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EFFECTS OF CALCIUM DEPLETION ON NOREPINEPHRINE- AND A23187-INDUCED STIMULATION OF INOSITOL PHOSPHATE FORMATION*

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Abstract—The role of calcium in the stimulation of phosphoinositide (PIn) hydrolysis by norepinephrine and the calcium ionophore A23187 was investigated in chopped cerebral cortex in which the PIns had been labeled previously with ([^{3}H]*myo*-inositol. The accumulation of the newly formed [^{3}H]inositol phosphate ([^{3}H]IPs) was used as an index of PIn hydrolysis. Norepinephrine produced a concentration-related increase in the accumulation of [^{3}H]inositol-phosphates ([^{3}H]IP), and this effect was only partially antagonized by omission of calcium from the incubation medium. Norepinephrine had relatively little effect on the accumulation of [^{3}H]inositol 1,4-bisphosphate [^{3}H]IP_2 and inositol 1,4,5-trisphosphate and/or inositol 1,3,4-trisphosphate ([^{3}H]IP_3). A23187 also increased the accumulation of [^{3}H]IP but this effect was not antagonized by omission of calcium from the incubation medium. When the tissue had been washed extensively with EGTA, the basal levels of [^{3}H]IP, [^{3}H]IP_2 and [^{3}H]IP_3 were decreased markedly, and the effects of both norepinephrine and A23187 were antagonized. Addition of calcium back to the depleted tissue led to an increase in the basal level of [^{3}H]IPs as well as restoration of the stimulation produced by norepinephrine and A23187. The calcium threshold for the PIn effect was 0.1 μM. Additional calcium did not affect markedly the stimulation of accumulation of [^{3}H]IP produced by norepinephrine and A23187. The results suggest that there is an absolute calcium requirement for PIn phosphodiesterase which is attained at 0.1 μM Ca^{2+}. A23187 can stimulate the accumulation of [^{3}H]IP perhaps by providing access of another form of the enzyme to artificially high concentrations (millimolar range) of calcium.

A large and diverse group of receptors share in common the ability to activate a series of events which results in the mobilization of intracellular calcium. It has become increasingly clear that receptor-activated mobilization of calcium is associated with an increase in phosphoinositide (PIn§) metabolism [1, 2]. In many peripheral tissues, a metabolite of PIn hydrolysis, inositol-1,4,5-trisphosphate (IP_3), has been demonstrated to release calcium from intracellular stores [3–6]. If PIn metabolism is to precede increased intracellular calcium, it should occur at basal intracellular calcium concentrations. In rat brain preparations, muscarinic–cholinergic stimulation of PIn metabolism can occur when calcium is omitted from the medium [7–10]. Stimulation of PIn hydrolysis can be blocked completely by the inclusion of 0.5 mM EGTA, as a calcium chelator. Repletion of calcium antagonizes the effects of prior incubation with EGTA [7–10]. Thus, a small amount of calcium appears to be required for activation of phosphodiesterases that are linked to muscarinic receptors.

Adrenergic agents also stimulate peripheral PIn metabolism primarily by activation of $alpha_1$-adrenoceptors [11–13]. In hepatocytes, epinephrine-stimulated incorporation of ^{32}P into phosphatidylinositol (PI) is decreased, but is not abolished, when calcium is omitted from the medium [14]. Adrenergic stimulation of hepatocytes is abolished, or nearly so, if EGTA (0.2 to 1.0 mM) is included in calcium-free medium [15, 16]. These data suggest that calcium is required for adrenergic-stimulated PIn hydrolysis in hepatocytes, but a small amount of calcium may be adequate. In another peripheral rat tissue, the parotid gland, 0.2 mM EGTA did not block epinephrine-stimulated PIn metabolism [17]. Thus, it seems that there are tissue differences in calcium sensitivity within adrenergic-stimulated PIn responses.

Since the calcium requirements for PIn metabolism appear to depend not only on the specific receptor coupled to this event, but also on the particular tissue within the same receptor type, it was of interest to investigate the involvement of calcium in norepinephrine-stimulated PIn metabolism in rat brain. We have used norepinephrine to investigate $alpha_1$-adrenoceptor-mediated PIn metabolism in brain tissue in the presence and absence of calcium in the incubation medium. Calcium–EGTA buffers

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§ Abbreviations: P_i, inorganic phosphate; IPs, inositol phosphates; IP, inositol l-phosphate; IP_2, inositol 1,4-bisphosphate; IP_3, inositol 1,4,5-trisphosphate and/or inositol 1,3,4-trisphosphate; PIn, phosphoinositide; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; and EGTA, ethyleneglycol-bis-(aminoethylether) tetra-acetate.

were also utilized to determine the calcium requirements for the norepinephrine-stimulated response.

