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Inositol Trisphosphate Mobilizes Intracellular Calcium in Permeabilized Adrenal Chromaffin Cells

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Abstract: Using permeabilized chromaffin cells and the fluorescent probe Quin 2 (an indicator of free Ca^{2+}), we found that inositol trisphosphate (IP_3) specifically triggered an immediate and dose-dependent release of Ca^{2+} from intracellular stores. Desensitization of the response was observed at nonsaturating concentrations of inositol trisphosphate and resequestration of Ca^{2+} was not observed. While representing only a small fraction of the total cellular Ca^{2+}, the amount released by IP_3 could significantly raise cytosolic Ca^{2+} and may account for muscarinic effects on Ca^{2+} metabolism in chromaffin cells.
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Exocytosis of catecholamine from the adrenal medulla has an absolute requirement for Ca^{2+} in the medium (Douglas, 1975). Activation of nicotinic receptors causes Ca^{2+} influx (Douglas and Rubin, 1961; Holz et al., 1982; Kilpatrick et al., 1982) and a rise in cytosolic Ca^{2+} (Knight and Kesteven, 1983) which triggers the sequence of events leading to exocytosis. Another possible pathway for increasing cytosolic Ca^{2+} is through inositol trisphosphate (IP_3). IP_3 is a product of phospholipase C activation, one of the earliest events following stimulation in many cell types. IP_3 releases Ca^{2+} from intracellular sites in pancreatic acinar cells (Streb et al., 1983), hepatocytes (Joseph et al., 1984), dimethyl sulfoxide (DMSO)-differentiated human leukocytes (Burgess et al., 1984), and neutrophils (Prentki et al., 1984). Evidence suggests that release occurs from a nonmitochondrial site such as endoplasmic reticulum (Joseph et al., 1984).

Previous studies in bovine adrenal medullary slices (Trifaro, 1969) demonstrated that phosphatidylinositol and phosphatidate turnovers were increased by the natural secretagogue acetylcholine. Subsequent studies, both in bovine adrenal medullary slices (Adnan and Hawthorne, 1981) and chromaffin cell cultures (Fisher et al., 1981), demonstrated that this effect was due primarily to activation of muscarinic receptors which are linked neither to Ca^{2+} influx nor to catecholamine secretion (Holz et al., 1982). Recent experiments indicate that inositol phosphates are released by muscarinic stimulation (D. Eberhard and R. W. Holz, unpublished observations). Nicotinic stimulation, which induces both Ca^{2+} uptake and catecholamine secretion, caused small but generally statistically insignificant increases in phosphatidylinositol or phosphatidate turnover.

Muscarinic stimulation of bovine adrenal chromaffin cells releases Ca^{2+} from intracellular sites (Oka et al., 1982) and raises cytosolic Ca^{2+} from approximately 0.1 μM to 0.15 μM in the absence of extracellular Ca^{2+} (Kao and Schneider, 1985). In the present study, we investigated the ability of IP_3 to induce Ca^{2+} release from intracellular sites within suspended bovine adrenal chromaffin cells permeabilized with digitonin. Although this rise in cytosolic Ca^{2+} is insufficient in itself to stimulate exocytosis (Knight and Kesteven, 1983), it may nevertheless have significant physiological consequences.

MATERIALS AND METHODS

Eagle's minimum essential medium and heat-inactivated fetal calf serum were purchased from Gibco (Grand

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Abbreviations used: DMSO, dimethyl sulfoxide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; PIPES, piperazine-*N*,*N'*-bis(2-ethanesulfonic acid).

Island, NY, U.S.A.). Digitonin and potassium glutamate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Potassium propionate was the product of K & K Rare and Fine Chemicals, Division of ICN (Plainview, NY, U.S.A.). Ionomycin was purchased from Calbiochem-Behring (San Diego, CA, U.S.A.). Quin 2 was a gift from Michael Welsh (Department of Anatomy and Cell Biology, University of Michigan, Ann Arbor, MI, U.S.A.). Inositol monophosphate (IP_1), inositol bisphosphate (IP_2), and IP_3 were isolated as previously described (Ballou, 1962; Agranoff and Seguin, 1974). All other materials were reagent grade.

