Cellular Responses to Stimulation of the M5 Muscarinic Acetylcholine Receptor as Seen in Murine L Cells*

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The neurotransmitter acetylcholine acting through muscarinic receptors has a wide variety of effects which are conditioned by the cell type and the molecular identity of both the receptor with which acetylcholine interacts and of the coupling proteins with which the receptors interact. Thus, acetylcholine may cause at the biochemical level the stimulation of phosphoinositide breakdown (2), which in turn leads through formation of the second messenger inositol trisphosphate to release Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores (3) or the inhibition of adenylyl cyclase (4) and, if present, concomitant stimulation of K\(^+\) channels (5, 6). Other cellular responses to muscarinic receptor stimulation are inhibition of the M-type K\(^+\) current in neuronal cells (7), inhibition of Ca\(^{2+}\) channels (8) and stimulation of Ca\(^{2+}\) influx (9), and may include increases in cAMP levels (10) and release of arachidonic acid (11). All these effects of acetylcholine are mediated by a set of muscarinic receptors which were subdivided on the basis of their pharmacological behavior with respect to blockers such as pirenzepine and AF-DX 116 and the primary tissues in which they are expressed into M1 or neuronal, M2 or cardiac, and M3 or glandular (12-14). More recently, purification and molecular cloning revealed the existence of a family of five distinct muscarinic receptors, termed M1 through M5 (reviewed in Ref. 15). Based on their structural similarity and biochemical effects (or lack thereof) on inhibition of adenylyl cyclase and on phosphoinositide turnover these receptors have been grouped as M2-like, which include the M2 and M4 receptors and cause inhibition of adenylyl cyclase in isolated membranes, and M1-like, which comprise the M1, M3 and M5 receptors and cause stimulation of phospholipase C. M1, M2, and M3 receptors are found rather widely distributed in both neuronal and non-neuronal tissues (16, 17). The M4 and M5 receptors have thus far been found only in neuronal tissues, the expression being most restricted for the M5 receptors (16-19). This makes the M5 receptor essentially inaccessible to biochemical analysis of its mode of action.

In a previous publication we showed that the M5 receptor\(^1\) when expressed in murine L cells stimulates phospholipase C activity as seen in intact cell studies but does not affect adenylyl cyclase as measured in isolated membranes (18). The present work was undertaken to explore other potential signaling pathways used by the M5 receptor as seen in the murine L cell, such as causing the release of arachidonic acid, as shown for M1 and M3 receptors (11), and changing intracellular Ca\(^{2+}\) concentrations. We also tested whether the M5 receptor would alter cAMP levels in the L intact cell, for it was reported have this effect in Chinese hamster ovary cells (20).

\(^1\) The M5 receptor cloned by us from a rat brain cDNA library (19), is the same as the m5 receptor cloned from rat and human genomic libraries by Bonner et al. (50).

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Changes in intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)) in response to hormones have been the center of attention of many laboratories. Earlier investigations studied 45Ca\(^{2+}\) fluxes (e.g., 21, 22) or \(^{86}\)Rb\(^+\) efflux, which reflected the activity of Ca-dependent K\(^+\) channels (23). With the advent of fluorescent Ca\(^{2+}\) indicators such as quin-2 (24) and fura-2 (25), transients in response to agonists have been accessed more directly and shown to follow a common biphasic pattern formed of a large and transient peak response and a lasting or sustained response. The peak response is independent of extracellular Ca\(^{2+}\) and is accepted as being the result of inositol triphosphate-induced release from an internal Ca\(^{2+}\) store. In contrast to the peak response, the sustained response is dependent on extracellular Ca\(^{2+}\) and due to influx of Ca\(^{2+}\) into the cell by mechanisms that are not well understood (reviewed in Ref. 26). The decreases in [Ca\(^{2+}\)]\(_i\) after the rise to the initial peak level is thought to be due to active extrusion of Ca\(^{2+}\) from the cells as deduced from 45Ca\(^{2+}\) efflux studies (22, 27). Quantitatively the two phases of the response observed in the various cells vary remarkably in their details. The sustained response arising in some cells faster than the peak response (28) and lagging behind the peak response in other cells (29). Further, the sustained response may maintain [Ca\(^{2+}\)]\(_i\), as high as 70% of that of the peak response and decay so slowly that it may be interpreted as a component of the peak response and decay to close to control values within a few minutes (vide infra). The mechanism by which Ca\(^{2+}\) enters cells during the sustained phase of the response varies as well with cell type. It may involve voltage-gated Ca\(^{2+}\) channels, as seen though their blockade with Ca\(^{2+}\) channel blockers (e.g. Ref. 30) or their oscillations concordant with changes in membrane potential (31), the activation of a sodium influx pathway in tandem with a compensatory Na\(^+\)/Ca\(^{2+}\) exchange mechanism (32), and it may enter through unknown pathways not involving electrophysiologically active ionic channels (e.g. Ref. 33). In view of the ability of the M5 receptor to stimulate phosphoinositide hydrolysis, we characterized its effects on intracellular Ca\(^{2+}\) transients as seen in murine L cells.

Murine Ltk\(^-\) cells were developed in 1963 by Kit et al. (34) while studying the functional importance of thymidine kinase and are easily transfected with genomic or cloned DNA (35). They have therefore been used rather widely as expression systems either to clone new genes (e.g. Refs. 36, 37) or, alternatively, to identify functional properties of cloned molecules, such as done recently by us when we identified a protein encoded in a newly cloned cDNA as the M5 acetylcholine receptor (15) and demonstrated voltage-gated Ca\(^{2+}\) channel activity for the protein encoded in a previously cloned cDNA (39). L cells appear to be useful in studying not only intrinsic activities such as binding and/or ionic channel activity of transfected molecules but also in studying the signaling pathways that such molecules may affect. With regards to G protein-coupled receptors, L cells contain both cholera and pertussis toxin subtypes, a prostaglandin (PG) stimulatible adenylyl cyclase, and a purinergic (ATP) stimulatable phospholipase C system which we showed in our previous publication to respond also to the transfected M5 acetylcholine receptor (15). We show below that L cells also contain the hereditary, genetically engineered machinery that allows it to respond to M5 and purinergic receptors by mobilizing Ca\(^{2+}\) from intracellular stores, by promoting Ca\(^{2+}\) influx from the extracellular space and by releasing arachidonic acid. In contrast to what is observed with other muscarinic receptors in other cells, the M5 receptor does not affect AMP levels in L cells. This indicates that regulation of adenylyl cyclase activity is not one of its primary effects.

**EXPERIMENTAL PROCEDURES**

**Materials**

Radiochemicals—myo-[\(^{3}H\)]inositol (40 Ci/mmol), [\(^{3}H\)]adenosine 3',5'-cyclic monophosphate (cAMP) (80-100 Ci/mmol) and [\(^{3}H\)]N-methylsphingosine (NMS, 82 Ci/mmol) were from Du Pont-New England Nuclear; \(^{151}\)-iodoacetyl-L-ascorbic acid (95 Ci/mmol) was from Amersham Corp.; [\(^{3}P\)]ATP (200-400 Ci/mmol) and [\(^{3}P\)]NAD* (400-1000 Ci/mmol) were synthesized according to Walseth and Johnson (39) and Cassel and Pfeuffer (40), respectively, and supplied by the Baylor College of Medicine Diabetes and Endocrinology Research Center.

Biochemicals—Serum-free bovine serum albumin (BSA), DNAse I, Lubrol PX, \beta-mercaptoethanol, PMA, cholate, bovine, neurotensin, thyrotropin-releasing hormone, bombesin, angiotensin II, bradykinin, vasoactive intestinal peptide, arginine-vasopressin, neuregulin, substance P, neuropeptide Y, thrombin, prostaglandin (PG) F2\(_a\), serotonin, atropine, carbobol (CCB), and (-)-epinephrine were from Sigma. Buserelin (a gonadotropin-releasing hormone receptor agonist), was from Hoechst (Frankfurt, N.M., Federal Republic of Germany). Hesper, ATP, and AMP-P(NH)P were from Boehringer Mannheim. BSA was from Aldrich. Analytical grade Dowex 1-X8 (AG 1-1X8), 200-400 mesh, formate form, polyacrylamide gel electrophoresis reagents and sodium dodecyl sulfate were from Bio-Rad. Fura-2/AM was from Molecular Probes (Eugene, OR) and PTX and CTX were from List (Campbell, CA).

