THE ROLE OF INOSITOL 1,4,5-TRISPHOSPHATE IN MOBILIZING CALCIUM FROM INTRACELLULAR STORES IN THE SALIVARY GLANDS OF *AMBLYOMMA AMERICANUM* (L.)

By

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(L.)

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ABSTRACT

Isolated tick salivary glands, permeabilized with digitonin in the presence of the mitochondrial and non-mitochondrial Ca^{2+} uptake inhibitors, sodium azide and vanadate, released Ca^{2+} in response to 20 μM inositol 1,4,5-trisphosphate (IP_3). Inositol 1-phosphate (IP_1) and inositol 1,4-bisphosphate (IP_2) appeared to stimulate an uptake of Ca^{2+} into whole glands. IP_3 caused release of Ca^{2+} from a 100,000 g microsome enriched pellet; however, IP_1 and IP_2 were ineffective in stimulating an uptake or efflux of Ca^{2+}. The combined 900 g and 11,500 g pellets showed no significant release of Ca^{2+} in response to addition of IP_3. IP_3 concentrations as low as 1 μM are capable of stimulating a significant release of Ca^{2+} from microsomes. Results suggest that intracellular Ca^{2+} is mobilized from microsomal intracellular stores in response to agonists which increase cytosolic IP_3 in tick salivary glands. Results also suggest a possible role for IP_1 or IP_2 or both in stimulating an uptake of Ca^{2+} into vanadate and azide-insensitive intracellular pools.

INTRODUCTION

Michell (1975) noted that plasma membrane-associated phosphoinositide metabolism correlates with many physiological agonists that raise intracellular calcium. Subsequently, a variety of intermediate products of hormone stimulated inositol lipid metabolism were proposed as agonists for mobilizing cytoplasmic calcium (Thomas et al., 1984). Inositol 1,4,5-trisphosphate (IP_3), a product of agonist-induced phosphatidylinositol 4,5-bisphosphate metabolism, was shown to release calcium rapidly from the endoplasmic reticulum of permeabilized cells such as rat pancreas and hepatocytes (Berridge, 1986; Streb et al., 1984). Kinetic analysis has shown that [^{32}P] IP_3 binds rapidly to the microsomal membranes (Spat et al., 1986). Spat et al. (1986) further demonstrated a specific, high affinity binding site for IP_3 on the microsomal fraction of rat liver which corresponds with the ability of IP_3 to release Ca^{2+}. Inositol 1,4-bisphosphate (IP_2) and inositol 4,5-bisphosphate at 1 μM had no effect on IP_3 binding (Spat et al., 1986). Berridge (1986) demonstrated that the vicinal phosphates on the 4 and 5 positions are essential for releasing calcium from intracellular stores and the phosphate on the opposite side of the ring enhances the affinity of IP_3 for its putative receptor. Unlike inositol 1,4,5-trisphosphate, neither inositol 1,3,4-trisphosphate nor inositol 1,3,4,5-tetrakisphosphate are able to mobilize

intracellular calcium (Tilly et al., 1987).

Activation of an endoplasmic reticulum Ca^{2+}-ATPase pump ensures rapid reloading of the endoplasmic reticulum with Ca^{2+} even at low extracellular Ca^{2+}. ATP dependent Ca^{2+} uptake into the endoplasmic reticulum of permeabilized IP_3-prestimulated cells is higher than that into the endoplasmic reticulum of unstimulated cells (Muallen et al., 1988). Non-mitochondrial calcium uptake in rat pancreatic acinar cells can be completely inhibited by 2 mM vanadate,(Streb and Schultz, 1983), and mitochondrial calcium uptake can be inhibited by azide (Streb et al., 1984). Ten micromolar GTP enhances IP_3-induced calcium release from liver microsomes (Dawson, 1985; Ueda, 1986).

Calcium is important in tick salivary gland function (Sauer and Essenberg, 1984). Dopamine and cyclic AMP stimulated fluid secretion by isolated salivary glands is inhibited by low extracellular Ca^{2+} (Needham and Sauer, 1979). Cyclic AMP-dependent phosphodiesterase activity is activated and inhibited by submicromolar and micromolar Ca^{2+} respectively (McMullen et al., 1980), and protein phosphatase activity is inhibited by micromolar Ca^{2+} (Williams et al., 1988). More recently a factor present in the tick's brain was shown to increase the level of inositol trisphosphate demonstrating the existence of the phosphoinositide signal transduction pathway in tick salivary glands (McSwain et al., 1989). This study demonstrates that IP_3 mobilizes Ca^{2+} from intracellular

stores of permeabilized tick salivary glands and that the subcellular location is likely the microsomal fraction. Unexpectedly, it appears that the immediate products of IP_3 metabolism, IP_1 and IP_2 may stimulate an uptake of Ca^{2+} into a non-mitochondrial component of the salivary glands.

MATERIALS AND METHODS

Materials. D-myo-inositol 1,4,5-trisphosphate (95% or greater purity) and D-myo-inositol 1-phosphate were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Indiana. D-myo-inositol 1,4-bisphosphate (98% pure), vanadium oxide, and digitonin were from Sigma Chemical Company, St. Louis, Missouri. TMB-8 [8-(diethylamino)-3,4,5-trimethoxybenzoate] was from Calbiochem Corporation, San Diego, California.

Tissue Preparation. Adult lone star ticks, Amblyomma americanum (L.), were reared following the methods of Patrick and Hair, (1975). Rapidly feeding female ticks (200-800 mg) were dissected at 4 °C in buffer containing 0.1 M MOPS and 20 mM EGTA at pH 6.8.

Calcium determination in whole salivary glands and subcellular fractions. The whole salivary glands or subcellular tissue fractions were placed in a 1 ml reaction medium containing 20 mM HEPES (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 10 μM GTP, 0.1 mM digitonin, 2 mM vanadate, and 0.1 M sodium azide following a modified procedure for measuring Ca^{2+} release from microsomes (Enouf et. al., 1987). With subcellular tissue fractions, digitonin was deleted from the reaction buffer. A miniature calcium electrode (MI-600, Microelectrodes, Inc., Londonderry, New

Hampshire) was pre-calibrated at 1 μM Ca^{2+} using a Corning calcium standard, and then placed into the medium with a micro-reference electrode (MI-402, Microelectrodes, Inc., Londonderry, New Hampshire). The reaction medium was continuously stirred. The IP_1, IP_2, IP_3, and calcium-free H_2O controls were separately added to the reaction medium to determine tissue responses. Inositol phosphates and H_2O were added sequentially at 4 minute intervals. Except for dose response experiments, 20 μM IP_1, IP_2, and IP_3 was used in all experiments. The free medium level of Ca^{2+} was monitored continuously with the use of a signal amplifier (Johnson Research Foundation, Philadelphia, Pa.) and a Bell and Howell oscillographic recorder (Figure 1).

Subcellular fractionation. Fractions were prepared according to a modified procedure of McSwain et al., 1987. Sixty glands were used in each assay. The glands were homogenized in 1 ml of medium containing 0.25 M sucrose, 10 mM tricine buffer (pH 7.2), 10 mM $MgCl_2$, 0.05% p-amino-benzamidine, and 5% (w/v) polyethylene glycol. The crude homogenate was centrifuged at 900 g for 10 min and the pellet was washed twice. The 900 g supernatant was centrifuged at 11,500 g for 10 min and the pellet was washed twice. The 11,500 g supernatant was centrifuged at 100,000 g for 60 min to yield the 100,000 g pellet.