To further investigate the role of calcium in the hydrolysis of PIns, we employed the calcium ionophore A23187 [18, 19] which increases the transmembrane movement of calcium [20]. It is known that A23187 is capable of stimulating PIn hydrolysis [9, 10, 21]. Experiments were designed to investigate the effects of varying calcium conditions on PIn hydrolysis stimulated by A23187. It is hoped that the comparison of the calcium requirements for receptor-mediated ($alpha_1$) and receptor-independent (A23187) hydrolysis of PIns will prove valuable in the future understanding of this important transduction mechanism.

MATERIALS AND METHODS

Tissue preparation and incubation conditions. Adult (>65 days of age) male Sprague–Dawley rats were obtained from Sasco, Inc. (Omaha, NE) or were from a colony bred in-house which originated from animals obtained from Sasco. Rats were decapitated, and the brains were rapidly excised and placed in ice-cold, freshly gassed (95% O_2, 5% CO_2) Krebs–Henseleit bicarbonate buffer [22]. All manipulations prior to incubation were performed at 4°. Final concentrations of the buffer (mM) were as follows: NaCl, 117.9; KCl, 4.72; $CaCl_2$, 2.54; $MgSO_4$, 1.18; Na_2EDTA, 0.045; KH_2PO_4, 1.19; $NaHCO_3$, 25.0; and glucose, 11.1 (pH 7.4). The cerebral cortex was dissected and cross-chopped twice at 0.3 mm intervals using a McIlwain tissue chopper. Chopped tissue was washed once with 6 ml buffer (per brain), by mixing and centrifuging for 5 min at 800 *g*. Pellets were resuspended in 3 ml buffer, and 3.5 ml (extra volume contributed by the tissue) was transferred to a polypropylene tube. [^{3}H]*myo*-Inositol was added (53 μCi in a volume of 0.265 ml H_2O, final concentration of 1.0 μM). Tubes were gassed, capped, and incubated for 60 min at 37° in a shaking water bath. Tissue was mixed approximately every 15 min and regassed approximately half way through the prelabeling period. Prelabeling was stopped by centrifuging the tissue for 5 min at 800 *g*. The pellet was washed five times in 10 ml of modified Krebs–Henseleit bicarbonate buffer containing 10 mM LiCl. Calcium was omitted in designated samples.

Washed, labeled tissue was resuspended in 3.2 to 3.5 ml of modified buffer with the appropriate calcium concentration. Aliquots (0.245 ml) were placed in polypropylene tubes, and 0.005 ml of norepinephrine, A23187 or vehicle was added. Norepinephrine was dissolved in 0.01 N HCl or buffer and A23187 was suspended in DMSO–H_2O (1:1) and mixed thoroughly before each addition. The final concentration of DMSO in the incubation mixture was 1%, but this did not alter basal [^{3}H]inositol phosphate accumulation. Each sample generally contained 3–5 mg protein. The accumulation of [^{3}H]inositol phosphates (IPs) was linear with time up to 30 min upon stimulation with 10^{-4} M norepinephrine. Tubes were gassed capped and incubated for 30 min at 37° and terminated by addition of 1 ml cold buffer. Samples were then homogenized in a ground glass homogenizer and centrifuged at 11,000 *g* for 10 min. Individual IPs were separated by methods adapted from Berridge *et al.* [23]. Supernatant fractions were passed over 5 ×20 mm Dowex AG 1-X8 (200–400 mesh, formate form) anion exchange resin columns, and free [^{3}H]-*myo*-inositol was eluted with two 5-ml water washes. Glycerophosphoinositol and inositol 1,2-cyclic phosphate were eluted with 5 ml of 5 mM sodium tetraborate/60 mM sodium formate. These two fractions were discarded. Inositol 1-phosphate (IP) was eluted with 5 ml of 0.1 M formic acid/0.2 M ammonium formate directly into a scintillation vial. Inositol 1,4-bisphosphate (IP_2) and IP_3 were eluted with 7 ml of 0.1 M formic acid/0.4 M ammonium formate and 5 ml of 0.1 M formic acid/1.0 M ammonium formate respectively. The IP_3 fraction would also be expected to contain [^{3}H]inositol-tetrakisphosphate. Although this extraction procedure does not yield optimum recovery of the polyphosphates [24], the relative recovery among the treatments should not be affected. Radioactivity was determined by liquid scintillation spectrometry in a Beckman LS100C spectrometer.