**Tissue Cultures**—Minimum essential medium (MEM) a medium, Hank's-buffered salt solution (HBSS), Dulbecco's buffered salt solution (PBS), penicillin/streptomycin, trypsin 0.5%, 5 mM EDTA, and fetal bovine and horse sera were from Gibco. Tissue culture plasticware (Falcon 24- and 6-well plates, Corning 10-cm culture plasticware) were from Fisher.

**Methods**

**Culture of Ltk\(^-\) and Transformed Ltk\(^+\) Cells—Mouse Ltk\(^-\) cells (clone c-\(-\)) were cultured at 5% CO\(_2\) in MEM a medium containing 10% heat-inactivated fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 \(\mu\)g/ml). Ltk\(^+\) cells lines (derived from Ltk\(^-\) by cotransfection of the cloned M5 receptor in vector pPH60731) and cells transfected with STAH (the wild-type thymidine kinase gene in pHSV-tk) were selected and maintained in 5% CO\(_2\) atmosphere in HAT selection medium as described previously (15).

**Phosphoinositide Hydrolysis—Receptor stimulation of cellular phosphoinositide-specific phospholipase C was tested for in intact cells by an adaptation of the procedures described by Borel et al. (41) and Kirk et al. (42) in which cells are labeled with myo-[\(^{3}H\)]inositol, incubated with agonist in the presence of LiCl, and the free \(^{3}H\)-labeled inositol phosphates accumulated during the incubation are extracted, separated on Dowex-1 columns, and quantified by liquid scintillation counting. Typically, \(-0.5 \times 10^6\) Ltk cells were seeded in 75-cm\(^2\) flasks or 6-well plates in 2.5 ml of HAT selection medium (see above) supplemented with 2 \(\mu\)Ci/ml [\(^{3}H\)]inositol and grown for 48 h to a density of about \(2 \times 10^5\) cells/well. Labeled cells were then placed into 2.5 ml of fresh HAT selection medium without fetal calf serum and without labeled inositol, and incubated for 1 h at 37°C. The cells then were rinsed twice at room temperature with 2.0 ml of
PBS supplemented with 5.5 mM glucose, 0.5 mM CaCl₂, and 0.5 mM MgCl₂. After addition of 2 ml of PBS with glucose, Ca²⁺, and Mg²⁺, cells were placed again at 37 °C for 20 min at which time 10 mM LiCl was added. CCH at various concentrations or other test agents were then added after a further 10 min, except for strontium which was added 5 min before CCH. Unless indicated otherwise the incubations were stopped 30 min after the additions of stimulatory agents by placing the dish on ice, removing anddiscarding the incubation medium, and addition of 1 ml of ice-cold 5% perchloric acid and 20 µl of 10% bovine serum albumin.

Arachidonic Acid Release—An adaptation of the protocols of Okajima and UI (43) and Burch et al. (44) was used. Briefly, cells were seeded and grown in 6-well plates as described for phosphoinositide hydrolysis. After addition of 20 pM of the acetoxymethyl ester of fura- (fura-P/AM, added of 2 x 10⁶ cells/ml, and incubated for another 30 min at 37 °C. The cells were washed twice with HBSS with 0.2% fatty acid-free BSA (HBBS-BSA) and incubated 2 min with 1 ml of HBBS-BSA, prior to addition of test agents in a fresh 1-ml aliquot of HBSS-BSA. After a final incubation of 10 min at 37 °C, the media were transferred to 1.5-ml microcentrifuge tubes, centrifuged for 1 min, and the re-leased radioactivity present in the supernatants was counted in a liquid scintillation counter after addition of 3.5 ml of Amersham's ACS counting fluid. The counts/minute obtained were taken as representing the arachidonic acid release. The cells that remained in the 1-ml of 0.05% BSA for 30 min were plated. The lysates were transferred to the counting vials and counted as described above. The counts/minute obtained plus those collected in the media represented the total arachidonic acid incorporated into the cells.

Measurement of cAMP Accumulation—Confluent L5 cells in 100-mm Petri dishes were washed with HBSS without Ca²⁺ and Mg²⁺ and incubated with 5 µl of PBS containing 5 mM EDTA at 37 °C for 5-10 min. The PBS was removed and the cells were detached by repeatedly flushing with 10 ml of growth medium. The cell suspensions were transferred to 50 ml of capped polyethylene tubes. After removing 0.1 ml for cell counting, the remnant was centrifuged at room temperature for 4 min at 100 x g, and the pellets were resus-pended in 1 ml of 0.05% BSA for 30 min. The lysates were transferred to the counting vials and counted as described above. The cells were then aliquoted into 12 x 75-mm glass tubes (106 cells/tube), incubated for 20 min at 32 °C to allow full equilibration with IBMX, and then exposed to test agents for 10 min. The incubations were terminated by centrifugation, removal of supernatant, addition of 500 µl of 50 mM sodium acetate, pH 5.8, boiling for 2-5 min and recentrifugation, followed by preparation of 10- and 100-fold dilutions of the resultant supernatants. The cAMP in 100-µl aliquots of the diluted extracts as well as standards in the same buffer were acetylated by addition/tube of the extract from 5 to 10 x 10⁶ cells.

Measurement of Cytosolic Calcium Concentration with Fura-2 Cells were grown to confluence in 6-well plates (Figs. 3 and 4) or on 150-mm Petri dishes (subsequent figures). They were detached and pelleted as described for the cAMPr measurement studies described above. The cell pellets were then suspended at room temperature in extracellular buffer (ECS buffer: 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 1.8 mM CaCl₂, 10 mM glucose, 0.1% BSA, 15 mM Hepes, pH adjusted to 7.4 at room temperature to a density of 10⁶ cells/ml. Two ml of the cell suspension were saved as nonloaded control. The remained of the cells were pelleted and resuspended in 2.5 ml of ECS buffer prewarmed to 37 °C and all subsequent manipulations were carried out keeping the cells in the dark to prevent bleaching of the fluorescent dye. To load cells with fura-2, the suspension (10⁶ cells/ml) was incubated for 30 min at 37 °C with 50 µM of the acetoxyethyl ester of fura-2 (fura-2/AM, added in 25 µl of dimethyl sulfoxide), diluted with ECS buffer to a density of 2 x 10⁶ cells/ml, and incubated for another 30 min at 37 °C. The cells were then pelleted, resuspended at a density of 2 x 10⁶/ml in ECS at room temperature, distributed in 2-ml fractions into 15-ml polystyrene tubes, and kept at room temperature until used. Prior to use the cells were washed twice with 2 ml of ECS buffer (100 x g, 4 min), resuspended in the same volume, and added to a quartz cuvette. Fluorescence (F) was monitored at 510 nm with a SLM 8600-C photon counting spectrophotometer (SLM Instruments, Urbana IL) with excitation wavelength of 340 nm. Cells were kept with a magnetic stirrer. After obtaining a few minutes long base-line record, experimental agents were added (arrows on figures) to give the indicated final concentrations. All experiments were terminated by adding first 20 µl of 10% Triton X-100 and then 30 µl of 500 mM EDTA, 1 mM Hepes-Na, pH 8.5, to obtain Fₘ and Fₐ respectively. Fluorescence intensities were recorded and an IBM XT computer (IBM, Boca Raton, FL) with SML software was used to correct for autofluorescence, which was determined on cells treated exactly as above but without fura-2/AM. The digitized records of F were imported into Symphony software (Lotus Development Corporation, Boston) for calculation of free intracellular Ca²⁺ concentration ([Ca²⁺]), and then into Sigma-Plot (Jandel Scientific, Corte Madera, CA) and plotted with a Hewlett-Packard Plotter (Palo Alto, CA). [Ca²⁺] was calculated from F values according to the equation:

$$[Ca^{2+}] = K_d (F - F_{min})/F_{max} - F$$

where the equilibrium dissociation constant (Kₐ) for the Ca²⁺-fura-2 complex is 135 mM at room temperature (25).