Electron microscopy. The 100,000 g pellet was collected

as described above and fixed according to the procedures of McSwain et al., 1987. Thin sections were obtained with a Sorvall MT-2 ultramicrotome and stained with methanolic uranyl acetate and lead citrate. Sections were examined and photographed with a JEOL cx 2 transmission electron microscope.

RESULTS

Determination of changes in free medium calcium in response to inositol phosphates added to permeabilized salivary glands and subcellular fractions

Changes in medium Ca^{2+} were continuously monitored for 4 minutes in the presence or absence of either single applications or sequential changes of IP_1, IP_2 and IP_3 (Figure 1). Water controls were subtracted from the observed changes. Each experiment was performed in triplicate except Figure 4b represents four replications. An analysis of variance procedure (Steel and Torrie, 1980) was used to evaluate statistical significance of data. A typical chart recording of one experiment after sequential additions of IP_3, H_2O, IP_2, H_2O, IP_1, and H_2O respectively at 4 minute intervals is indicated in Figure 2. Few oscillations in levels of medium Ca^{2+} (Berridge and Galione, 1986) were observed possibly because the Ca^{2+} uptake inhibitors vanadate and azide were present in the bathing medium.

Inositol phosphate effects on whole permeabilized salivary glands

IP_3 stimulated a significant increase in bathing medium Ca^{2+} ($p < 0.05$) whereas IP_1 and IP_2 produced a significant decrease ($p < 0.05$) (Figure 3). Additions of IP_3 followed by H_2O, IP_2 and H_2O at 4 minute intervals stimulated a release of Ca^{2+} in response to IP_3 (Figure 4a), but not IP_2. TMB-8, a

calcium release inhibitor (Lydan and O'Day, 1988), completely inhibited the effect of IP_3 (Figure 4b). When IP_2 was added to whole permeabilized glands prior to adding IP_3 (Figure 5), a significant ($p < 0.05$) decrease in medium Ca^{2+} (tissue uptake) was observed while subsequent addition of IP_3 stimulated a release of Ca^{2+} ($p < 0.05$).

Inositol phosphate effects on fractionated salivary gland tissue

The 100,000 g pellet (Figure 6) contained a homogenous mixture of free or attached ribosomes and small membrane-bound vesicles (microsomes). These results are comparable to those observed by McSwain et al. (1987) in the microsomal fraction of tick salivary glands. The addition of IP_3 to the 100,000 g pellet (Figure 7) resulted in a significant release of Ca^{2+}. There were no significant changes in the bathing medium Ca^{2+} after adding IP_1, IP_2 or IP_3 to combined non-microsomal fractions (900 g and 11,500 g pellets) (Figure 8). Sequential additions of increasing concentrations of IP_3 to microsomal tissue (Figure 9) indicated that 1 μM IP_3 stimulated a significant ($p < 0.05$) release of Ca^{2+} from microsomes although 20 μM concentrations were more effective. IP_3 was ineffective in stimulating a release of Ca^{2+} from non-microsomal tissue (Figure 10).

Figure 1. Method for determining release of calcium from tick salivary glands with the use of a microelectrode. Changes in free medium levels of calcium from fourteen permeabilized tick salivary glands (0.1 mM digitonin) or non-permeabilized microsomes prepared from sixty glands were monitored with a Ca^{2+} sensitive microelectrode in response to adding IP_1, IP_2, IP_3 and H_2O.

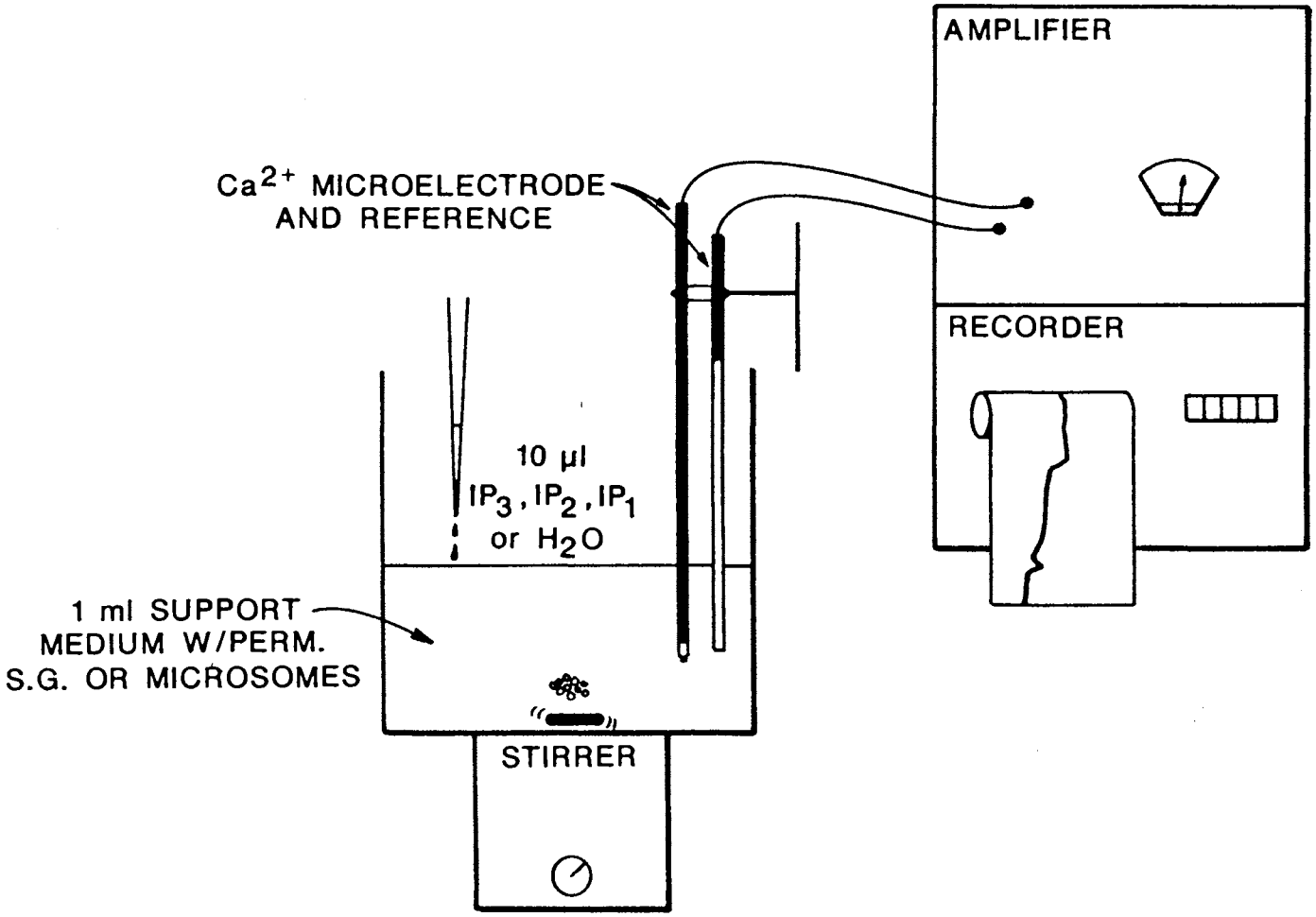

AMPLIFIER
Ca^{2+} MICROELECTRODE
AND REFERENCE
RECORDER
10 µl
IP_3, IP_2, IP_1
or H_2O
1 ml SUPPORT
MEDIUM W/PERM.
S.G. OR MICROSOMES
STIRRER

Figure 2. A representative temporal trace of changes in free medium calcium after adding 20 μM IP_1, IP_2, IP_3 and H_2O to tick salivary gland microsomes. The times of IP_1, IP_2, IP_3 and H_2O additions are indicated at arrows and experiments were performed sequentially, left to right. A 10 mm recorder deflection corresponded to a 5 nmole change in bathing medium Ca^{2+}.