Free calcium concentration–effect relationships. In these experiments, the concentration of free calcium during stimulation was varied using calcium–EGTA buffers. Stimulation was terminated by the addition of 1 ml of ice-cold water rather than buffer as previous data indicated that more complete lysis and release of IPs were obtained with this procedure. Tissue was prepared as described above, and [^{3}H]-*myo*-inositol (30.2 to 35.6 μCi) was added to a final concentration of 1.0 μM and a final incubation volume of 2.5 ml. Tissue was incubated for 60 min as in the prelabeling experiments above. Pellets were washed five times in a modified Krebs–Henseleit bicarbonate buffer. This buffer contained 10 mM LiCl, but $CaCl_2$ and EDTA were omitted so that the free calcium concentration could be determined and controlled. EGTA (1 mM) was included to chelate calcium and to buffer calcium that was to be added just prior to incubation with norepinephrine or A21387.

The washed pellet was resuspended in 3.5 to 4.0 ml of buffer, and 0.24 ml aliquots were transferred to polypropylene tubes. To these samples, 0.005 ml norepinephrine or A23187 (final concentration of each, 10^{-4}M) and 0.005 ml of various concentrations of $CaCl_2$ were added. Calcium concentrations were calculated as described by Raaflaub [25], using 11.0 at pH 7.4 as the log of the equilibrium constant for EGTA and calcium. Samples were incubated and terminated as above except that 1 ml of water, rather than buffer, was used to terminate the stimulation.

Materials. *l*-Norepinephrine bitartrate and calcium ionophore A23187 were obtained from the Sigma Chemical Co. (St. Louis, MO). Dowex AG 1-8X anion exchange resin was purchased from BioRad (Richmond, CA) and 3a70B scintillation mixture from Research Products International (Mount Prospect, IL). *myo*-[2-^{3}H(N)]Inositol (14.0 to 15.5 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO) and was purified and diluted prior to use. This was accomplished by passing the material over a 5 × 10 mm anion exchange column and washing with several aliquots of deion-

ized water. The radioactivity per volume was determined by liquid scintillation spectrometry and the tracer diluted as needed, generally to 0.2 μCi/μl water.

Statistics. A one-way analysis of variance was used in conjunction with the Student–Newman–Keuls test for comparison of means. When non-homogeneity of variance occurred, the Games and Howell method for approximate test of equality of means was used [26]. A $P < 0.05$ was considered significant.

RESULTS

Norepinephrine concentration–effect curves. The effect of 3–300 μM norepinephrine on the accumulation of [^{3}H]IPs in chopped rat cerebral cortex was evaluated in the presence or absence of calcium (2.5 mM) in the incubation medium. Norepinephrine stimulated the accumulation of [^{3}H]IP in brain slices prelabeled with [^{3}H]*myo*-inositol (Fig. 1). Stimulation occurred both in the absence and presence of medium calcium. Maximal stimulation was lower in the absence of calcium (268 ± 11% of control) than in its presence (341 ± 40% of control). Removal of calcium from the medium did not affect the basal [^{3}H]IP accumulation. Norepinephrine stimulated the accumulation of [^{3}H]IP_2 (maximal, 170 ± 16% of control), although the effect was not statistically significant due to the large variability. The response did not appear to reach a maximum, even at 300 μM. Norepinephrine was unable to stimulate [^{3}H]IP_2 accumulation in the absence of calcium, and basal accumulation was also decreased in this condition. Norepinephrine did not stimulate the accumulation of [^{3}H]IP_3. Removal of calcium from the medium resulted in a decrease in basal [^{3}H]IP_3 accumulation, just as it did for [^{3}H]IP_2. Accumulation of [^{3}H]IP_3 in the absence of calcium at various concentrations of norepinephrine resulted in a nearly identical curve to that seen in its presence, except that the curve is displaced to a lower level due to lowered initial values.

A23187 concentration–effect curves. The divalent cation ionophore A23187, in concentrations varying from 30 μM to 1 mM, was incubated in the presence or absence of medium calcium with rat brain slices prelabeled with [^{3}H]-*myo*-inositol. [^{3}H]IP was observed to accumulate nearly as well in the absence of medium calcium as in its presence (Fig. 2). When expressed as percent of control, the maximal level of stimulation was slightly higher when calcium was deleted (274 ± 8%) than when it was included (247 ± 18%). Basal accumulation was not affected by deletion of calcium. Accumulation of [^{3}H]IP_2 was also stimulated by the ionophore but, in contrast with [^{3}H]IP, it was sensitive to calcium omission. Maximal stimulation occurred at 100 μM. No significant stimulation of [^{3}H]IP_3 formation was produced by A23187. Basal accumulation of both labeled inositol polyphosphates was decreased upon omission of calcium in the medium.