ADP-ribosylation of Cellular PTX Substrates with [35P]NAD— For ADP-ribosylation with PTX, cells grown to confluence were detached with EDTA in PBS as described for cAMP measurements, centrifuged at 4 °C, and resuspended at a density of 1 x 10⁶ cells/100 µl in ice-cold homogenization buffer (10 mM Na-Hepe, 1 mM EDTA, 27% (w/w) sucrose, pH 8.0) and homogenized in a Dounce homoge-nizer in the presence of 1% Lubrol PX added at the moment of homogenization (20 strokes with the tight pestle). The homogenate was centrifuged for 1 min. The supernatant was distributed in 50 µl of 2% fatty acid-free BSA (HBSS-BSA), and incubated 2 min with 1 ml of HBSS-BSA. The supernatants were then added 5 min before CCh. Unless indicated otherwise the incubations were imported into the ADP-ribosylation reactions at 37 °C. ADP-ribosylations were carried out in a final volume of 30 µl containing 10 µl of homogenate, 10 µl of 45 mM Tris-HCl, pH 8.0, 3 mM EDTA, 30 mM thymidine, 0.45 mM ATP, 0.3 mM GTP/BS, and 0.05% BSA, 5 µl of 1 mM [35P]NAD° (5 x 10⁶ cpm), and 5 µl of activated and diluted PTX. PTX activation was at 300 µg/ml in 50 mM dithiothre-itol, 1 mM AMP-P(NH)P for 20 min at 32 °C followed by a 5-fold dilution to 60 µg/ml toxin with 0.05% BSA and 10 mM Tris-HCl, pH 8.0. After 30 min of incubation, the ADP-ribosylation reactions received 5 µl of 5 µg/ml DNase I, 10 mM MgCl₂ and 60 mM NAD°, and diluted homogenates were prepared and stopped by addition of 50 µl of Laemmli's sample buffer (45) containing 10% mercaptoethanol and 100 mM NAD°. For analysis, the totality of the samples were loaded into the sample wells of 10% polyacrylamide gel slabs and electrophoresed at 100 mV. The gels were stained with Coomassie Blue for 30 min, checked for even transfer of the BSA of the samples, dried, and visualized with 10% acetic acid, dried, and autoradiographed.

Other—Muscarinic receptor sites were determined on intact cells as described (15). Proteins were assayed by the method of Lowry et al. (46) using BSA as standard.

**Gifts—Mouse Ltk-a-HT, a subclone of the mouse fibroblast cell line originally established by Kit et al. (34), selected for high frequency of transformation, was a generous gift from Dr. Frank Ruddle (Department of Biology, Yale University).

**RESULTS**

Survey of Signaling Pathways of L Cells Affected by the M5 Receptor

In our previous work we demonstrated that the M5 receptor stimulated phosphoinositide hydrolysis as seen through the accumulation of 3H-labeled inositol phosphates from prelabeled pools of phosphoinositides in the presence of 10 mM LiCl. Fig. 1 confirms and expands those finding by showing that even though levels of the three main forms of inositol phosphates that accumulate in the presence of LiCl vary as a function of time, the accumulation of the sum of inositol mono-, bis-, and triphosphates is essentially linear over a time period of 10 min. This indicates that as far as phosphoinositide hydrolysis is concerned, the effect of the M5 receptor does not undergo desensitization during the first 3 min of incubation and that losses of other responses that may occur as a function of time must be due to reasons other than receptor uncoupling from the phospholipase C system. The L cells expressing the transfected M5 receptor accumulated inositol phosphates about 20-fold faster in the presence of
response to the purinergic ligands 2-2.3-fold with respect to ysis or to promote increases in $[\text{Ca}^{2+}]_i$. Significant effects were genie amines, serotonin, and epinephrine, which are all known phosphate (ZP), inositol bisphosphate (ZP$p$), and inositol trisphosphate (ZP$3$) of the activity of the receptor-sensitive phospholipase C, this enzyme 20-fold over basal.

of inositol phosphates under these conditions is a reflection L cells express receptors for other agonists acting similarly to this enzyme and to either stimulate phosphoinositide hydrol- obtained only with ATP (Table I) and was mimicked by AMP-ergic phosphates accumulated. Inset, distribution of radioactivity in fractions eluting from the Dowex 1 columns with standard inositol phosphate (IP$3$), inositol bisphosphate (IP$2$), and inositol trisphosphate (IP$1$). For further details see Ref. 15. Results such as these were obtained in two additional experiments.

saturating carbachol than in its absence. Since accumulation of inositol phosphates under these conditions is a reflection of the activity of the receptor sensitive phospholipase C, this indicates that the M5 receptor occupancy is able to stimulate this enzyme 20-fold over basal.

We tested whether in addition to the transfected receptor L cells express receptors for other agonists acting similarly to stimulate formation of inositol phosphates. These agonists were the purinergic receptor ligands ATP and AMP-P(NH)$_2$P, a variety of peptides, thrombin, prostaglandin F$_2$-a, and bio-
genic amines, serotonin, and epinephrine, which are all known in other systems to either stimulate phosphoinositide hydrolysis or to promote increases in [Ca$^{2+}$]. Significant effects were obtained only with ATP (Table I) and was mimicked by AMP-P(NH)$_2$P (not shown). Inositol phosphates accumulated in response to the purinergic ligands 2-2.3-fold with respect to control in the parent Ltk$^-$ cell line and a similar 2.3-2.8-fold in the transfected LM5.36 cell line (not shown). Since phosphoinositide hydrolysis can be increased in these cells by as much as 20-fold, this indicates that the resident purinergic receptor(s) affect but a fraction of the intrinsic cellular poten-
tial for hydrolyzing phosphoinositides.

The potential of M5 and purinergic receptors to affect arachidonic acid release from cells prelabeled with $[^{14}C]$arachidonic acid was assessed for both LM5 and Ltk$^-$ cells. As illustrated in Fig. 2, both types of receptors caused release of free arachidonic acid, and like phosphoinositide hydrolysis, M5 receptors had a much higher efficacy eliciting this re-
sponse than the purinergic receptors. Incubation of cells for 15 min (Fig. 2) or 30 min (not shown) with protein kinase stimulator PMA did not mimic the effects of receptor stimulation on arachidonic acid release by LM5 (Fig. 2) or by Ltk$^-$ cells (not shown). This indicated that stimulation of this enzyme alone cannot be solely responsible for the receptor-stimulated arachidonic acid release.