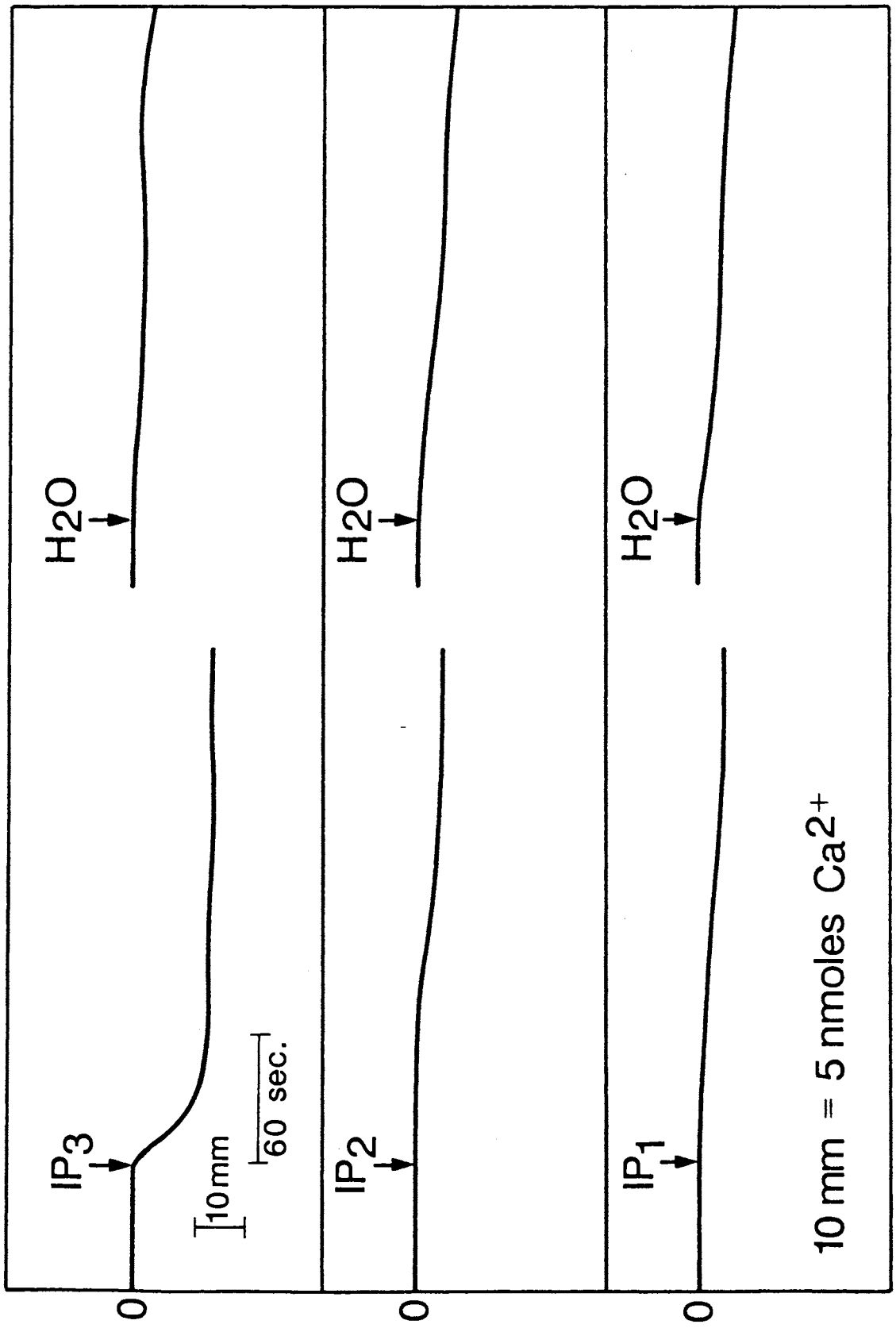

H2O
H2O
H2O
IP3
IP2
IP1
10mm
60 sec.
0
0
0
10 mm = 5 nmoles Ca2+

Figure 3. Changes in free medium Ca^{2+} (see Figures 1 and 2) after adding IP_1 (□), IP_2 (○) and IP_3 (△) to whole permeabilized tick salivary glands. Additions of the three inositol phosphate analogs were monitored in separate experiments and compared with a H_2O control. An analysis of variance was used to determine significant differences at the $p < 0.05$ level. The effects of IP_1 and IP_2 were significantly different from all other additions after 2 minutes. The effect of IP_3 was significantly different from the effects of IP_1 and IP_2 at all times. Values represent the mean $\pm$ S.E.M.

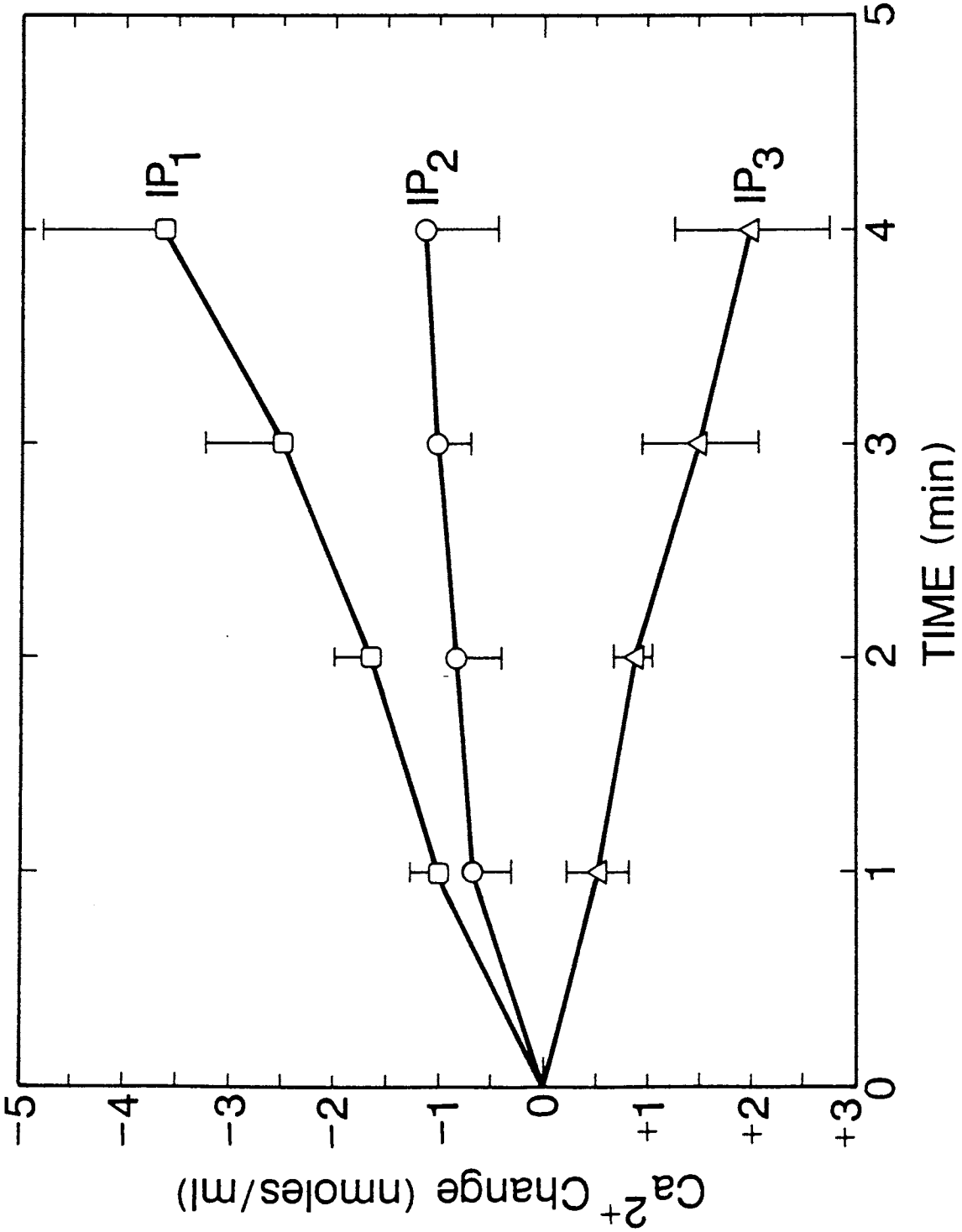

IP1
IP2
IP3
-5
-4
-3
-2
-1
0
+1
+2
+3
0
1
2
3
4
5
TIME (min)
Ca2+ Change (nmoles/ml)