Effect of various concentrations of calcium upon norepinephrine stimulation of IP accumulation. Cerebral cortical slices were prelabeled with [^{3}H]*myo*-inositol and depleted of calcium by repeated washing with EGTA. Tissue was then incubated in the presence or absence of norepinephrine, in calcium–EGTA buffers with free calcium concentrations from zero through 1.5 mM. Calcium levels of 0.1 μM and higher significantly stimulated the accumulation of

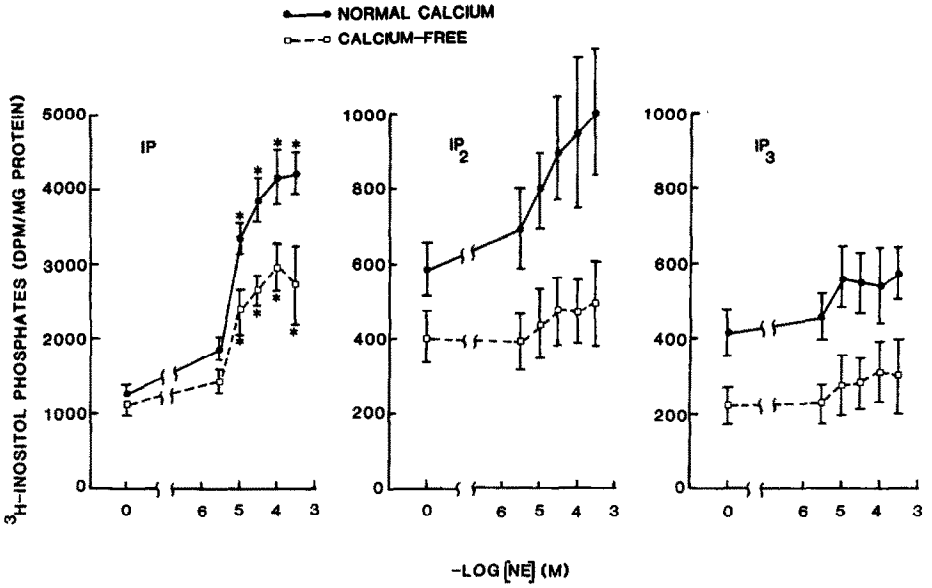

Fig. 1. Norepinephrine-stimulated accumulation of [^{3}H]inositol phosphates. Chopped rat cerebral cortical tissue was prelabeled in normal buffer with [^{3}H]*myo*-inositol. Tissue was washed five times in modified buffer containing 10 mM LiCl with or without added calcium (2.5 mM). Washed slices were incubated for 30 min in appropriate buffer with various concentrations of norepinephrine. Individual IPs were separated by anion exchange chromatography, and radioactivity was determined by liquid scintillation spectrometry. Each value is the mean of four to five determinations ± SEM. Key: (*) significantly different from control (absence of norepinephrine), $P < 0.05$.