In our previous report we reported that the M5 receptor did not mediate inhibition or stimulation of adenyl cyclase as assessed in membranes isolated from LM5 cells. However, in view of a report that the M5 receptor increases cAMP levels in intact Chinese hamster ovary cells (20), we investigated a potential cAMP-elevating role for the M5 receptor in intact

### Table I

Phosphoinositide hydrolysis in Ltk$^-$ cells in response to compounds with potential stimulatory activity

| Addition to incubations | Total $[^{3}H]$inositol phosphates accumulated$^*$ |
|-------------------------|-----------------------------------------------|
|                         | cpm x $10^3$/well                           |
| None                    | 1.5 ± 0.1                                    |
| ATP (100 μM)            | 3.4 ± 0.1                                    |
| Colectokinin (100 nM)   | 1.5 ± 0.1                                    |
| Angiotensin II (100 nM) | 1.6 ± 0.3                                    |
| Bradykinin (100 nM)     | 1.6 ± 0.2                                    |
| Thyrotrpin releasing hormone (100 nM) | 1.3 ± 0.2 |
| Substance P (100 nM)    | 1.4 ± 0.1                                    |
| Buserelin (100 nM)      | 1.5 ± 0.1                                    |
| Bombesin (100 nM)       | 1.6 ± 0.1                                    |
| Vasoactive intestinal peptide (100 nM) | 1.4 ± 0.2 |
| Arginine-vasopressin (100 nM) | 1.5 ± 0.1 |
| Neuromedin C (100 nM)   | 1.7 ± 0.1                                    |
| Neuropeptide Y (100 nM) | 1.4 ± 0.2                                    |
| Neurotensin (100 nM)    | 1.3 ± 0.1                                    |
| Thrombin (10 units/ml)  | 1.7 ± 0.1                                    |
| Prostaglandin F$_2$-a (10 μM) | 1.6 ± 0.1 |
| Serotonin (10 μM)       | 1.4 ± 0.1                                    |
| (–)-Epinephrine (10 μM) | 1.6 ± 0.2                                    |
| Carbachol (100 μM)      | 1.5 ± 0.1                                    |

$^*$ Values are means ± ½ range (rounded off to the nearest 100 cpm/ well) of the sum of water-soluble inositol phosphates accumulated in 30 min as determined in duplicate wells.
Effects of the Cloned M5 ACh Receptor

L cells by assessing cAMP levels measured by radioimmunoassay. To this effect LM5.36 cells were incubated 10 min in the absence and presence of the adenyl cyclase stimulator PGE1, under conditions where PGE1 increased intracellular cAMP levels ~10-fold, and tested for an effect of CCh either increasing or decreasing basal or PGE1-stimulated cAMP levels. We found that CCh, and by inference the M5 receptor, was without effect. Stimulation of the purinergic receptor was also without effect (not shown).

Because both M5 and purinergic receptors promote IP3 formation, and in other cell systems this leads to release of Ca2+ from endogenous stores (3), we loaded Ltk- and LM5 cells with the fluorescent Ca2+ indicator dye fura-2 and tested the effect of ATP and CCh. As shown in panels A–D of Fig. 3, we indeed found that intracellular Ca2+ levels were transiently elevated in response stimulation of these receptors, following a time course that is typical for these type of responses (e.g. 27, 28, 33). As expected from the phosphoinositide hydrolysis and arachidonic acid release studies, only the LM5 cells responded to CCh while ATP triggered a response in both the Ltk- and the LM5 cells. In agreement with the known pharmacological properties of these type of receptors, the effect of CCh but not that of ATP was blocked by the muscarinic blocker atropine (Fig. 6, B and E). However, while the ratio of responsiveness CCh/ATP was about the same for phosphoinositide hydrolysis and arachidonic acid release (CCh stimulating about 8-fold more than ATP), the peak concentrations of intracellular Ca2+ ion ([Ca2+]i) in the LM5.36 cell in response to saturating CCh were about 3-fold higher than those obtained with ATP. Fig. 4 presents the results from four experiments in which the accumulation of inositol phosphates, arachidonic acid release, and the maximal peak increase in [Ca2+]i were studied as a function of CCh concentration. Half-maximal effects for the last two of these effects were obtained at 0.4–0.5 μM CCh. In contrast between 1.5 and 2 μM CCh were required for half-maximal effects on accumulation of inositol phosphates.

The above experiments are consistent with the interpretation that both the L cell purinergic receptor and the neuronal M5 receptors trigger cellular responses by stimulating phospholipase C with consequential formation of IP3 and that the IP3 thus formed causes release of Ca2+ from intracellular stores, and that in addition these receptors stimulate the liberation of free arachidonic acid. The nature of the lipid from which arachidonic acid is hydrolyzed has not been investigated, but similar studies from other laboratories suggest that it is a phospholipid and the release is due to stimulation of phospholipase A2 activity (47). The relative responses to purinergic and M5 receptors suggest that if the increase in [Ca2+], is required for triggering the arachidonic acid release response, it is not the sole intracellular second mediator of this effect. Our data are consistent with the proposal of Burch et al. (44) that the arachidonic acid release responses triggered may be mediated through a signaling pathway parallel to that responsible for inositol trisphosphate production.

Complexity of the Ca2+ Response Triggered by the M5 and Purinergic Receptors in L Cells

The [Ca2+]i, transients caused by CCh and ATP in Fig. 3 agree with those described for other phospholipase C-stimulating agonists in other cell systems in that they are formed of a transient peak and a sustained phase. The peak response has a rapid rise and a relatively fast decay (t1/2 ~0 s) that is slower than that of the ATP-triggered response (see Fig. 3, A versus D and E). The sustained response appears to rise more slowly with CCh than ATP (see third and fourth panels from the top in Fig. 5, and others where the effects of subsaturating concentrations of CCh are shown to result in a clear biphasic response). As pointed out earlier the reduction of [Ca2+]i, after the peak rise is not due to a homologous receptor desensitization reaction (inositol phosphates are formed at a constant rate during this time) and is therefore a reflection of a combination of factors that include exhaustion of the pool of Ca2+ from which the initial increase in [Ca2+]i, is derived

![Fig. 3. Stimulation of Ca2+ mobilization by carbachol and ATP in LM5.36 cells, and Ltk- cells. Intracellular free Ca2+ concentrations, [Ca2+]i, were measured at room temperature using 4 × 104 fura-2 loaded cells suspended in 2 ml of buffer for each trace. CCh, ATP, and atropine were added to the LM5.36 cells (A–C) and Ltk- cells (D–F) as shown. The changes in fluorescence intensity were measured and converted to [Ca2+]i values as described under "Experimental Procedures." The traces are computer generated and representative of similar curves obtained in two to three repeat experiments. Peak [Ca2+]i values elicited in LM5.36 cells upon addition of 100 μM CCh were (mean ± S.D.) 333 ± 43 nM (n = 13) and those obtained in the same cells with 100 μM ATP were 203 ± 13 nM (n = 12).](http://www.jbc.org/)
the setting in of homeostatic mechanisms that lead to a final leveling off of [Ca\(^2+\)]\(_i\), at levels that, elevated over the preagonists [Ca\(^{2+}\)]\(_i\), are but a fraction of the peak value of [Ca\(^{2+}\)].

Several lines of evidence were obtained that indicate that the fast decay in the initial [Ca\(^{2+}\)]\(_i\), is associated with an exhaustion of the pool of Ca\(^{2+}\) from which the initial peak had been derived. The first was that postaddition of saturating (100 \(\mu\)M) ATP after a saturating concentration (100 \(\mu\)M) of CCh (Figs. 3A and 5) had no effect, the second was that stimulation of the less effective purinergic receptor with 100 nM CCh (Figs. 3A and 5) had no effect, the second was that postaddition of saturating ATP 4 to 5 min later had the same effect as in naive cells (cf. Figs. 3 and 6).

The left panels of Fig. 3 show the effect of varying the concentrations of CCh on the first and second phase responses as well as on the availability of Ca\(^{2+}\) for a subsequent response to saturating ATP. In these particular experiments, carried out 4 months after the dose-response curves shown in Fig. 4 using cells grown to confluence in 150-mm Petri dishes instead of 6-well plates, the concentrations of CCh giving half-maximal effects were 2 \(\mu\)M, 1, and 0.6 \(\mu\)M for eliciting, respectively 1) the initial peak increase of [Ca\(^{2+}\)]\(_i\), 2) the peak increase of [Ca\(^{2+}\)]\(_i\), of the second phase response (as seen 1.2 min after CCh with 0.3 and 1.0 \(\mu\)M CCh), and 3) the inhibition of the ATP response. While in this and two repeat experiments we always found a slight (2-2.5-fold) left shift in the dose-response curve for inhibition of a second first-peak response in [Ca\(^{2+}\)]\(_i\), as compared with that with which the first peak [Ca\(^{2+}\)]\(_i\), response was obtained, the dose-response curves for the first and second peak responses to the initial addition of CCh did not differ in a statistically significant manner.