Figure 4. Changes in free medium Ca^{2+} (see Figures 1 and 2) after adding IP_2 (○) and IP_3 (△) to permeabilized tick salivary glands. IP_3, H_2O, IP_2 and H_2O; respectively, were added to the medium at 4 minute intervals. TMB-8 (75 μM) was used to inhibit IP_3-sensitive calcium release. An analysis of variance was used to determine significant differences at the $p < 0.05$ level. Effects of IP_3 were significantly different with and without TMB-8 after 1 minute. Values represent the mean $\pm$ S.E.M.

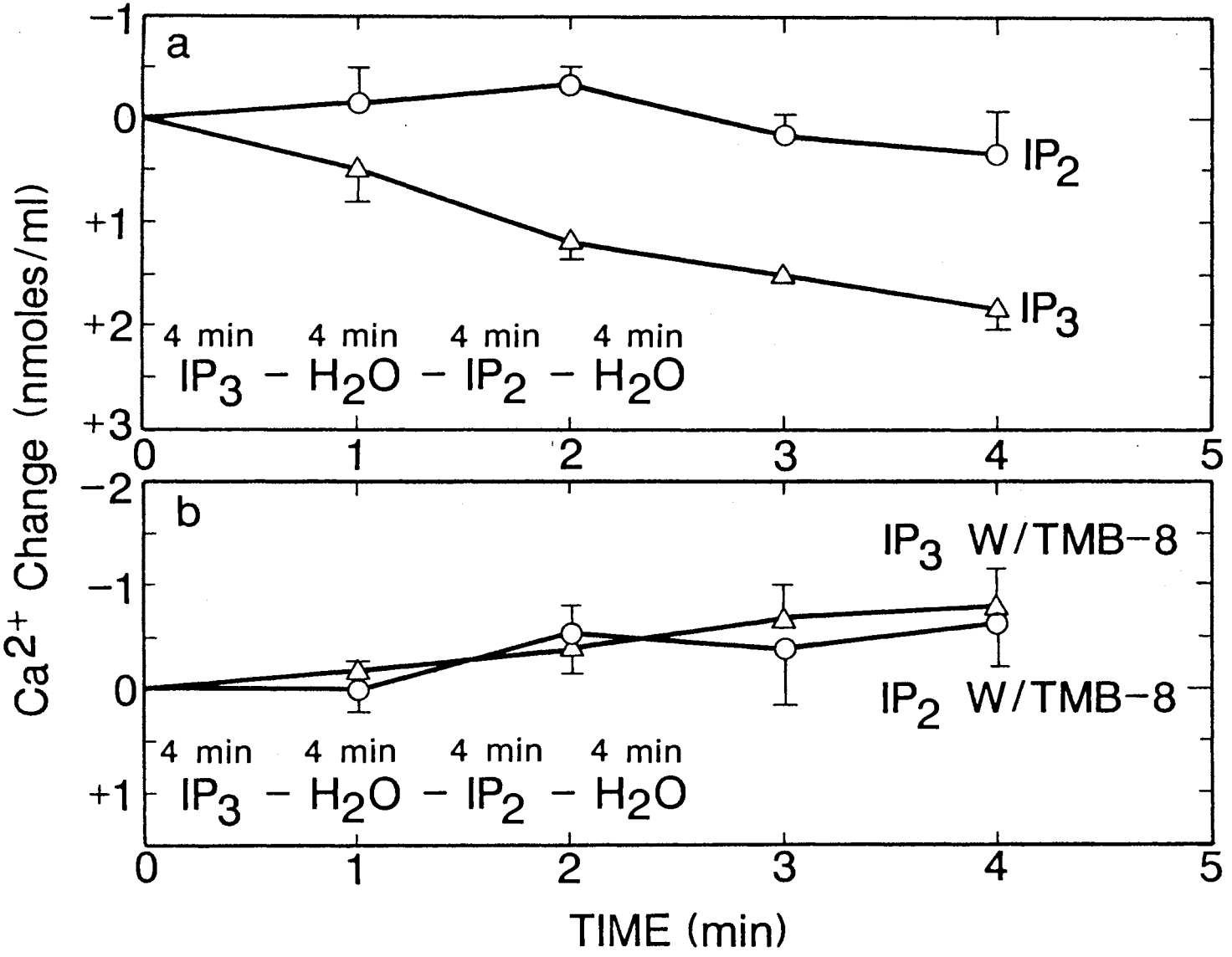

a
b
IP_3
IP_2
IP_3 W/TMB-8
IP_2 W/TMB-8
4 min 4 min 4 min 4 min
$IP_3 - H_2O - IP_2 - H_2O$
Ca^{2+} Change (nmoles/ml)
TIME (min)
−1
0
+1
+2
+3
−2
−1
0
+1
0 1 2 3 4 5

Figure 5. Changes in free medium Ca^{2+} (see Figures 1 and 2) after adding IP_2 (○) and IP_3 (△) to permeabilized tick salivary glands. IP_2, H_2O, IP_3 and H_2O; respectively, were added to the medium at 4 minute intervals as illustrated in Figure 2. An analysis of variance was used to determine significant differences at the $\bar{p} < 0.05$ level. This order of addition produced a significant difference with the IP_2 when added before IP_3. Values represent the mean $\pm$ S.E.M.

