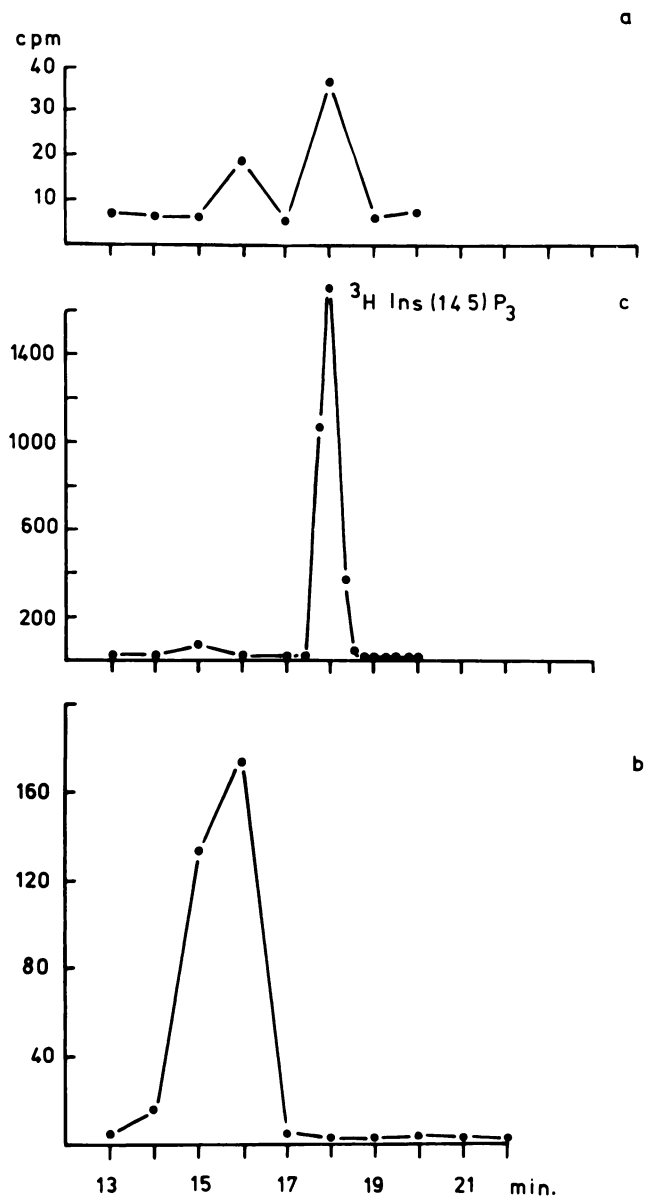

Fig. 7. Separation of inositol trisphosphate isomers by HPLC using elution procedure A. a shows the chromatogram where ^{3}H-Ins(1,4,5)P_3 was incubated with a homogenate of guinea pig ileum for 5 min and the ^{3}H-InsP_3 fractions purified from Dowex columns were analyzed by HPLC as described in Materials and Methods. b shows a similar experiment using a homogenate from rat brain and an incubation time of 10 min. c shows the chromatogram from ^{3}HIns(1,4,5)P_3 obtained using the same technique.

Ins(1,3,4,5)P_4, probably via Ins(1,4,5)P_3-3-kinase, and by dephosphorylation to InsP_2. Finally, in keeping with the proposed role of diacylglycerol as a second messenger, a rapid increase in this lipid could occur upon receptor stimulation which should in turn be followed by activation of protein kinase C and hence phosphorylation of the light chain of myosin, similar to the process reported in other tissues (30). The observation of a very rapid rise in Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 is consistent with there being some causal relationship between this early production of inositol phosphates and development of the mechanical response. The maximal production of Ins(1,4,5)P_3 on stimulation with histamine and carbachol occurs after the maximal contraction has developed. One possible explanation for this