We obtained an idea as to the time required for full reappearance of a first phase response (as seen with ATP) after causing its partial disappearance with an initial dose of CCh when we blocked the sustained response to CCh with excess atropine, and then added saturating ATP at varying times thereafter. Fig. 6 shows the results of one of three such experiments indicating that the depletable pool of intracellular Ca\(^{2+}\) is refilled in 3-4 min under the standard incubation conditions used here. The data are consistent with the first peak response being due to IP\(_3\)-mediated release of a Ca\(^{2+}\) from a limiting intracellular pool. Panels G and H are from another experiment and demonstrate that the replenishment of this limiting pool does not occur for as long as agonist is present, consistent with continued production and action of IP\(_3\).

The experiments of Fig. 6 (see also Fig. 7C) indicate further that the [Ca\(^{2+}\)]\(_i\), response is continuously dependent on agonist occupancy of the receptor. As has been seen in other systems, the peak height of the first peak response is essentially unaltered or diminished only slightly by removal of Ca\(^{2+}\) from the medium as obtained when, for example, 4 mM EGTA is added together with the agonist (not shown). In contrast, the sustained response is abolished. Continued incubation in the presence of high levels of EGTA led to a reduction in [Ca\(^{2+}\)]\(_i\), to levels below control. The dependence of the sustained response on extracellular Ca\(^{2+}\) was established further in the experiments shown in Fig. 7, in which fura-2-loaded cells were resuspended in low, nominally Ca\(^{2+}\)-free buffer containing 0.3 mM EGTA. Under these conditions, basal levels of [Ca\(^{2+}\)]\(_i\), while lower than in Ca\(^{2+}\)-containing buffers, were nevertheless stable for up to 12-15 min. It can be seen that under these conditions the sustained response is absent, both at high (Fig. 7B) and low (Fig. 7C) agonist, but can be readily restored by addition of extracellular Ca\(^{2+}\). The experiment in Fig. 7C confirms the conclusion drawn on the basis of results shown in Fig. 6 that the sustained phase Ca\(^{2+}\) entry is dependent on continued receptor activation by agonist.

Factors That Do and Do Not Alter the Agonist-induced [Ca\(^{2+}\)]\(_i\): Transients in Murine L Cells

Nature of the Agonist—The right panels of Fig. 5 show the results of experiments akin to that shown in the right panels but in which cells were stimulated with varying concentrations of the P2 purinergic agonist instead of CCh. It is clear that regardless of the concentration of ATP used patterns of sustained [Ca\(^{2+}\)]\(_i\), increases such as seen with CCh are not obtained with ATP. These results indicate that the temporal pattern of [Ca\(^{2+}\)]\(_i\), changes may have receptor specific components.

Effect of Cholera Toxin (CTX) Treatment—CTX (3 \(\mu\)g/ml for 24 h) reduced both the peak and the sustained responses to sub saturating as well as saturating concentrations of ATP or CCh. This is shown for the response to 1 \(\mu\)M CCh in Fig. 8B. Although reduced in magnitude, the Ca\(^{2+}\) entry was still agonist-dependent. The effect of CTX was mimicked by forskolin treatment (Fig. 8C), indicating that it is most likely mediated by cAMP rather than being due to an adenylyl cyclase-independent effect of Ga. In contrast, determination of the total number of N methyl neopaleamine specific binding sites were either unchanged (n = 1) or increased by 10 ± 4% (n = 1) upon treatment with CTX and were unchanged upon treatment with forskolin (n = 2) (not shown). When tested for effects on phosphoinositide hydrolysis, CTX had a minor (23%) but significant stimulatory effect on the basal rate (agonist absent) and did not affect the agonist-stimulated rates of phosphoinositide hydrolysis (not shown), indicating that the CTX- and forskolin-induced reduction in the responses of [Ca\(^{2+}\), to agonists is unrelated to IP\(_3\) formation. The inhibitory effect of CTX and forskolin are likely to be related to the findings of Supattapone et al. (48) indicating that the brain IP\(_3\) receptor is a substrate for cAMP-dependent protein kinase and that IP\(_3\) is 10-fold less potent in releasing
Effects of the Cloned M5 ACh Receptor

FIG. 5. Dose-response relationships for stimulation of the Ca\textsuperscript{2+} mobilization by carbachol (left set of panels) and ATP (right set of panels) and distinct kinetics of the responses to the two agonists. All the traces were obtained with the same batch of cells. The individual tracings were obtained in random order, but the traces are rearranged in the order of decreasing CCh and ATP concentrations. The results are representative of a total of three experiments of this type. Note that at none of the concentrations did ATP elicit a peak within the sustained phase of the response of the kind seen with CCh.

Ca\textsuperscript{2+} from phosphorylated than non-phosphorylated microsomes.

Effect of PTX Treatment—Depending on the cell and the type of receptor examined, Capon and collaborators (10, 49) found that PTX inhibits to variable degrees the action of M1 muscarinic receptors to stimulate phosphoinositide hydrolysis. In initial experiments we found no effect of PTX (0.1 µg/ml, overnight) on either CCh-stimulated phosphoinositide hydrolysis or on the CCh-induced [Ca\textsuperscript{2+}]\textsubscript{i} transients. Upon testing for the effectiveness of this type of treatment in L cells, we noted that it resulted in no more than 80% of the endogenous PTX substrates being affected. We found subsequently that full ADP-ribosylation of L cell PTX substrates required a more prolonged treatment (36 h) with repeated additions of PTX (once every 12 h) at a minimum of 0.5 µg/ml each time. This led to a reduction of unaffected PTX substrate(s) to ~0.05% of control (Fig. 9). Phosphoinositide hydrolysis in such PTX-treated cells was unaltered in the absence of agonist and slightly (26%) enhanced in the presence of CCh (not shown) and specific N-methyl-scopolamine binding was reduced by 12 ± 3% (n = 2) (not shown). Under these conditions, we found that while the peak [Ca\textsuperscript{2+}]\textsubscript{i} responses to CCh or ATP were essentially unchanged, the sustained responses to the two agonists were clearly enhanced (Fig. 10). This last effect is best seen in Fig. 10B, where the stimulation of the sustained increase in [Ca\textsuperscript{2+}], obtained at 1 µM CCh is occurring earlier in PTX-treated than in control cells.

On the Mechanism of the Sustained Agonist-stimulated Ca\textsuperscript{2+} Entry into L Cells. Agonist-stimulated Ca\textsuperscript{2+} entry into L cells was blocked by La\textsuperscript{3+}, suggesting that it enters through a Ca\textsuperscript{2+}-specific pathway but was unaffected by nitrendipine, a dihydropyridine with Ca\textsuperscript{2+} channel blocking activity (not shown). This indicated that Ca\textsuperscript{2+} entry during the sustained response was not secondary to voltage-dependent Ca\textsuperscript{2+} channel activation and thus different from the mechanism by which
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Fig. 6. Time-dependence for the recovery of a second peak [Ca\(^{2+}\)] response, as seen after blocking the first response with atropine and eliciting the second response with the heterologous agonist ATP. Traces obtained with two batches of fura-2-loaded LM5.36 cells are shown. Note that the time for recovery is about the same whether the sustained response is interrupted early (panels A–F) or late (panels G and H). The results are representative of a total of four experiments of this type.

The sustained Ca\(^{2+}\) entry is promoted in pituitary GH\(_2\)C\(_1\) cells (30, 31). The finding that Ca\(^{2+}\) entry was unaffected by the dihydropyridine blocker is consistent with our independent observation that L cells do not exhibit voltage-gated Ca\(^{2+}\) currents (38). Although, the detailed kinetics of agonist-induced [Ca\(^{2+}\)] transients are affected somewhat by substitution of the extracellular monovalent cation, replacement of Na\(^+\) with K\(^+\), N-methylglucamine, Tris, or Li\(^+\) did not interfere with the basic changes induced by agonist in [Ca\(^{2+}\)]. (not shown). This indicated that agonist-induced Ca\(^{2+}\) entry is not second-

Fig. 7. Recovery of the sustained [Ca\(^{2+}\)] response but not the peak [Ca\(^{2+}\)], response upon addition of extracellular Ca\(^{2+}\) to agonist-stimulated cells. All traces were obtained with the same batch of fura-2-loaded LM5.36 cells except that cells were resuspended in normal medium (panel A) and in medium with no added Ca\(^{2+}\) and 0.3 mM EGTA (panels B and C). The results are representative of a total of three experiments of this type.