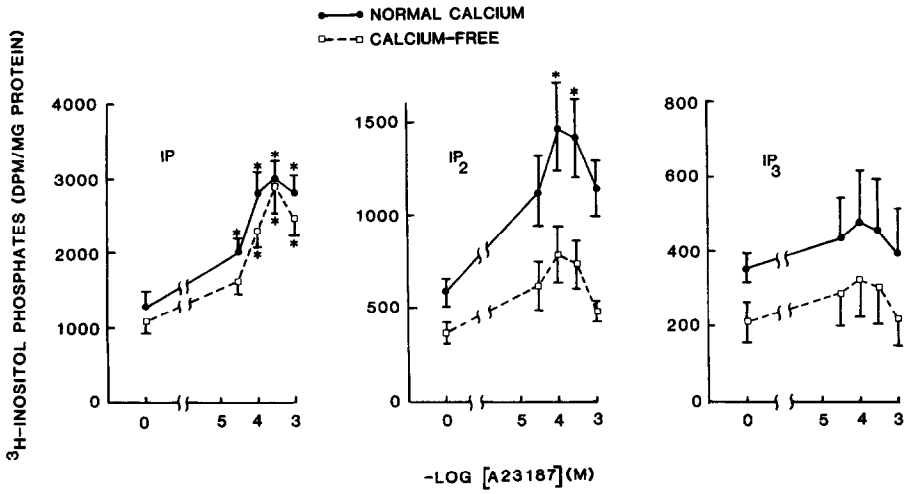

Fig. 2. A23187-stimulated accumulation of [^{3}H]IPs. Chopped rat cerebral cortical tissue was prelabeled in normal buffer with [^{3}H]*myo*-inositol. Tissue was washed five times in modified buffer containing 10 mM LiCl with or without added calcium (2.5 mM). Washed slices were incubated for 30 min in appropriate buffer with various concentrations of A23187. Individual IPs were separated by anion exchange chromatography, and radioactivity was determined by liquid scintillation spectrometry. Each value is the mean of five determinations ± SEM. Key: (*) significantly different from control (absence of A23187), $P < 0.05$.

[^{3}H]IP, [^{3}H]IP$_2$ and [^{3}H]IP$_3$ when compared to controls containing no added calcium (Fig. 3). This was true for both basal and norepinephrine-stimulated accumulation of [^{3}H]IPs. The concentration–effect curve for each inositol phosphate displayed a very steep slope between 0.01 μM free calcium, at which no stimulation was observed, and 0.1 μM. Very small increases in norepinephrine-stimulated [^{3}H]IP accumulation were seen at calcium concentrations of 0, 0.001 and 0.010 μM (142–154% of control). Larger increases were observed at 0.1 μM calcium, but increasing free calcium concentrations above 0.1 μM had little additional effect on norepinephrine stimulation of [^{3}H]-IP accumulation (212–247% of control), and no general pattern of increased stimulation with increasing calcium levels was observed. No norepinephrine-stimulated accumulation of either [^{3}H]IP$_2$ or [^{3}H]-IP$_3$ occurred in the absence of calcium. Significant increases in [^{3}H]IP$_2$ accumulation were seen when free calcium levels were raised to 1 μM and above, but no significant accumulation of labeled [^{3}H]IP$_3$ was observed at any of the calcium concentrations. Norepinephrine stimulation of [^{3}H]IP$_2$ accumulation was not enhanced when the free calcium concentration was varied from 1 μM to 1.5 mM. The percent stimulation (127–156% of control) showed no progressive increase with increasing free calcium concentration.

Free calcium concentration–effect curve with A23187. Experiments similar to those described in the previous section were conducted on prelabeled rat brain slices incubated in the presence or absence of A23187 (100 μM). Free calcium concentrations of 0.1 μM and above significantly stimulated the accumulation of all three labeled [^{3}H]IPs (Fig. 4). A23187 did not stimulate the formation of IPs in the absence of calcium in the incubation medium. A23187 caused slight, but statistically significant, stimulation of the formation of all three IPs at low calcium concentrations (0.001 to 0.010 μM). Maximal stimulation of [^{3}H]IP and [^{3}H]IP$_2$ formation occurred at 0.1 μM free calcium, and a higher concentration of calcium had little effect on A23187-mediated stimulation. A23187-stimulated accumulation of [^{3}H]IP$_3$ occurred only at 0.1 μM, 1.0 μM and 1.5 mM free calcium (167, 178 and 140% of control respectively).

DISCUSSION

The hydrolysis of PIns in chopped cerebral cortex has an absolute requirement for calcium. Prolonged incubation of tissues with EGTA markedly decreases the level of intracellular calcium [27, 28]. This is accompanied by a substantial decrease in basal PIn hydrolysis and complete antagonism of the norepinephrine-stimulated PIn response. However, replenishment of calcium by incubation with concentrations as low as 0.1 μM restored basal activity as well as norepinephrine-induced stimulation of [^{3}H]-inositol phosphate formation. Kendall and Nahorski [10] observed that incubation of rat cerebral cortical slices in the absence of calcium and in the presence of 0.5 mM EGTA abolishes stimulation of inositol phosphate formation by a variety of substances including norepinephrine. When ^{32}Pi was used to label PI in guinea pig synaptosomes, low concentrations of calcium in the incubation medium (0.1 μM and above) were required to obtain a carbachol-mediated PIn response [7].