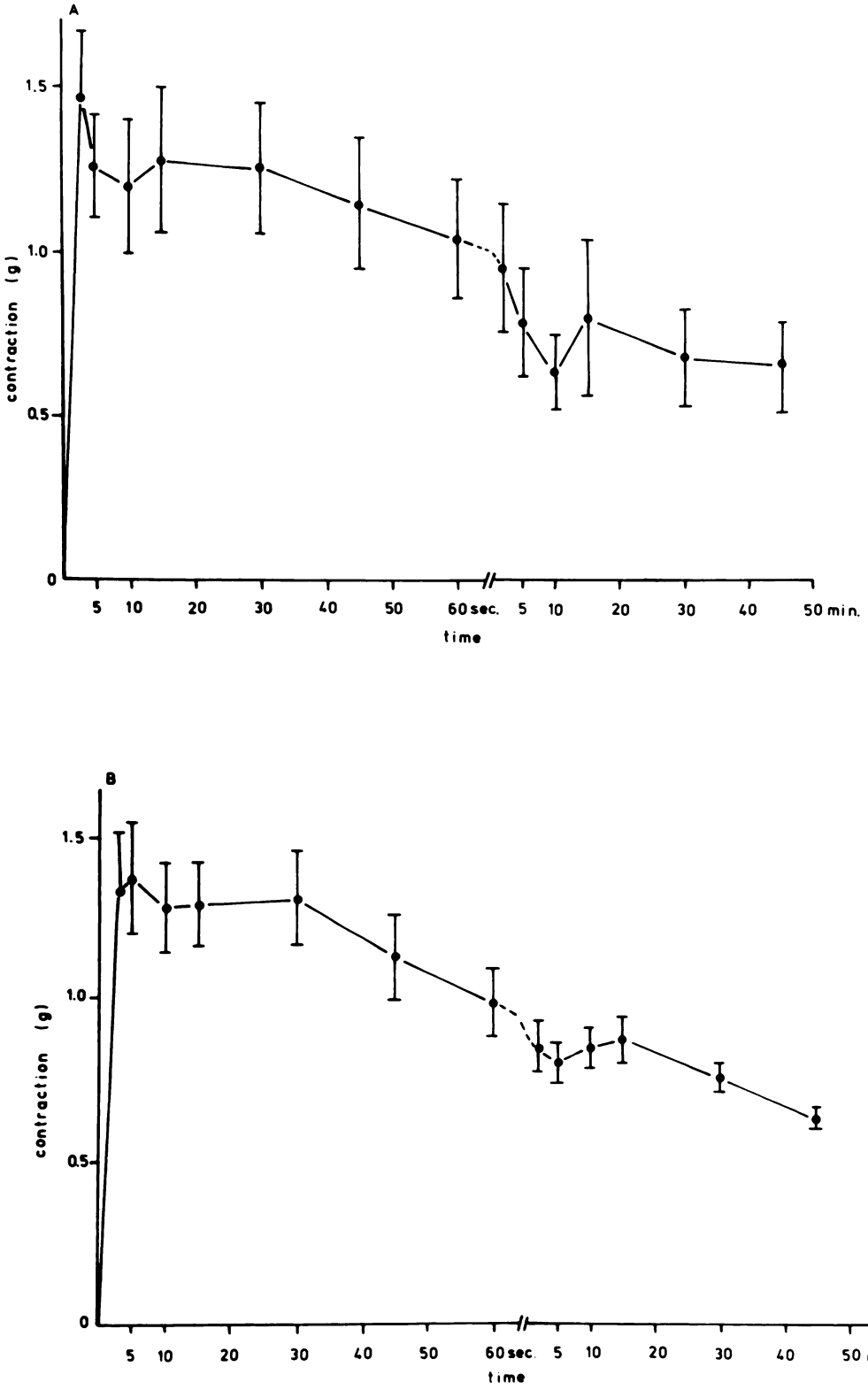

Fig. 8. Time course of contractile response to carbachol (10^{-4} M) and histamine (10^{-4} M). Responses are mean response (in grams tension) ± SE; n = 6. Agonists were added at time zero. A, Carbachol; B, histamine.

finding is the presence of a substantial receptor reserve for both these substances in smooth muscle. If, indeed, there are spare receptors for histamine and acetylcholine, it is possible that rapid activation of a small proportion of them could achieve the maximal response and that, if there were no "receptor reserve" for Ins(1,4,5)P_3 formation, then it might be anticipated that peak Ins(1,4,5)P_3 production and peak tension development might not occur at the same time. The receptor reserve hypothesis mentioned above may also provide an explanation for the wide discrepancy in concentrations between the EC_{50} for contraction (10^{-7} M) and for Ins(1,4,5)P_3 formation (6 × 10^{-6} M) (31, 32). It would be supposed that at the low concentration a sufficient proportion of receptors would be occupied to generate the contractile response, but a much

higher concentration would be required to generate maximal production of $Ins(1,4,5)P_3$, the latter requiring greater receptor occupancy. This suggestion is supported by a recent study of polyphosphoinositide levels in response to carbachol in airway smooth muscle. The maximal contractile response to carbachol was obtained when less than 20% of the high affinity muscarinic receptors were occupied, and under these circumstances less than 30% of the maximum production of inositol phosphates was achieved (33). It is also possible that the receptor reserve is not for the agonist *per se*, but resides in the receptors that respond to the second messenger and which mediate the release of Ca^{2+}. Thus, a small amount of $Ins(1,4,5)P_3$ could effect maximal release of Ca^{2+} and maximal contraction despite the ability of a higher concentration of agonist to continue to generate $Ins(1,4,5)P_3$. Indeed, it has been suggested that a single molecule of $InsP_3$ can cause the release of about 20 calcium ions (2). This suggestion leads to a number of testable conclusions, some of which are currently being investigated.