Fig. 8. Effect of cholera toxin (CTX) and forskolin on stimulation of Ca\(^{2+}\) mobilization by the transfected M5 receptor. LM5.36 cells were seeded and grown in a 150-mm Petri dish with 30 ml of HAT medium. CTX (5 \(\mu\)g/ml) or forskolin (30 \(\mu\)M) was added to the cells 24 h before the cells were detached for fura-2 loading. Control cells, CTX-treated, or forskolin-treated cells were loaded with fura-2 as described under “Experimental Procedures.” CCh and atropine were added to the control (A), CTX-treated (B), or forskolin-treated (C) cells as shown. The traces were obtained from the same set of experiments. Each trace is representative of similar traces obtained in three to six experiments.
FIG. 9. Autoradiogram of homogenates from control or PTX-treated LM5 cells labeled with PTX and \[^{32}P\]NAD\(^{+}\). Cell homogenates prepared from PTX-treated (0.1 \(\mu\)g/ml, three times at 12-h intervals) or untreated LM5.36 cells (control) were labeled with \[^{32}P\]NAD\(^{+}\) in the presence (+) or absence (−) of activated PTX, electrophoresed, and autoradiographed as described under "Experimental Procedures." The samples in the left eight lanes are from serial dilutions of a replicate labeling reaction of the same sample as in lane A. The dilution factors are shown on the top of each lane with 1.0 representing the ADP-ribosylation of the homogenate of 10\(^{7}\) cells. Molecular weight standards (\(x 10^{2}\)) are indicated on the left side of the autoradiogram; ori, the origin of the separating gel. The band migrating at an \(M_r\) of \(\approx 40,000\) represents the \[^{32}P\]ADP-ribosylated \(\alpha\)-subunits of PTX-sensitive G-proteins. The identity of the material(s) with an approximate \(M_r\) of 44,000 labeled independently of the presence of PTX is unknown. Note that treatment of cells with PTX prior to homogenization resulted in a decrease in the PTX substrate(s) available for \[^{32}P\]ADP-ribosylation to a level that is between 1/8 and 1/16 of control.

ary to \(Na^+\) influx as may be the case in smooth muscle cells (32).

Other—Attempts to dissociate arachidonic acid release from phosphoinositide hydrolysis have failed. These included addition of 1 \(mM\) neomycin sulfate (\(n = 3\)), shown to inhibit inositol phosphate production in Fisher rat thyroid cell line cells in response to \(\alpha\)-adrenergic receptor stimulus (44) and in Madin-Darby canine kidney cells in response to bradykinin (51) and PTX treatment of L cells, which inhibited the agonist-stimulated arachidonic acid release in Fisher rat thyroid cell line cells (44) and macrophages (43). Neither had an effect on the L cells studied in the present report. i.e. neomycin failed to affect CCh-stimulated phosphoinositide hydrolysis and PTX failed to affect basal or CCh-stimulated arachidonic acid release (not shown).

Addition of 20-40 \(\mu M\) arachidonic acid 2-4 min prior to agonist was without effect on the [Ca\(^{++}\)]\(_{\text{in}}\); transients (not shown). Addition of PMA (1 \(\mu M\)) added 1 or 10 min prior to 1 \(\mu M\) CCh had no effect on basal [Ca\(^{++}\)], or on the CCh-induced [Ca\(^{++}\)], transients (not shown).

**DISCUSSION**

On the Signaling Properties of the M5 Receptor and the Use of L Cells to Study Cellular Signaling—The experiments presented here were carried out to characterize whether in addition to stimulating phosphoinositide turnover (15, 50), the M5 receptor is able to trigger other cellular responses. Of these, some, like IP\(_3\)-mediated release of Ca\(^{++}\) from intracellular stores seems to be an obligatory consequence if the cellular biochemistry supports it, while others such as the arachidonic acid release, or elevation of cAMP levels are often but not always associated with receptors capable of stimulating phospholipase C. For a receptor with a cellular distribution as limited as that of the M5 receptor, this may be the only way to learn about its properties. We found the receptor to trigger a complex Ca\(^{++}\) mobilization response and arachidonic acid release, but it caused no changes in cAMP levels. Unfortunately, we have been unable to discern whether any of the effects other than inositol phosphate accumulation are due to an effect of the M5 receptor independent of phospholipase C stimulation. Thus, while in some studies it has been possible by the use of neomycin to dissociate the arachidonic acid release, i.e. phospholipase A\(_2\) stimulation, from phospholipase C stimulation (44, 51), our studies were not informative in this regard. It may be that in L cells the release of arachidonic acid is a consequence of increased Ca\(^{++}\) levels plus a protein kinase C-mediated phosphorylation event, as opposed to a true receptor G-protein-mediated stimulation of phospholipase A\(_2\) activity. Consistent with this interpretation is the finding that arachidonic acid release was stimulated by 60-100% with CCh and not more than 15-18% with ATP which is in close proportion to their relative effect on phosphoinositide hydrolysis. Likewise, PTX, which abolishes the arachidonic acid response in FRTL cells without affecting phospholipase C stimulation by \(\alpha\)-adrenergic receptors (44), was without effect on arachidonic acid release in our studies.

We failed to observe effects of CCh on intracellular cAMP levels in the LM5 cells. This indicated that it is unlikely that either the transfected M5 or the resident purinergic receptors are direct regulators of adenyl cyclase and suggests that the cAMP-elevating role of the M5 receptor in other cells (20) is likely to be indirect and conditioned by the particular biochemistry of that cell. Indeed while this manuscript was under consideration for publication, Felder et al. (52) reported that accumulation of cAMP in A9 cells in response to M1 receptor
stimulation is indirect, depending on phosphatidylinositol hydrolysis and occurring possibly via an increase in cytosolic 
Ca** as a result of IP3 formation, followed by an action of 
Ca**-calmodulin on calmodulin-dependent adenyl cyclase.

Although we have no evidence that M5 receptors may alter 
cAMP levels or have an effect on arachidonic acid formation 
in L cells, this is not to say that in its natural neuronal 
environment it may not have these effects. Both effects are 
dependent not only on the nature of the receptor but also on 
the biochemistry of the effector cell. Thus, our descriptions 
of M5 receptor properties is limited by the nature of the cell 
in which we have chosen to express it. In view of the paucity 
of L cell receptors of the type that would be expected to 
 promote phospholipase C activation and Ca** mobilization 
(Table I) and the robust response in these two parameters 
obtained with the transfected M5 receptor, we believe that 
the non-excitable murine L cell may be a good model for the 
characterization of receptors that may have this type of effects 
but which cannot as yet be studied in their natural environ-
ment. Overall the M5 receptor resembles in its cellular sign-
aling properties the M1 and M3 receptors, which also trigger 
intracellular Ca** transients (53) and promote arachidonic 
acid release (52). Our studies indicate further, the L cell may 
also be good to study [Ca**]i regulations, such as may occur 
in other non-excitable cells, none of which exists as an estab-
lished cell line.