Suspended bovine adrenal chromaffin cells were prepared and maintained as previously described (Holz et al., 1983). Experiments were performed within 24 h of isolation of the cells. For each experiment, the cells (1×10^7/ml) were gently centrifuged (100 *g*) and resuspended in KGP buffer [145 m*M* potassium glutamate, 20 m*M* piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 2 m*M* Mg-ATP, and 1 m*M* EGTA, pH 6.6] supplemented with 20 μ*M* digitonin (Dunn and Holz, 1983). The cells were incubated for 5 min at 25°C, gently centrifuged, and resuspended (7×10^6/ml) in KPH buffer [100 m*M* potassium propionate, 40 m*M* KCl, 20 m*M* NaCl, 30 m*M* K^+-*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), and 2 m*M* Mg-ATP, pH 7.0]. The cell suspension (5.6×10^6 in 0.8 ml) was transferred to a fluorescence cuvette and supplemented with Quin 2 (150 μ*M*). Fluorescence of the cell suspension was continuously monitored using a Perkin Elmer Model 650-10S spectrofluorimeter (excitation of 326 nm, emission 492 nm, slit widths 5, 25°C) equipped with a stirrer and a temperature-controlled cuvette holder. Maximal fluorescence signal was selected using a combination of high gain and zero suppression of the baseline. Once a baseline was established (1–3 min), the cells were exposed to 1- to 10-μl aliquots containing IP_3 or other agents to be tested, and the change in fluorescence due to release of Ca^{2+} was monitored on the recorder. At the end of each run, sequential additions of 1 nmol of $CaCl_2$ were used to calibrate the fluorescence signal internally.

For measurement of the Ca^{2+} content of intact and permeabilized cells, 2×10^6 cells were resuspended in KPH buffer, lysed with 0.2% Triton X-100, and centrifuged in a Beckman microfuge. The increase in fluorescence of an aliquot of the supernatant was measured in KPH buffer containing Quin 2.

RESULTS

IP_3 released Ca^{2+} from digitonin-permeabilized adrenal chromaffin cells. Figure 1 illustrates typical traces obtained when 4.4 μ*M* IP_3 was added to intact cells (Fig. 1A) and to permeabilized chromaffin cells (Fig. 1B). Intact chromaffin cells did not release Ca^{2+} following two separate additions of IP_3 (Fig. 1A). In contrast, permeabilized cells released 1.25 nmol of $Ca^{2+}/10^7$ cells following the initial addition of IP_3; however, a second dose of IP_3 failed to elicit further Ca^{2+} release (Fig. 1B). Ca^{2+} release from permeabilized cells was immediate following the addition of IP_3 and leveled off in seconds (Fig. 1B). In some experiments, the permeabilization buffer was supplemented with 10^{-7} or 10^{-6} *M* free