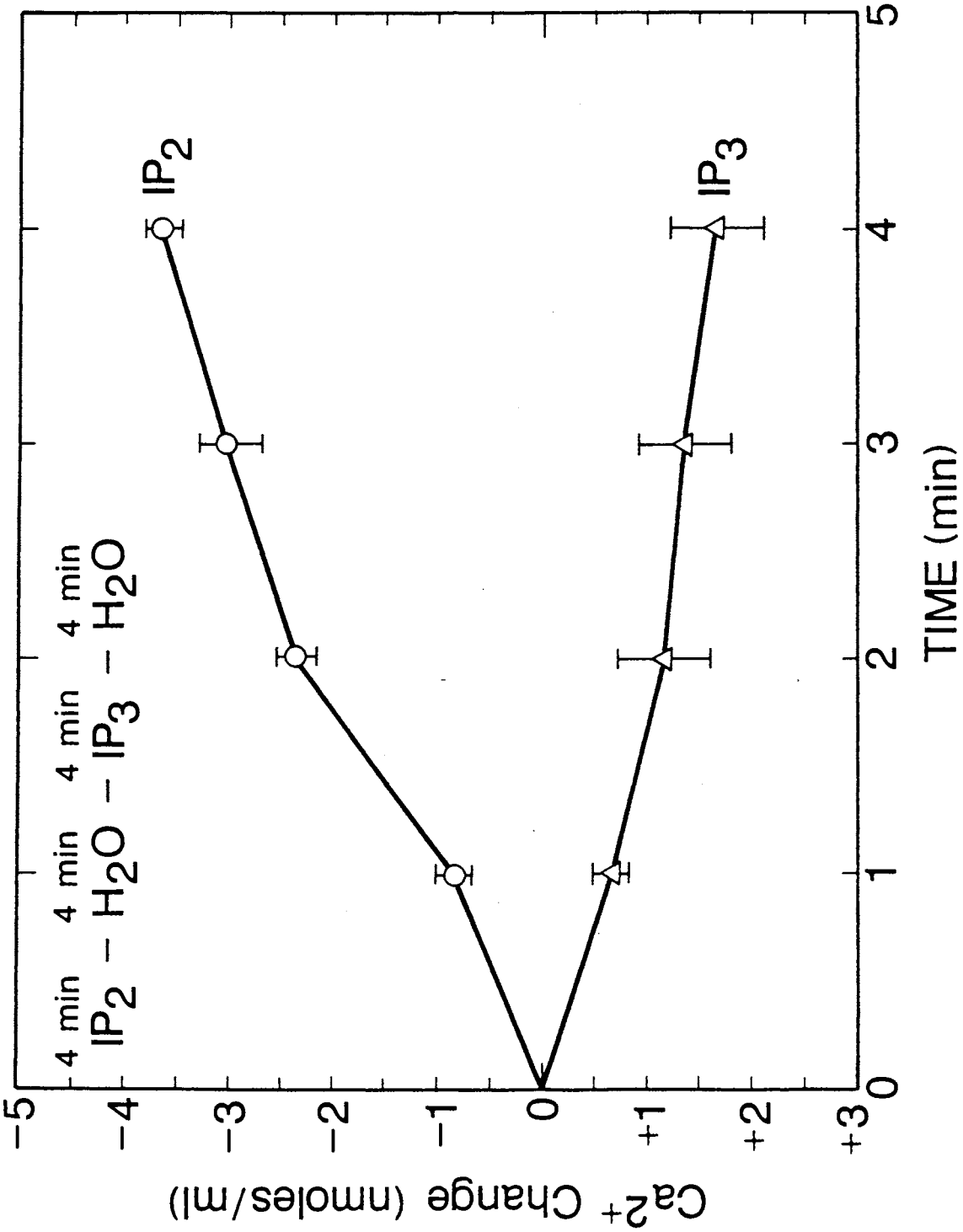

4 min 4 min 4 min 4 min
IP2 – H2O – IP3 – H2O
IP2
IP3
Ca2+ Change (nmoles/ml)
TIME (min)
−5
−4
−3
−2
−1
0
+1
+2
+3
0
1
2
3
4
5

Figure 6. Electron micrograph of the 100,000 g pellet from feeding lone star tick salivary gland tissue. A large number of free or attached ribosomes (R) and membrane-bound vesicles (►) were present in the total 100,000 g pellet. Figure B represents an enlargement of the indicated portion in Figure A (X19,268).

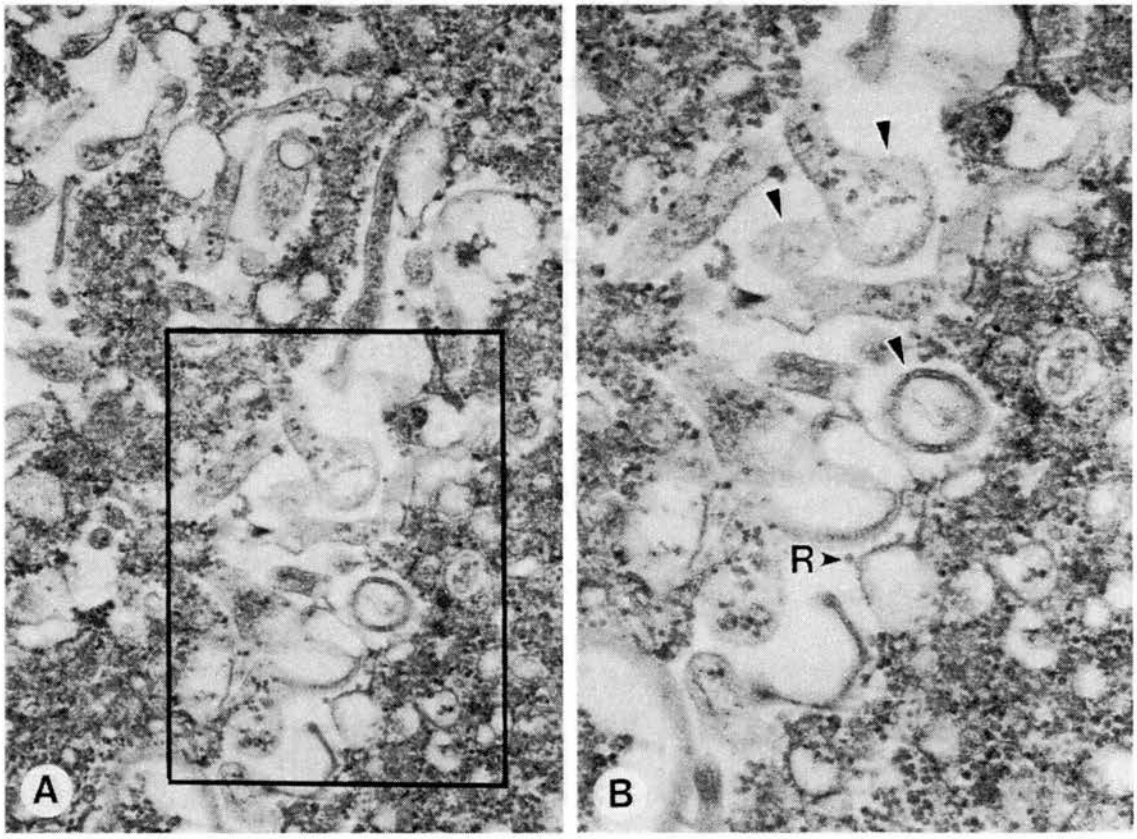

A
B
R

Figure 7. Changes in free medium Ca^{2+} (see Figures 1 and 2) after adding IP_1 (□), IP_2 (○) and IP_3 (△) to tick salivary gland microsomes. IP_3, H_2O, IP_2, H_2O, IP_1 and H_2O; respectively, were added at 4 minute intervals as illustrated in Figure 2. An analysis of variance was used to determine significant differences at the $p < 0.05$ level. The effect of IP_3 was significantly different from the effects of IP_1 and IP_2 after 2 minutes. Values represent the mean $\pm$ S.E.M.