On the Interpretation of the Changes in [Ca**]i. Observed 
upon Stimulation of the L Cell by Agonists—The pattern of 
change in [Ca**]i, observed in L cells upon addition of agonists 
could be described as being the result of two distinct effects 
of receptors, one to release via IP3 formation Ca** from a 
depletable intracellular compartment and the second to cause 
entry of Ca** from the extracellular space possibly through 
activation of receptor (G-protein?) or IP3/IP4 operated Ca** 
channels located on the plasma membrane of the cell. How-
ever, as summarized in Fig. 11, an analysis of the literature 
suggests that the two phases of the Ca** mobilization response 
may have a single underlying mechanism consisting at all 
times of an IP3-mediated release of Ca** from an internal 
store. This is based on both the oscillatory responses to 
agonists as seen in single cells and the biochemical resolution 
of distinct types of Ca** accumulating and releasing vesicles.

It has been shown that application of low concentrations 
of agonists to cells results in sustained trains of periodic 
increases of [Ca**]; that are independent of changes in volt-
age-gated ion channels (54–57). These periodic increases, 
referred to as oscillations (58) or spikes (54), are of relatively 
constant amplitudes and may vary in frequency between 0.25/ 
min to 4/min, depending on agonist concentration (54). At 
high agonist concentrations these spikes become too frequent 
and coalesce into what probably corresponds to the transient 
or peak response observed in our studies. As predicted from 
studies of cell populations such as reported here, the [Ca**]; 
oscillations become dependent on extracellular Ca** in cells 
from which the intracellular IP3-releasable pool has been 
depleted (26, 55). Thus a sustained response at the cell pop-
ulation level, having as its basis in nonsynchronized cells 
discrete periodic [Ca**]i oscillations, could simply be the result 
of a pulsatile activation of an entry mechanism through the 
plasma membrane from the extracellular space into the cyto-
plasm (e.g. via receptor-operated (59) or IP3/IP4-operated 
(60, 61) Ca** channels). Arguments have been presented that 
the intrinsic oscillator lies in the microsomal site of action of 
IP3 (56, 60–65).

Refilling of the IP3-sensitive Ca** store has been proposed 
to occur by a path that bypasses the cytosolic [Ca**]; com-
partment (26), triggered by the lowering of its Ca** content. 
Biochemically, the refilling process that bypasses the cytosolic 
Ca** compartment appears to involve two non-mitochondrial 
Ca** compartments of which one is IP3 sensitive and the other 
is IP3-insensitive (66, 67). These have been proposed by Gill 
and co-workers (67) to constitute a vesicular Ca** transport 
system, akin to the vesicular translocation system operating 
between Golgi stacks (68), in which the IP3-sensitive com-
partment receives Ca** both from the plasma membrane and 
from the cytosol and transfers its contents into the IP3- 
sensitive compartment (67). These two subcompartments 
have recently been localized to separable membrane vesicles 
(67).

Based on the findings with single cells, it is thus possible 
to propose that the sustained response, dependent on extrac-
tellular Ca** for its continuity, is due to accumulation of 
threshold levels of IP3 (66, 63, 64), release of Ca** from the 
IP3-sensitive store (3), Ca**-mediated inhibition of continued 
action of IP3 (65), and refilling of the Ca** store triggered by 
lowering of its Ca** content (69), lowering of cytosolic Ca** 
by ATP-dependent extrusion from the cell (21, 22). ATP-
dependent re-uptake into both the transfer pool and the IP3-
releasable pool (66, 67, 70), and reintiation of the cycle (Fig. 
11). The lowering of [Ca**]; through ATP-dependent extru-
sion, could be responsible for a time- and agonist-dependent 
shift in the source of Ca** for re-refilling of the IP3-sensitive 
pool from cytosolic to extracellular and for a gradual increase 
in the role for the transfer pool, thought to be submembranous 
(66, 67, 70), in maintaining of continuously oscillating re-
sponse.

Although many details of this cycle are missing and subject 
of intense work in several laboratories, many of our results 
are in good agreement with this scheme. It predicts the 
existence of a delimited and exhaustible pool of IP3-releasable 
Ca** (Fig. 3, 5, and 6) and requires a continued role for IP3 
throughout the agonist response such as seen here (Fig. 6). 
Our finding that increasing cAMP levels in cells, a condition 
that affects the affinity of IP3 for its receptor and increases 
its EC50 for Ca** release from microsomal stores (48), results 
in a decrease in the agonist response for both the peak and 
the sustained phases of the Ca** mobilization response (Fig. 
8) supports a role for IP3 throughout the sustained phase of 
the agonist response.

However, some of the patterns of the sustained responses 
obtained in the present study are difficult to interpret in 
terms of amounts of IP3 formed acting on a single pool of 
stored [Ca**], and lead us to the argument that oscillations 
seen at very low agonist concentrations may in fact be due to 
a combination of the above mechanisms plus a pulsatile entry 
of Ca** through the plasma membrane into the cytosol by a 
mechanism that does not involve IP3.

First, we observed that the pattern of the Ca** mobilization 
response varied with the agonist (compare the shape of the 
[Ca**]; responses to CCh to those obtained with ATP in Fig. 
5). This variation could not be compensated for by varying 
the concentration of the agonist and or be correlated with the 
degree of depletion of the IP3-sensitive pool of Ca**. A similar 
result was reported by Cobbold and collaborators (71), who 
found with aqueorin-loaded hepatocytes that the individual 
Ca** spikes had shapes (e.g. narrow and smooth versus wider 
and jagged; varying rise and relaxation times) that differed 
depending on whether they were elicited by vasopressin, phen-
ylephrine, or angiotensin II. Our results therefore indicate 
that the Ca** response depends not only on the absolute level 
of cytosolic IP3 but also the rate and/or cellular site of 
formation of the second messenger. An explanation for this
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FIG. 11. Scheme of some of the signal transduction pathways leading to the changes in [Ca^{2+}]i in LMG cells in response to stimulation of acetylcholine M5 and purinergic P2 receptors. The pathways by which the M5 and the P2 receptors are thought to activate phospholipase C with resultant formation of DAG and IP3, are denoted along the upper surface of the cell as is the metabolic conversion of IP3 to IP1. Three forms of Ca^{2+} entry through non-voltage-dependent Ca^{2+} channels, activated either directly by the receptor or indirectly through a G-protein or products of phospholipase C activation (60, 61), which would all behave according to the macroscopic properties of the sustained response and fall under the classification of receptor-operated Ca^{2+} channels or ROCCs (58), are depicted along the left side of the cell. Two possible modes of Ca^{2+} entry, stimulation of Na+/Ca^{2+} antiport activity, and direct or indirect stimulation of voltage-gated Ca^{2+} channels or VOCCs (58), which were ruled by the experiments reported here, are depicted along the right side of the cell. The IP3-sensitive intracellular Ca^{2+} pool is represented in the center of the cell delimited by a membrane containing the IP3 receptor/Ca^{2+} release channel, a Ca^{2+} pump responsible at least partially for the refilling of the pool, and a GTP-dependent transfer mechanism (63, 70) also responsible for the refilling of the IP3-sensitive Ca^{2+} pool by transfer from a second vesicular, IP3-insensitive pool of Ca^{2+} derived which can derive its Ca^{2+} both from extracellular Ca^{2+} space (lower edge of the cell) and from the cytosolic space (Ca^{2+} pump). The figure also depicts locations of Ca^{2+} pumps responsible for the lowering of [Ca^{2+}]i by extrusion from the cell and uptake into both the IP3-sensitive transfer and the IP2-sensitive storage pools but does not detail mechanisms by which the muscarinic and purinergic receptors cause arachidonic acid release. 

**Abbreviations:** ACh, acetylcholine; M5 AChR, muscarinic acetylcholine receptor subtype M5; P2R, purinergic receptor subtype P2; Gi, G-protein responsible for mediating the activation of phospholipase C by the muscarinic and purinergic receptors; a, an activate subunit of Go protein; PIP2, phosphatidylinositol 4,5-bisphosphate; PhLC, phospholipase C; DAG, diacylglycerol; Ca^{2+}o, Ca^{2+}i, Ca^{2+}s, extracellular, intracellular, and stored Ca^{2+}; Na^{+}o, Na^{+}i, extracellular, and intracellular Na^{+}; DHP, dihydropyridine. For further details see text.

may be that different receptors use a different complement of G-proteins to stimulate IP3 formation. Precedents for this were provided by Ewald et al. (72), who have shown in sensory rat dorsal root ganglion cells that bradykinin and type-B γ-aminobutyric acid receptors differ in their ability to use exogenously added Gi and Go to cause inhibition of Ca^{2+} currents, a common effector system not unlike Ip3 mediated Ca^{2+} release. Likewise, Ashkenazi et al. (49) showed in Chinese hamster ovary cells that to stimulate phosphoinositide hydrolysis different receptors use a different complement of G-proteins (subclassified in terms of pertussis toxin sensitivity).