The decline in $Ins(1,4,5)P_3$ after 60 sec which is observed in the case of both carbachol and histamine corresponds to a decline in developed tension, but the subsequent elevation at 10 and 30 min may be a different phenomenon. As mentioned earlier, anion exchange chromatography cannot distinguish $Ins(1,4,5)P_3$ from other isomers of $InsP_3$, and recently it has been suggested that a significant proportion of total $InsP_3$ is not the active $Ins(1,4,5)P_3$ but $Ins(1,3,4)P_3$, whose biological role is not yet well understood. Studies by Irvine *et al.* (7) and by Burgess *et al.* (8) show that there is a latent period before the 1,3,4-isomer appears. Moreover, Burgess *et al.* (8) have found that, in pancreatic acinar cells, the accumulation of $Ins(1,4,5)P_3$ and secretion from these cells were unaffected by the presence of Li^+, whereas this cation increased the levels of the 1,3,4-isomer. On the basis of this finding they suggest that the 1,3,4-isomer neither releases Ca^{2+} nor inhibits the action of the 1,4,5-isomer and that the enzymes degrading the two isomers are different. They also suggested that $Ins(1,3,4)P_3$ does not regulate the rate of formation or degradation of $Ins(1,4,5)P_3$ (8). Our HPLC analysis revealed that the predominant isomer of $InsP_3$ formed during the first seconds of stimulation was $Ins(1,4,5)P_3$, and the only isomer at 10 min of stimulation was identified as $Ins(1,3,4)P_3$. It is thus possible that the secondary accumulation of $InsP_3$ induced by carbachol and histamine actually represents the appearance of the 1,3,4-isomer. This observation is consistent with the proposal of Burgess *et al.* (8) that the 1,3,4-isomer may be formed as a means of desensitizing the cellular component which interacts with Ca^{2+}. This is also consistent with the loss of tone to a sustained response of only about 50% of the maximal response. Alternatively, protein kinase C may be physiologically involved in homologous desensitization, as it has been shown for hormone-mediated responses in a variety of cells (34). Rapid accumulation of ^3H-$Ins(1,3,4,5)P_4$ on stimulation with histamine or carbachol coincided with the early increase in ^3H-$Ins(1,4,5)P_3$ formation and with maximal contraction. The early increase in ^3H-$Ins(1,3,4,5)P_4$ formation and the gradual accumulation of $Ins(1,3,4)P_3$ suggests that the latter may be formed as a consequence of $Ins(1,3,4,5)P_4$ breakdown. Batty *et al.* (11) proposed that $Ins(1,3,4,5)P_4$ is the precursor for $Ins(1,3,4)P_3$ and that the most likely source of the tetrakisphosphate is hydrolysis of phosphatidylinositol-3,4,5-trisphosphate by phosphodiesterase. If $Ins(1,3,4,5)P_4$ is indeed derived from this phospholipid, then the former could be the precursor of $Ins(1,4,5)P_3$ with the proportion of the other $InsP_3$ isomers dependent on the respective activities of appropriate 5- and 3-phosphatases (10, 11). The only obvious alternative route of synthesis of $Ins(1,3,4,5)P_4$ is by phosphorylation of $Ins(1,4,5)P_3$, which has recently been shown to be possible by the demonstration of the existence of a novel $Ins(1,4,5)P_3$-3-kinase that converts $Ins(1,4,5)P_3$ to $Ins(1,3,4,5)P_4$ (24). The function of $Ins(1,3,4,5)P_4$ is as yet unknown. It may be that it is a messenger with a distinct function, and this is a possibility that requires further investigation. However, it may represent merely an intermediate in the production or metabolism of another second messenger, or may have no significant role at all. The $InsP_1$ and $InsP_2$ continue to rise under circumstances where a steady contraction equal to about 50% of the maximal response is maintained. There is also the possibility that phosphatidylinositol and phosphatidylinositol-4-phosphate may be the potential sources of the appropriate inositol phosphates $InsP_1$ and $InsP_2$, respectively (10). Also, the sequence of $Ins(1,3,4)P_3$ dephosphorylation has not yet been defined; thus, different inositol bis- and monophosphates may arise from the two isomers of $InsP_3$. Our findings of the rapid release of ^3H-$Ins(1,4,5)P_3$ on stimulation of histamine H_1 receptors and muscarinic cholinoceptors, together with the simultaneous formation of $Ins(1,3,4,5)P_4$, both of which precede the formation of $InsP_2$ and $InsP_1$, support the view that $Ins(1,4,5)P_3$ plays a role as a second messenger in the initiation of contractions in response to histamine and carbachol in the longitudinal muscle of guinea pig ileum.

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