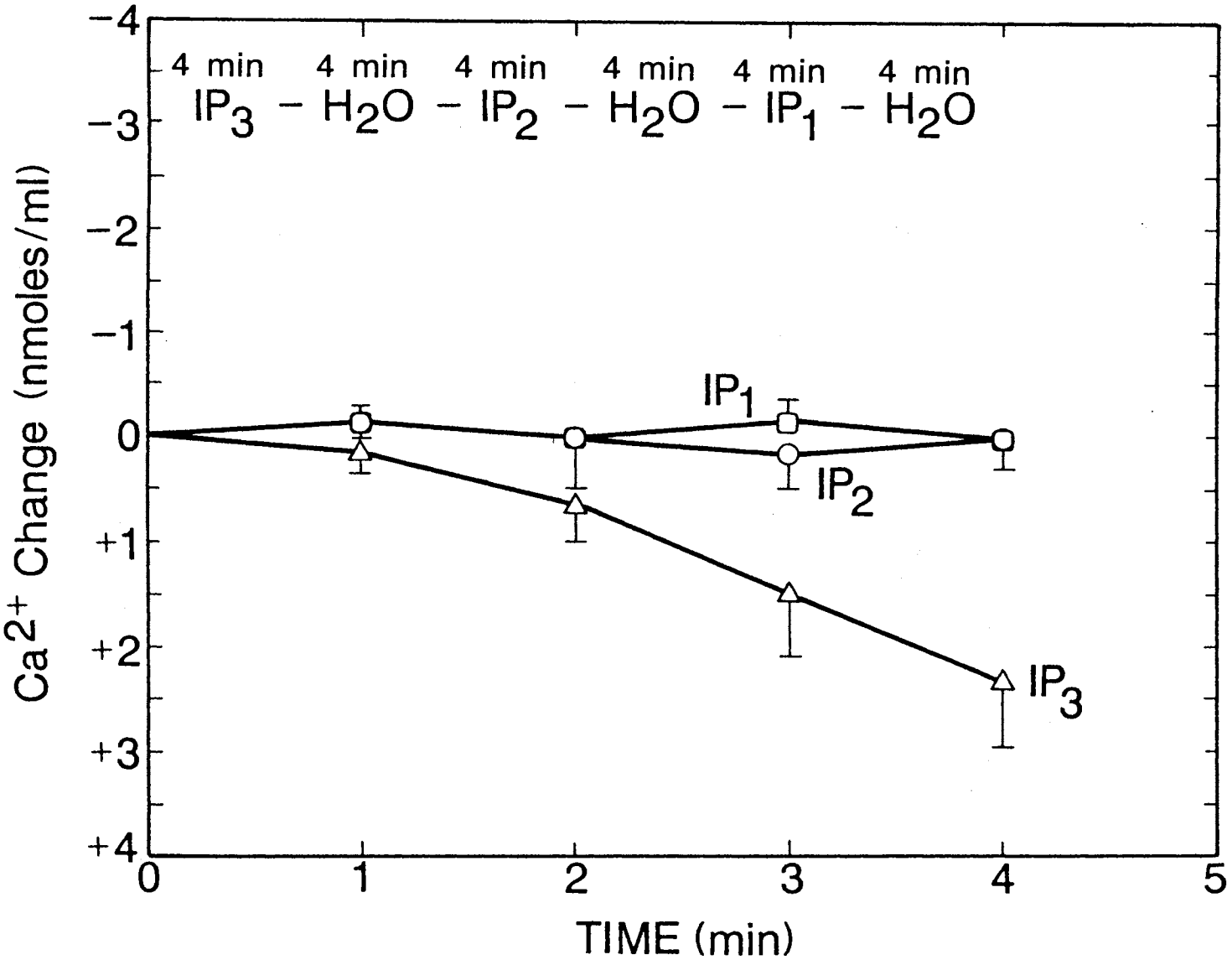

4 min
4 min
4 min
4 min
4 min
4 min
IP3 – H2O – IP2 – H2O – IP1 – H2O
Ca2+ Change (nmoles/ml)
−4
−3
−2
−1
0
+1
+2
+3
+4
IP1
IP2
IP3
0
1
2
3
4
5
TIME (min)

Figure 8. Changes in free medium Ca^{2+} (see Figures 1 and 2) after adding IP_1 (□), IP_2 (○) and IP_3 (△) to non-microsomal (900 g and 11,500 g combined pellets) tick salivary gland tissue. Water, IP_3, H_2O, IP_2, H_2O, and IP_1; respectively, were added at 4 minute intervals as illustrated in Figure 2. Values represent the mean ± S.E.M.

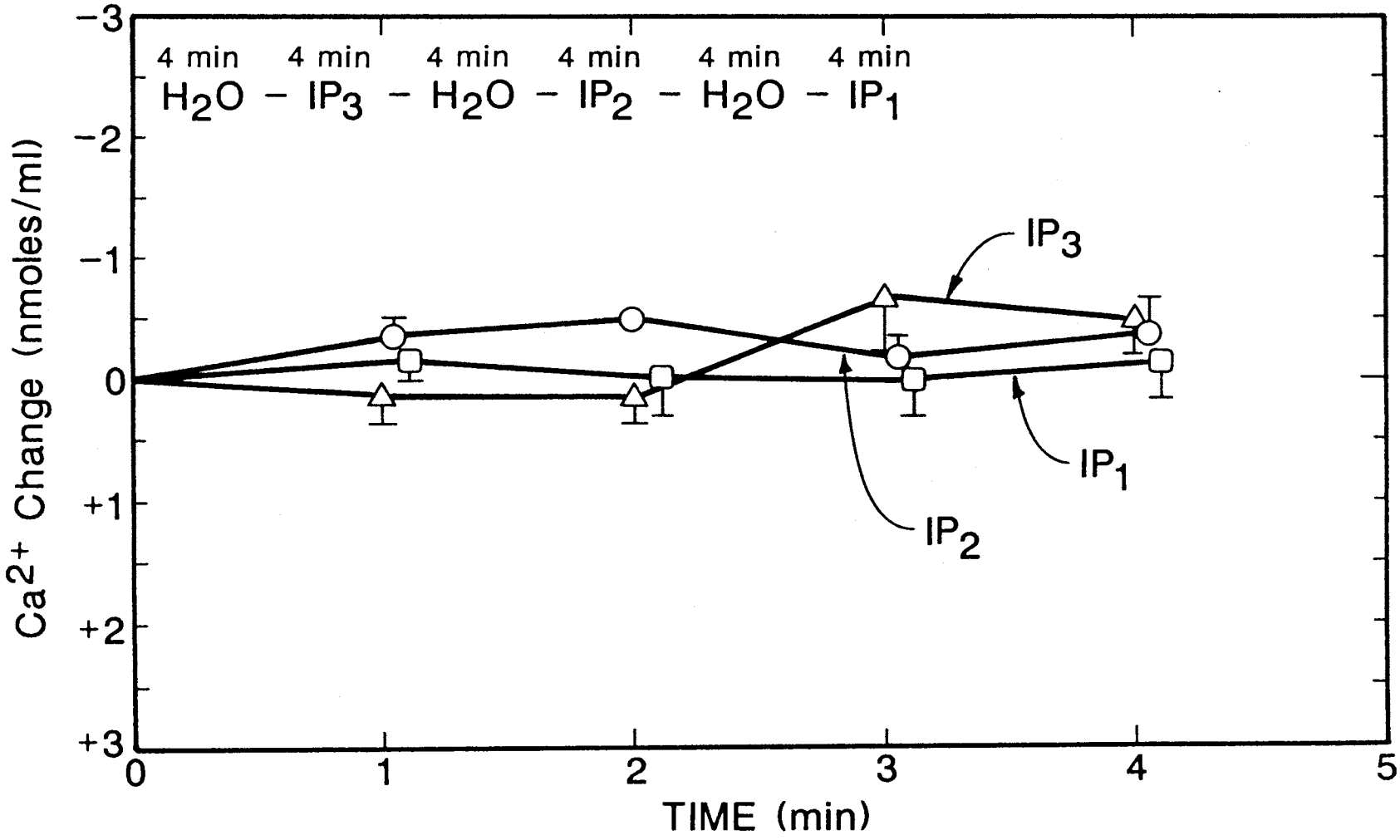

4 min 4 min 4 min 4 min 4 min 4 min
H2O – IP3 – H2O – IP2 – H2O – IP1
Ca2+ Change (nmoles/ml)
−3
−2
−1
0
+1
+2
+3
0
1
2
3
4
5
TIME (min)
IP3
IP1
IP2

Figure 9. Dose response changes in free medium Ca^{2+} (see Figures 1 and 2) after adding IP_3 to tick salivary gland microsomes. The IP_3 concentrations of 1,2,5,10 and 20 μM; respectively, were added sequentially at 4 minute intervals as illustrated in Figure 2. An analysis of variance was used to determine significant differences at the $p < 0.05$ level. The effect of 1 μM IP_3 was significantly different from the H_2O control. Values represent the mean $\pm$ S.E.M.