Second, we found that the shape of the Ca^{2+} response, the underlying nature of which we are assuming to be a sum of individual Ca^{2+} spikes, is altered upon treatment of cells with PTX (Fig. 10). This is also in agreement with the possibility that different G-proteins may be involved in the activation of phospholipase C by one agonist as compared with another. ADP-ribosylation of all PTX-sensitive G-protein molecules is likely to alter the activation/deactivation kinetics of other G-proteins that share the same pool of βγ dimers (for review see Ref. 73), even if they are themselves insensitive to PTX. For example we have noted that PTX treatment of cells tends to potentiate stimulatory responses of adenyl cyclase. Alternatively, rather than being a reflection of temporal or kinetic aspects of IP3 formation, the agonist-specific aspects of the Ca^{2+} response could also be due to existence of a Ca^{2+} entry path that is regulated by one or more G-proteins or by a site of IP3 and/or IP2 action distinct from that causing the release of Ca^{2+} from the internal store.

Third, we noticed that readmission of Ca^{2+} to cells in which we had depleted the IP3-sensitive Ca^{2+} compartment in the absence of extracellular Ca^{2+} (Fig. 7) results within seconds in restoration of the average intracellular Ca^{2+} levels, interpreted as resumption of periodic oscillations of [Ca^{2+}]i, equivalent to what would have been seen if the agonist response would have been allowed to run its course in the presence of extracellular Ca^{2+}. This would indicate that the refilling process reaches steady state very rapidly and that the turnover of agonist-sensitive intracellular Ca^{2+} is very fast. In contrast,
in Fig. 6, in which the action of CCh is interrupted shortly after the peak response, and ATP is added at varying times therefor to measure the refilling of depletable IP₃-sensitive pool of Ca²⁺, we found that this pool is refilled only gradually. This would indicate that the turnover of agonist-sensitive intracellular Ca²⁺ is very slow. Delaying the interruption of the sustained response did not alter this result (Fig. 8, A-F versus G and H). This raises the question as to whether indeed there is only one Ca²⁺ store responsible for both the sustained elevation of [Ca²⁺], which presumably represents the sum of non-synchronous low frequency [Ca²⁺] oscillations, and the peak increases in [Ca²⁺], which presumably represents increases in [Ca²⁺], under conditions where individual [Ca²⁺] oscillations have coalesced, are one and the same.

Taken together our results indicate that the Ca²⁺ response of L cells is both under continuous dependence of IP₃ and under a continuous control either of the temporal pattern of IP₃ formation or of how Ca²⁺ entry is regulated. Although the overall sources of Ca²⁺ (intra-versus extracellular) are known, the immediate origin of the free intracellular Ca²⁺ ([Ca²⁺]I) is not clear and deserving of further investigation.

REFERENCES

1. Nathanson, N. M. (1987) Annu Rev Neurosci 10, 185-236
2. Hokin, L. E., and Hokin, M. R. (1971) J. Biol. Chem. 246, 540-558
3. Strehl, H., Hirvenoja, A., and Saksela, E. (1982) J. Biol. Chem. 257, 1233-1238
4. Pfaffinger, P. J., Martin, J. M., Hunter, D. D., Nathanson, N. M., and Hille, B. (1986) Nature 327, 530-532
5. Yatani, A., Codina, J., Brown, A. M., and Birnbaumer, L. (1987) Science 235, 207-211
6. Brown, D. A., and Adams, P. R. (1980) J. Biol. Chem. 255, 14711-14717
7. Malhotra, V., Serafini, T., Orci, L., Shepherd, J. C., and Rothman, J. (1989) J. Biol. Chem. 264, 7328-7337
8. Jones, S. V., Barker, J. L., Banner, T. I., Buckley, N. J., and Brann, M. R. (1989) Pfluegers Arch. 410, 12801-12805
9. Kuhn, L. C., McClelland, A., and Ruddle, F. H. (1984) Cell 37, 95-103
10. Chilton, F. H., and Connell, T. R. (1980) J. Biol. Chem. 256, 3260-3260
11. Putney, J. W. (1976) J. Biol. Chem. 251, 12859-12866
12. Meritt, J. E., and Rink, T. J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2669-2673
13. Albert, P. R., and Tashjian, A. H. (1984) J. Biol. Chem. 259, 15350-15363
14. Schindler, W., Wiesiger, B. P., Mollard, P., Vachter, P., Wazny, F., Zahnd, G. W., Golzheim, C. B., and Dufy, B. (1987) Nature 329, 719-721
15. Kherson, K., Kitamura, K., and Kurijama, H. (1987) Pfluegers Arch. 410, 12801-12805
16. Schilling, W., Lajdonin, G., and Strbik-Jager, E. (1989) J. Biol. Chem. 264, 12385-12389
17. Kub, S., Fubini, D. R., Piekarz, L. J., and Hsu, T. C. (1963) Exp. Cell Res. 31, 297-312
18. Wipper, M., Pellerin, A., Silverstein, S., Axel, R., Ursula, G., and Chassin, 1979 Proc. Natl. Acad. Sci. U. S. A. 76, 1523-1526
19. Kuhn, L. C., Barry, J. A., Kamarck, M. E., and Ruddle, F. H. (1983) Mol. Biol. Med. 1, 355-360
20. Kuhn, L. C., McClelland, A., and Ruddle, F. H. (1984) Cell 37, 95-103
21. Perez-Reyes, E., Kim, H. S., Lacerda, A. F., Horne, W., Xi, R., Rampe, D., Campbell, K. P., Brown, A. M., and Birnbaumer, L. (1989) Nature 340, 203-206
22. Walseuth, T. F., and Johnson, R. A. (1979) Biochem. Biophys. Acta 662, 111-131
23. Casset, D., and Pfeiffer, T. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 2669-2675
24. Brierley, A., Fretton, P., Palmer, S., Kirk, C. J., and Michell, R. H. (1984) Biochem. J. 221, 833-831
25. Kirk, C. J., Gallion, G., Balestre, M. N., and Jard, S. (1986) Biochem. J. 236, 207-211
26. Putney, J. W. (1976) J. Biol. Chem. 251, 13863-13871
27. Burch, R. M., Lucent, A., and Axelrod, J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7293-7295
28. Laemmli, U. (1970) Nature 227, 680-685
29. Lefebvre, D., Rosemberg, J. Z., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
30. Sletter, J. H., and Connell, T. R. (1980) J. Biol. Chem. 256, 3260-3260
31. Schindler, W., Wiesiger, B. P., Mollard, P., Vachter, P., Wazny, F., Zahnd, G. W., Golzheim, C. B., and Dufy, B. (1987) Nature 329, 719-721
32. Kherson, K., Kitamura, K., and Kurijama, H. (1987) Pfluegers Arch. 410, 12801-12805
33. Brown, D. A., and Adams, P. R. (1980) J. Biol. Chem. 255, 14711-14717
34. Hoger, D. M., and Brann, M. R. (1989) J. Biol. Chem. 264, 11284-11290
35. Jones, S. V., Barker, J. L., Lanter, T. J., and Brann, M. R. (1988) Mol. Pharmacol. 34, 421-426
36. Jones, S. V., Barker, J. L., Bonner, T. I., Buckeley, N. J., and Brann, M. R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4056-4060
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J. Biol. Chem. 1990, 265:11273-11284.

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