It is assumed that the internal stores of calcium are relatively intact if calcium is simply removed from the incubation medium. Under these conditions, the basal activity of [^{3}H]IP formation is not reduced, and the stimulation produced by norepinephrine is only partially antagonized. This has also been observed in cerebral cortex by Kendall and Nahorski [10] with a

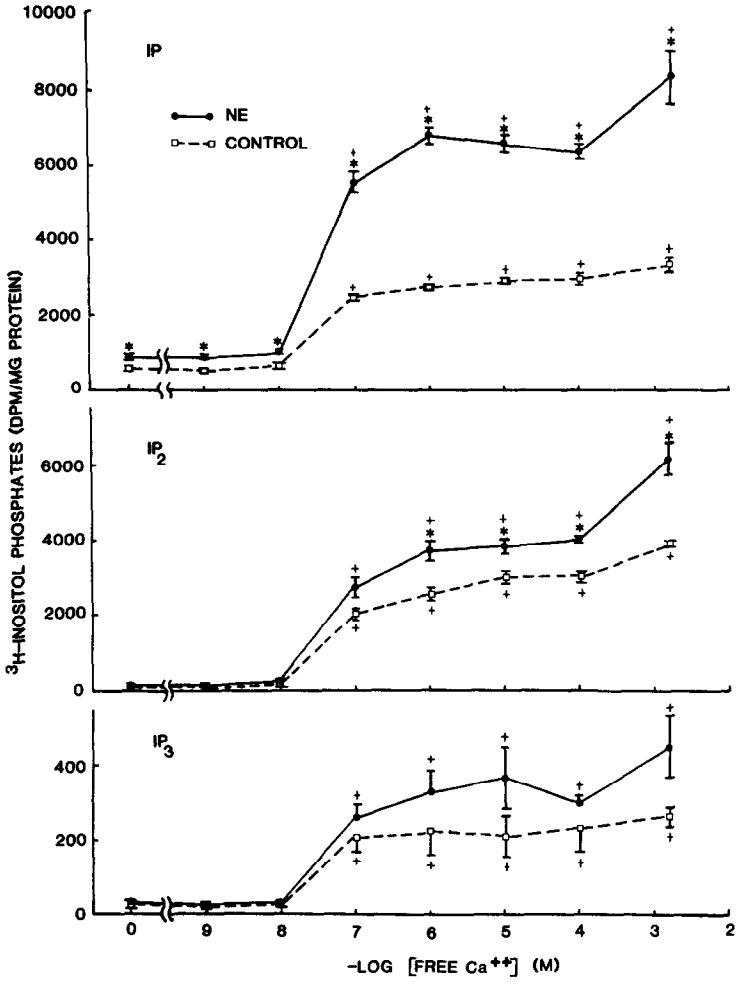

Fig. 3. Effect of free calcium concentration on norepinephrine-stimulated accumulation of [^{3}H]inositol phosphates. Chopped rat cerebral cortical tissue was prelabeled in normal buffer with [^{3}H]-*myo*-inositol. Tissue was washed five times in modified buffer containing 10 mM LiCl with no calcium. EGTA (1 mM) was included in the buffer. Washed slices were incubated for 30 min in buffer with various concentrations of free calcium in the presence or absence of norepinephrine (10^{-4} M). Incubations were terminated with water. Individual IPs were separated by anion exchange chromatography, and radioactivity was determined by liquid scintillation spectrometry. Each value is the mean of three determinations ± SEM. Key: (*) significantly different from control (absence of norepinephrine), $P < 0.05$ and (†) significantly different from O-calcium, $P < 0.05$.

variety of agonists. Similar observations have been made in a number of peripheral tissues [29]. Since the intracellular concentration of calcium is approximately 0.1 μM [30, 31] and since the phosphodiesterase is fully active at this concentration of calcium, it is generally accepted that a change in intracellular calcium is not the initiating event in stimulating PIn hydrolysis upon exposure to a number of neurotransmitters and hormones [32–35].

The hydrolysis of phospholipids in chopped cerebral cortex which have been labeled previously with [^{3}H]inositol demonstrates a calcium sensitivity similar to that seen with the phosphodiesterase isolated from rat brain [36]. In both the isolated enzyme and the chopped tissue preparations, the enzyme was relatively inactive at concentrations of calcium less than 0.01 μM. There was a marked increase in activity of the isolated enzyme between 0.001 and 0.01 μM Ca^{2+}, and the enzyme activity–calcium concentration curve was shallow or flat up to a concentration of 100 μM. A similar calcium requirement has also been observed for soluble phosphodiesterase isolated from seminal vesicles [37] in that approximately 0.1 μM Ca^{2+} was sufficient to activate the enzyme. In some studies, very high calcium concentrations (100 μM to 1 mM) were required to stimulate phosphodiesterase [38–40]. The isolated enzyme has been difficult to study since it is sensitive to changes in both the ionic composition of the medium and the lipid microenvironment of the substrate [40, 41]. It is also not clear whether the substrate present in the membrane is properly presented to the soluble enzyme in experiments of this type [36, 37]. Since it is possible to stimulate the enzyme with very low concentrations of calcium and since the calcium sensitivity is similar to that observed in preparations with intact cells, it is