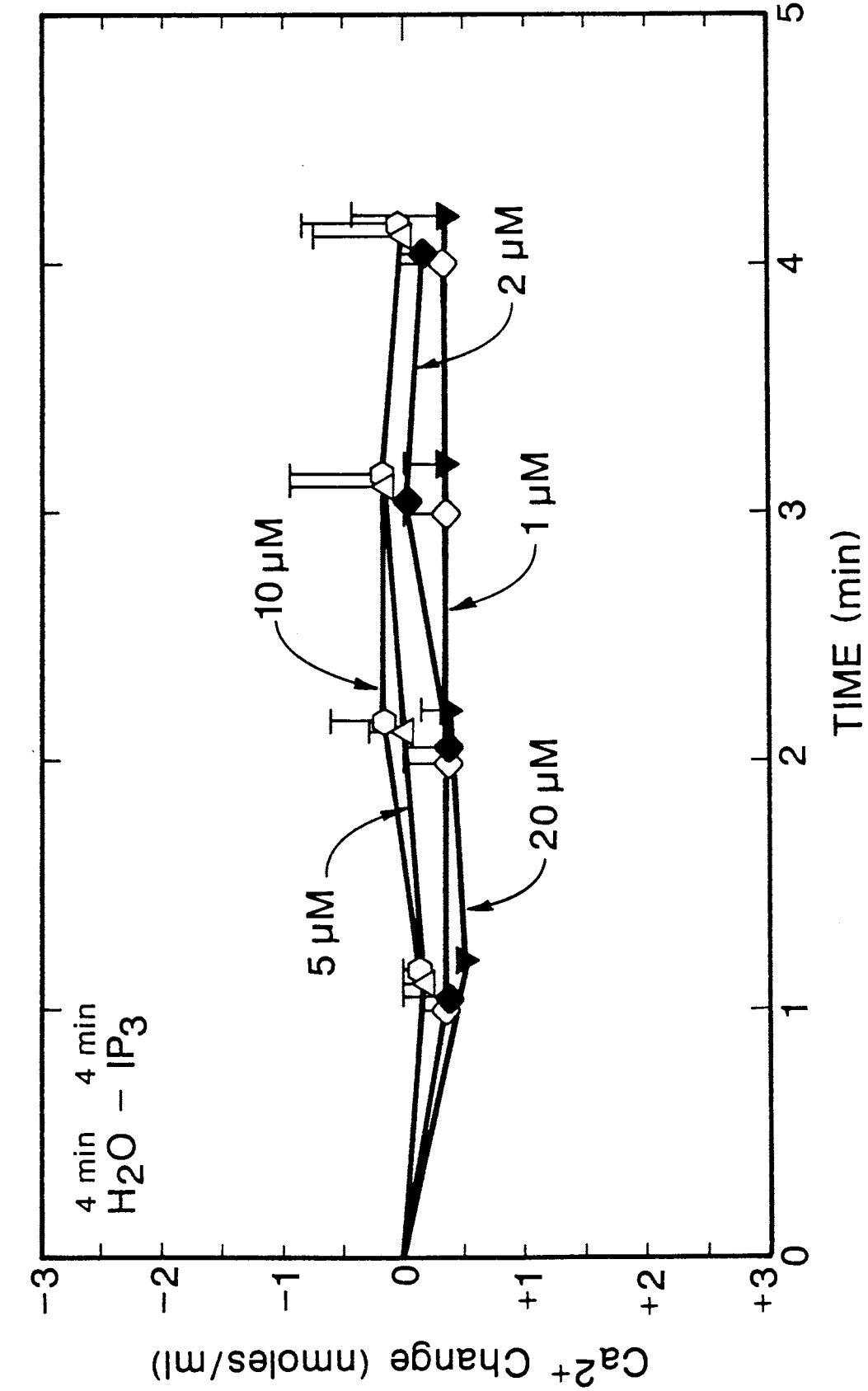
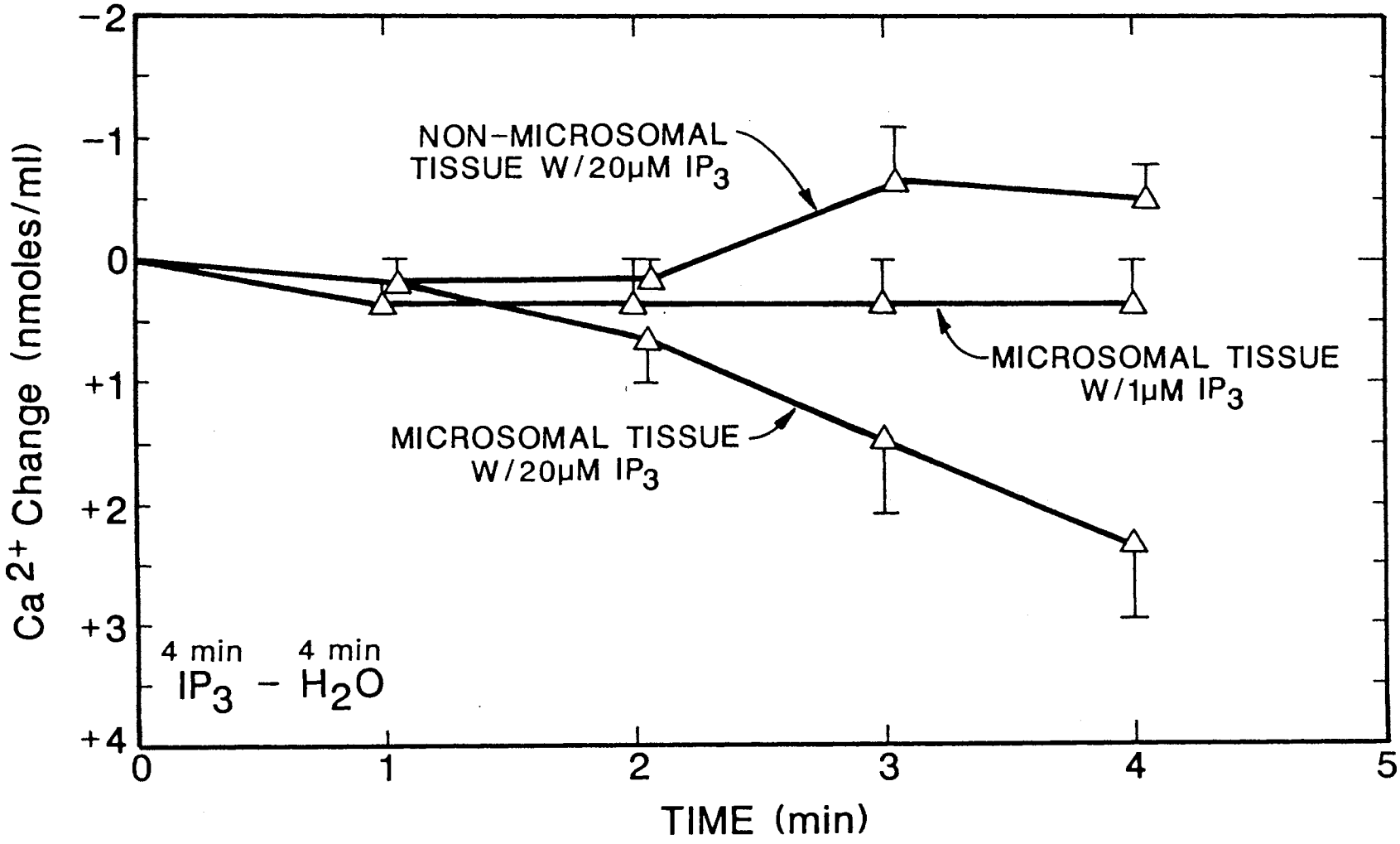