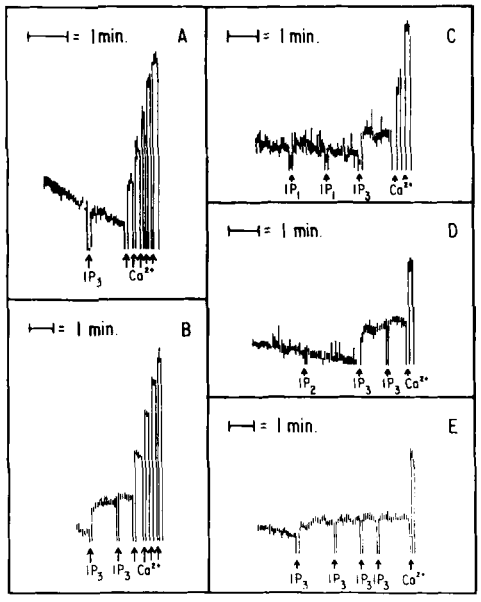

FIG. 1. Ca^{2+} release from adrenal chromaffin cells induced by IP_3 and its analogs. The fluorescence of Quin 2 in adrenal chromaffin cell suspensions was monitored as detailed in Materials and Methods. Following measured responses with IP_3 or one of its analogs, sequential additions of 1 nmol $CaCl_2$ were used to calibrate the fluorescence signal. (At least one $CaCl_2$ addition is shown for each trace.) **A:** Response of cells, incubated in KGP buffer without digitonin, to IP_3 (4.4 μ*M*). **B:** Responses of permeabilized cells exposed to two sequential additions of IP_3 (4.4 μ*M*). Cell concentrations = 7×10^6 in 1 ml. **C:** Responses of permeabilized cells to two sequential additions of IP_1 (5 μ*M*), followed by the addition of IP_3 (5 μ*M*). **D:** Responses of permeabilized cells to the addition of IP_2 (5 μ*M*), followed by two sequential additions of IP_3 (5 μ*M*). **E:** Responses of permeabilized cells to four sequential additions of IP_3 (0.55 μ*M*).

Ca^{2+}; this increase in free Ca^{2+} did not enhance the subsequent IP_3-induced response (data not shown).

The effect of analogs of IP_3 on the release of Ca^{2+} from permeabilized chromaffin cells was also tested. Two analogs, IP_1 and IP_2, at concentrations of 5 and 10 μ*M* failed to trigger Ca^{2+} release from chromaffin cells (Fig. 1C and D). However, the subsequent addition of IP_3 (5 μ*M*) revealed that the cells functioned normally and released the anticipated amounts of Ca^{2+}.

The release of Ca^{2+} from chromaffin cells was dependent on the concentration of IP_3 (Fig. 2). Release could be initiated by concentrations of IP_3 as low as 0.55 μ*M*, with half-maximal release occurring at approximately 1 μ*M* IP_3. Maximal release, aproximately 1.2 n*M* $Ca^{2+}/10^7$ cells, occurred at 4 μ*M* IP_3. When submaximal release of Ca^{2+} was induced by low levels of IP_3, subsequent additions of IP_3 failed to elicit additional Ca^{2+} release from the cells (Fig. 1E).

We attempted to assess both total Ca^{2+} and Ca^{2+} pools available for release in chromaffin cells. Total

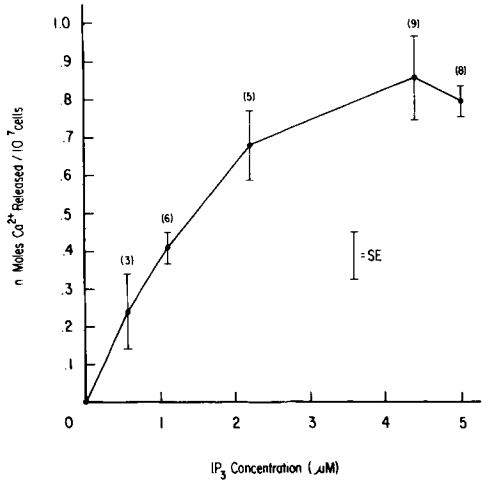

FIG. 2. Effect of IP_3 concentration on Ca^{2+} release from permeabilized adrenal chromaffin cells. The fluorescence of Quin 2 in permeabilized adrenal chromaffin cell suspensions was monitored as detailed in the Materials and Methods. IP_3 (1–10 μl aliquots) was added to the cells at the indicated final concentrations. The number of determinations for each concentration is shown in parentheses, and the error bars represent SEM.

Ca^{2+} in intact cells was approximately 40 nmol/10^7 cells. Permeabilization reduced the total Ca^{2+} to approximately 30 nmol/10^7 cells. The Ca^{2+} ionophore ionomycin (1 μ*M*) released 3–4 nmol Ca^{2+}/10^7 cells. Exposure of the permeabilized cells to ionomycin (1 μ*M*) completely inhibited subsequent IP_3-induced Ca^{2+} release. Thus, IP_3 caused Ca^{2+} release which represented approximately 30% of the ionomycin-sensitive and 3% of the total cellular Ca^{2+}.