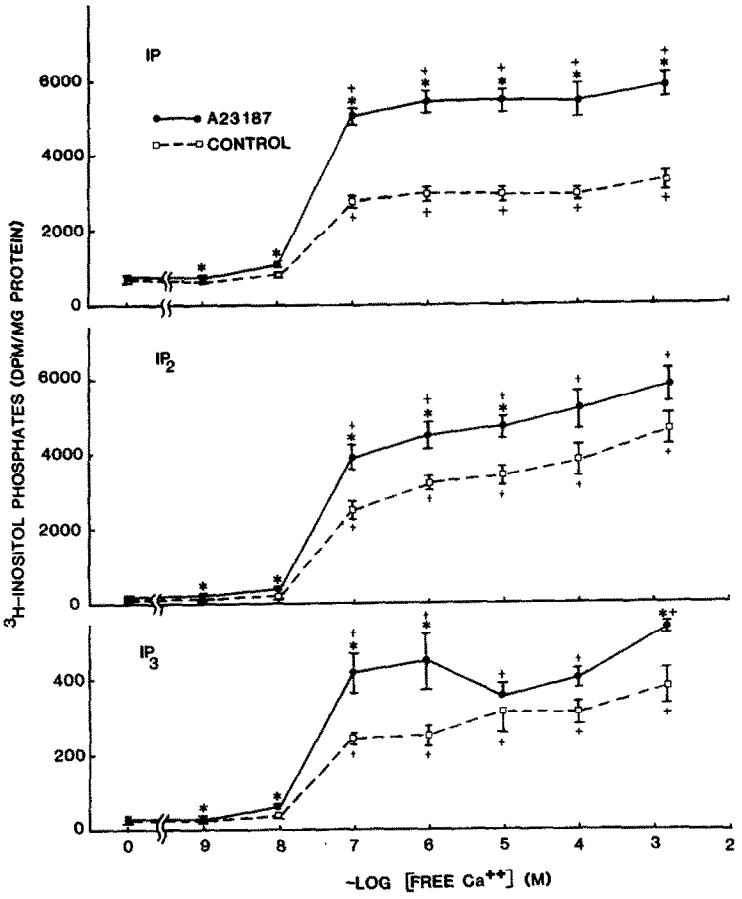

Fig. 4. Effect of free calcium concentration on A23187-stimulated accumulation of [^{3}H]IPs. Chopped rat cerebral cortical tissue was prelabeled in normal buffer with [^{3}H]*myo*-inositol. Tissue was washed five times in modified buffer containing 10 mM LiCl with no calcium and 1.0 mM EGTA. Washed slices were incubated for 30 min in buffer with various concentrations of free calcium in the presence or absence of A23187 (10^{-4} M). Individual IPs were separated by anion exchange chromatography, and radioactivity was determined by liquid scintillation spectrometry. Each value is the mean of four to five determinations ± SEM. Key: (*) significantly different from control (absence of A23187, $P < 0.05$; and (†) significantly different from O-calcium, $P < 0.05$.

suggested that the absolute requirement for calcium which is satisfied by about 0.1 μM Ca^{2+} represents the true calcium sensitivity under normal physiological conditions. On the other hand, when the concentrations of calcium at the active site of the enzyme are artificially elevated to the millimolar range, it is possible that another form of phosphodiesterase is activated, leading to a further increase in PIn hydrolysis [42].

Another issue which has presented difficulty in interpretation of the calcium sensitivity of phosphodiesterase is whether or not PI, phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) are hydrolyzed by the same enzyme. It is apparent that there are multiple forms of phosphodiesterase [42–44] and that all three PIns are substrates [37, 40, 43]. The relative affinity of the three substrates for the enzymes is affected markedly by the intracellular K^+ concentration. Potassium is known to inhibit phosphodiesterase when PI is the substrate [45, 46]. Recent experiments suggest that PIP_2 is the preferred substrate [47] especially under conditions that are characteristic of the cell cytoplasm and when the substrate is presented to the enzyme in an appropriate lipid environment [36, 40].