Ca^{2+} Change (nmoles/ml)
−3
−2
−1
0
+1
+2
+3
0
1
2
3
4
5
TIME (min)
4 min 4 min
H_2O – IP_3
5 μM
10 μM
20 μM
1 μM
2 μM

Figure 10. Comparison of changes in free medium Ca^{2+} (see Figures 1 and 2) after adding IP_3 to microsomal and non-microsomal tick salivary gland tissue. The IP_3 and H_2O were added at 4 minute intervals as illustrated in Figure 2. An analysis of variance was used to determine significant differences at the $p < 0.05$ level. A comparison of tissue fractions showed a significant difference in the effects with 20 μM additions of IP_3. Values represent the mean $\pm$ S.E.M.


−2
−1
0
+1
+2
+3
+4
Ca 2+ Change (nmoles/ml)
0
1
2
3
4
5
TIME (min)
NON-MICROSOMAL TISSUE W/20μM IP3
MICROSOMAL TISSUE W/1μM IP3
MICROSOMAL TISSUE W/20μM IP3
4 min
4 min
IP3 − H2O

DISCUSSION

A peptidergic factor from tick brain increases inositol phosphates in isolated whole salivary glands providing evidence that plasma membrane associated phosphoinositides are metabolized in response to an agonist in tick salivary glands (McSwain et al., 1989). The final physiological consequence of this neuropeptide's action is unknown at this time. Inositol 1,4,5-trisphosphate (IP_3), an immediate product of agonist induced metabolism of membrane associated phosphatidylinositol 4,5-bisphosphate, mobilizes Ca^{2+} from intracellular stores, possibly the endoplasmic reticulum, in many cells (Berridge, 1987). Physiological levels of Ca^{2+} affect the activities of several key regulatory enzymes in tick salivary glands (Sauer et al., 1989). We sought to test the hypothesis that intracellular Ca^{2+} is mobilized by inositol phosphates in tick salivary glands.

Digitonin selectively binds to membrane cholesterol which produces micelle channels allowing substances to move freely through the plasma membrane (Peppers and Holz, 1986; Tsien et al., 1982). Since the salivary glands of ticks are relatively small, Ca^{2+} released from whole salivary glands can be monitored in a support medium with the use of a Ca^{2+}-sensitive microelectrode. Data indicate that release of Ca^{2+} takes place in response to IP_3 but not to IP_1 or IP_2 when added to whole salivary glands and microsomes isolated from rapid feeding adult female ticks. TMB-8, a specific

inhibitor of IP_3 stimulated release of Ca^{2+} from intracellular stores in Dictyostelium discoideum (Lydan and O'Day, 1988), blocks IP_3-stimulated Ca^{2+} release from salivary gland microsomal tissue. Sequential dose response experiments indicate that 1 μM IP_3 can effect a significant release of Ca^{2+} from microsomes.

The activation of Ca^{2+} mobilizing receptors can rapidly increase the levels of cytoplasmic Ca^{2+} by both releasing stored Ca^{2+} and stimulating an uptake (Hughes and Putney, 1988). Berridge and Galione (1988) suggest that there are both receptor-controlled and second messenger oscillator mechanisms that effect Ca^{2+} uptake and Ca^{2+} release. In the presence of Ca^{2+} and ATP, the endoplasmic reticulum Ca^{2+} pumps form a 100 kDa phosphorylated intermediate which can be blocked by $VO_4^{=}$ (Imamura and Schulz, 1985). Vanadate prevents non-mitochondrial Ca^{2+} uptake by inhibiting Ca-ATPase activity in pancreatic acinar cells and rabbit iris-ciliary body (Simons, 1979; Streb et al., 1983; Socci and Delamere, 1988). The uptake of mitochondrial calcium can be inhibited by sodium azide (Streb and Schulz, 1983). Although vanadate and azide were included in the medium in all experiments it was surprising that IP_1 and IP_2 stimulated an apparent uptake of Ca^{2+} into whole tick salivary glands. Of all the inositol phosphates identified in cells to date, only IP_3 has been clearly shown to have a "second messenger" function (Berridge, 1987). These results suggest a role for either or both IP_1 and IP_2 in stimulating an uptake of Ca^{2+}

into Ca^{2+} sequestering pools unaffected by vanadate or azide. IP_1 and IP_2 did not stimulate an uptake of Ca^{2+} into microsomal tissue. Clearly more research is needed to elucidate the physiological roles of IP_1 and IP_2.

The sequential additions of IP_2 and IP_3 to whole glands produced a significant difference only when IP_2 was added first. This could be due to unmetabolized IP_3 remaining in the medium which continues to stimulate a release of Ca^{2+} when IP_2 is subsequently added. When IP_1 and IP_2 were added without prior addition of IP_3 to the bathing medium a significant uptake of Ca^{2+} into permeabilized whole glands was observed. GTP and polyethylene glycol (PEG) were included in the bathing medium when testing the effects of inositol phosphates on Ca^{2+} release in microsomal tissue. Preliminary data indicated that without their inclusion results were inconclusive (data not shown). Dawson et al. (1986) suggested that a protein, phosphorylated by GTP, binds to the microsomal membranes before IP_3 can stimulate Ca^{2+} release and that the binding of this protein is favored by the presence of PEG, a fusogen that forms membrane bound vesicles. Thomas (1988) concluded that GTP increases the proportion of the sequestered Ca^{2+} which is available for release by IP_3, either by unmasking latent IP_3-sensitive Ca^{2+} release sites or by allowing direct Ca^{2+} movement between IP_3-sensitive and IP_3-insensitive Ca^{2+} storage pools.

The role of IP_3 in stimulating a release of Ca^{2+} from feeding tick salivary glands has been clearly demonstrated.

Although the formation of inositol phosphates and the signal transduction pathway exists in tick salivary glands (McSwain et al., 1989), a possible physiological function for the breakdown products of IP_3 is of additional interest. Functions for inositol phosphates other than IP_3 are mostly unknown; however, because the enzymes which effect their formation are quite specific (Majerus, 1986), it is speculated that the various inositol phosphates should control or effect vital cellular functions. As discussed earlier, IP_1 and IP_2 appear to stimulate an uptake of Ca^{2+} into vanadate and azide insensitive pools of permeabilized whole salivary glands. Many secretory cells use IP_3 to generate the calcium signal required to control either ion permeability or the release of granules by exocytosis (Berridge, 1986). Sequential events in the latter process are poorly understood. Tick salivary glands perform an array of functions during tick feeding (Kaufman, 1989). Many of its cells are filled with granules which later disappear and are likely secreted as the tick progresses through feeding. If IP_1 and IP_2 stimulate an uptake of Ca^{2+} into the vesicular granules of tick salivary glands, one might speculate that there is a possible role for these molecules in granular secretion. Another possible explanation for the apparent uptake of Ca^{2+} in response to IP_1 and IP_2 is the complexing of free Ca^{2+} with phosphates derived from the metabolism of inositol phosphates. In this case the IP_3 stimulated release of free Ca^{2+} would be much greater than observed. These

highly speculative proposals await further investigation to determine the cellular functions of Ca^{2+} in response to IP_1 and IP_2.

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