DISCUSSION

IP_3 induced a rapid release of Ca^{2+} from digitonin-permeabilized chromaffin cells, but not from untreated cells. Thus, IP_3 probably acted at an intracellular site to trigger the release of Ca^{2+}. IP_3 acted rapidly, within a few seconds, was half-maximally effective at 1 μ*M*, and released approximately 1 nmol of Ca^{2+}/10^7 cells. These characteristics are similar to those found in other systems (Streb et al., 1983; Joseph et al., 1984; Prentki et al., 1984). However, in some of these systems, it has been noted that the released Ca^{2+} was immediately resequestered (Streb et al., 1983; Joseph et al., 1984); we did not observe this phenomenon. Dawson and Irvine (1984) also failed to observe Ca^{2+} resequestration after IP_3 stimulated Ca^{2+} release in mitochondrial fractions of rat liver, even in the presence of an ATP-regenerating system. Additionally, sequential additions of nonsaturating concentrations of IP_3 suggested that desensitization was taking place in our system, as occurred in the mitochondrial rat liver fractions (Dawson and Irvine, 1984).

Muscarinic stimulation increases the turnover of phosphatidylinositol (Adnan and Hawthorne, 1981; Fisher et al., 1981) and releases inositol phosphates (D. Eberhard and R. W. Holz, unpublished observations) from bovine chromaffin cells. The effects of IP_3 in the present experiments suggest that muscarinic stimulation of Ca^{2+} release from intracellular stores in adrenal chromaffin cells (Oka et al., 1982) and the rise in cytosolic Ca^{2+} (Kao and Schneider, 1985) may be caused by release of IP_3. The permeabilization method used here, which allows IP_3 to reach the intracellular space, masks the concentrations of both IP_3 and Ca^{2+} which could be reached within the confined volumes of intact cells. Without accounting for the Ca^{2+}-buffering capacity of the cell, Ca^{2+}, if released by a maximal concentration of IP_3 and confined to the cell, could possibly trigger exocytosis in bovine chromaffin cells or potentiate exocytosis when nicotinic stimmulation is suboptimal.[1]

Derome et al. (1981) have reported that muscarinic stimulation inhibits rather than enhances nicotinic-induced secretion from cultured bovine chromaffin cells. We have not observed this inhibition in a series of experiments (not shown) in which various concentrations of muscarinic and nicotinic agonists were varied systematically. Under physiological conditions, acetylcholine is released from nerves and interacts with chromaffin cells for periods probably considerably less than 1 s compared with the 2–15 min in tissue culture experiments. It remains possible that during brief exposure of chromaffin cells to acetylcholine in the functioning bovine adrenal medulla, both the muscarinic receptor-induced release of IP_3 and the nicotinic-receptor-induced Ca^{2+} influx contribute to the rise in cytosolic Ca^{2+} and the triggering of exocytosis. In species such as cat and guinea pig, where muscarinic agonists directly stimulate catecholamine secretion (Role et al., 1981; Kirpekar et al., 1982), IP_3 production may be greater than in bovine chromaffin cells and/or may result in greater Ca^{2+} mobilization than we have observed in this system.

REFERENCES

Adnan N. A. M. and Hawthorne J. N. (1981) Phosphatidylinositol labeling in response to activation of muscarinic receptors in bovine adrenal medulla. *J. Neurochem.* **36**, 1858–1860.

[1] Chromaffin cells (10^7) have a volume of approximately 0.01 ml (Hampton and Holz, 1983). Chromaffin cells release 1 nmol Ca^{2+}/10^7 cells in response to maximal concentrations of IP_3 (5 μ*M*). The released Ca^{2+} would result in an intracellular Ca^{2+} concentration of 100 μ*M* if the Ca^{2+} released were confined to the cell interior without rapid resequestration. Resequestration would reduce this concentration.

Agranoff B. W. and Seguin E. B. (1974) Preparation of inositol triphosphate from brain: GLC of trimethylsilyl derivative. *Prep. Biochem.* **4**, 359–366.

Ballou C. E. (1962) 1-myo-Inositol 1-phosphate (cyclohexammonium salt), in *Biochemical Preparations*, Vol. 9, (Coon M. E., ed), pp. 99–102. John Wiley & Sons, New York.

Burgess G. M., Godfrey P. P., McKinney J. S., Berridge M. J., Irvine R. F., and Putney J. W. Jr. (1984) The second messenger linking receptor activation to internal Ca release in liver. *Nature* **309**, 63–66.

Dawson A. P. and Irvine R. F. (1984) Inositol (1,4,5)-trisphosphate-promoted Ca^{2+} release from microsomal fractions of rat liver. *Biochem. Biophys. Res. Commun.* **120**, 858–864.