Of the three IPs which were isolated, the formation of IP was stimulated to the greatest extent by norepinephrine. There was much less IP_2 formed and an even smaller amount of IP_3, although this may be due to incomplete extraction of the polyphosphates. This is similar to what has been found in brain slices [23] and several peripheral tissues [23, 48–50]. When blowfly salivary glands were examined at very early times (within 1 min) after addition of the agonist 5-hydroxytryptamine, it was observed that IP_3 was formed first followed by IP_2 and finally by IP_3 [51]. The accumulation of IP is due to inhibition of inositol-1-phosphatase by lithium [52, 53]. Lithium is much less effective as an inhibitor of the hydrolysis of IP_2 and IP_3 [54, 55]. It is known that IP_3 phosphatase is a soluble enzyme that requires Mg^{2+} for activity [54–57]. The presence of 1.18 mM Mg^{2+} in the incubation medium would assure maximum activity, and therefore the small amount of IP_3 isolated after

30 min of incubation would be expected. It is also possible that PIP_2 is hydrolyzed by a phosphomonoesterase [58, 59] to form PIP and PI and that these latter compounds are then hydrolyzed to form IP. Because of the ionic conditions required for the monoesterases, it is generally assumed that IP is formed by hydrolysis of PIP_2 and subsequent metabolism of the IPs [35, 60]. Evidence has accumulated that the IP_3 fraction contains a mixture of inositol 1,3,4-trisphosphate and inositol 1,4,5-trisphosphate with the 1,4,5-isomer predominating at early time periods and the 1,3,4-isomer accumulating later [61, 62]. Furthermore, it has been observed that inositol 1,3,4,5-tetrakisphosphate can be formed in rat cerebral cortex slices upon stimulation with carbachol [63], and it is suggested that the two IP_3 isomers are derived from this compound [63–65]. The relationships, among the hydrolysis of inositol tetrakisphosphate, the formation of the two inositol trisphosphates, and calcium mobilization have not yet been clearly established [65].

The calcium ionophore A23187 produced a concentration-related increase in [^{3}H]IP formation. This effect was independent of the calcium in the incubation medium but was completely antagonized by prolonged incubation with EGTA. Since EGTA produces a marked depletion of intracellular calcium [27, 28], it is suggested that A23187 produces its effect by releasing intracellular calcium to activate an intracellular phosphodiesterase. A direct demonstration that A23187 can release calcium from intracellular storage sites was obtained in skinned single cells of porcine artery [6]. Activation of PIn hydrolysis in brain tissue by A23187 was first shown by Griffin and Hawthorne [21]. They observed that A23187 decreased radioactivity in PIP and PIP_2 from guinea pig synaptosomes previously labeled by incubation with $^{32}P_i$. This effect was antagonized by omission of calcium from the incubation medium. It was concluded that an increase in cytoplasmic calcium activates polyphosphoinositide diesterase but has relatively little effect on the hydrolysis of PI. These observations were extended by Fisher and Agranoff [9] who observed that A23187 increased the loss of ^{32}P from PIP_2 and that the rate of loss was further increased by acetylcholine. Kendall and Nahorski [10] observed that A23187 increases the formation of [^{3}H]IP from cerebral cortex slices that had been labeled previously with [^{3}H]inositol. Similar effects on PIn metabolism were observed in human erythrocytes [66], rabbit iris [27, 49], and rat lacrimal acinar cells [34]. A23187 produced an increase in IP and IP_2 without having an effect on IP_3. This is similar to the effect of the ionophore in GH_3 pituitary cells [67] and may indicate that a high concentration of intracellular calcium leads to a greater hydrolysis of PI and PIP relative to PIP_2.

The mechanism by which A23187 increases PIn hydrolysis is most likely related to activation of a form of phosphodiesterase that requires very high calcium concentrations, in the 1–2 mM range [42]. It is known that the phosphodiesterase can be stimulated by these concentrations of calcium [40, 68, 69]. By markedly increasing the transport of calcium across the plasma membrane [20] and by releasing calcium from internal stores [6], it is possible that local calcium concentrations of this magnitude can be achieved. Another mechanism by which A23187 could stimulate PIn hydrolysis is the release of neurotransmitter which could subsequently produce receptor-mediated PIn response. Evidence for this mechanism was obtained in the rabbit iris where it was shown that the effect of A23187 could be blocked by prazosin, indicating that norepinephrine was released and subsequently activated alpha$_1$-receptors which are coupled to PIn hydrolysis [49]. A final mechanism by which A23187 could produce its effect would be to mobilize calcium to sites containing phosphodiesterase molecules that have not been activated previously by low concentrations of calcium. This mechanism cannot be excluded in the present study. Further experiments are needed in less complex systems of neural tissue to clarify the relationship between intracellular calcium ions and the activity of the PIP_2 phosphodiesterase.

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