Derome G., Tseug R., Mercier P., Lemaire I., and Lemaire S. (1981) Possible muscarinic regulation of catecholamine secretion mediated by cyclic GMP in isolated bovine adrenal chromaffin cells. *Biochem. Pharmacol.* **30**, 855–860.

Douglas W. W. (1975) Secretory control of adrenal medullary secretion: membrane and ionic events in stimulus-secretion coupling, in *Handbook of Physiology Endocrinology, Section 7*, pp. 367–388. American Physiological Soc., Washington, DC.

Douglas W. W. and Rubin R. P. (1961) The role of calcium in the secretory response of the adrenal medulla to acetylcholine. *J. Physiol. (Lond.)* **159**, 40–57.

Dunn L. A. and Holz R. W. (1983) Catecholamine secretion from digitonin-treated adrenal medullary chromaffin cells. *J. Biol. Chem.* **258**, 4989–4993.

Fisher S. K., Holz R. W., and Agranoff B. W. (1981) Muscarinic receptors in chromaffin cell cultures mediate enhanced phospholipid labeling but not catecholamine secretion. *J. Neurochem.* **37**, 491–497.

Hampton R. Y. and Holz R. W. (1983) Effects of changes in osmolality on the stability and function of cultured chromaffin cells and the possible role of osmotic forces in exocytosis. *J. Cell Biol.* **96**, 1082–1088.

Holz R. W., Senter R. A., and Frye R. A. (1982) Relationship between Ca^{2+} uptake and catecholamine secretion in primary dissociated cultures of adrenal medulla. *J. Neurochem.* **39**, 635–646.

Holz R. W., Senter R. A., and Sharp R. R. (1983) Evidence that the H^+ electrochemical gradient across membranes of chromaffin granules is not involved in exocytosis. *J. Biol. Chem.* **258**, 7506–7513.

Joseph S. K., Thomas A. P., Williams R. J., Irvine R. F., and Williamson J. R. (1984) myo-Inositol 1,4,5-trisphosphate. A second messenger for the hormonal mobilization of intracellular Ca^{2+} in liver. *J. Biol. Chem.* **259**, 3077–3081.

Kao L. S. and Schneider A. S. (1985) Muscarinic receptors on bovine chromaffin cells mediate a rise in cytosolic calcium that is independent of extracellular calcium. *J. Biol. Chem.* **260**, 2019–2022.

Kilpatrick D. L., Slepetis R. J., Corcoran J. J., and Kirschner N. (1982) Calcium uptake and catecholamine secretion by cultured bovine adrenal medulla cells. *J. Neurochem.* **38**, 427–435.

Kirpekar S. M., Prat J. C., and Schiavone M. T. (1982) Effect of muscarine on release of catecholamines from the perfused adrenal gland of the cat. *Br. J. Pharmacol.* **77**, 455–460.

Knight D. E. and Kesteven N. T. (1983) Evoked transient intracellular free Ca^{2+} changes and secretion in isolated bovine adrenal medullary cells. *Proc. R. Soc. Lond. B.* **218**, 177–199.

Oka M., Isosaki M., and Watanabe J. (1982) Calcium flux and catecholamine release in isolated bovine adrenal medullary cells: effects of nicotinic and muscarinic stimulation. *Adv. Biosci.* **36**, 29–36.

Prentki M., Wollheim C. B., and Lew P. D. (1984) Ca^{2+} homeostasis in permeabilized human neutrophils. Characterization of Ca^{2+}-sequestering pools and the action of inositol 1,4,5-trisphosphate. *J. Biol. Chem.* **259**, 13777–13782.

Role L. W., Leeman S. E., and Perlman R. L. (1981) Somatostatin and substance P inhibit catecholamine secretion from isolated cells of guinea pig adrenal medulla. *Neuroscience* **6**, 1813–1821.

Streb H., Irvine R. F., Berridge M. J., and Schulz I. (1983) Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature* **306**, 67–69.

Trifaro J. M. (1969) Phospholipid metabolism and adrenal medullary activity. 1. The effect of acetylcholine on tissue uptake and incorporation of orthophosphate-^{32}P into nucleotides and phospholipids of bovine adrenal medulla. *Mol. Pharmacol.* **5**, 382–393.