Stimulation of muscarinic receptors in dissociated embryonic chick heart cells promotes the hydrolysis of the phosphoinositides resulting in accumulation of the breakdown products inositol trisphosphate, bisphosphate, and monophosphate (InsP$_3$, InsP$_2$, and InsP, respectively). $[^{3}H]$InsP$_3$ and $[^{3}H]$InsP$_2$ are significantly elevated within 10 seconds of carbachol addition, while there is a lag in the accumulation of $[^{3}H]$InsP. The time courses of the formation of the inositol phosphates suggest that carbachol activates a polyphosphoinositide-specific phospholipase C resulting in the formation of InsP$_3$, which is subsequently metabolized to InsP$_2$ and InsP. High-performance liquid chromatography analysis demonstrates the formation of both naturally occurring InsP isomers (Ins-1,4,5-P$_3$ and Ins-1,3,4-P$_3$) and of inositol tetrakisphosphate (InsP$_4$) as well. To investigate whether a guanine nucleotide–binding protein couples receptor stimulation to phosphoinositide (PI) hydrolysis in the heart, we developed a saponin-permeabilized cell preparation that would allow external manipulation of the intracellular guanosine triphosphate (GTP) concentration. In the permeabilized cell preparation, guanosine-5'-O-(3-thiotriphosphate) (GTPyS) stimulates the accumulation of $[^{3}H]$InsP$_3$, $[^{3}H]$InsP$_2$, $[^{3}H]$InsP, and $[^{3}H]$InsP$_4$. The effect of GTPyS is half-maximal at 1 $\mu$M and maximal above 100 $\mu$M. In contrast, GTPyS is ineffective in promoting PI hydrolysis in the nonpermeabilized cell except at high concentrations. Other guanine nucleotides also lead to the accumulation of $[^{3}H]$InsP in the permeabilized cell, while 5'-adenylylimidodiphosphate does not. Carbachol also stimulates PI hydrolysis in the permeabilized cell preparation although it is less effective than in the intact cell. The response to carbachol is at least additive with the response to GTPyS. The observation that GTPyS stimulates the accumulation of $[^{3}H]$InsP$_3$ provides evidence that a guanine nucleotide–binding protein is involved in the regulation of the polyphosphoinositide-specific phospholipase C in the heart. The data also demonstrate the formation of the biologically active isomer of InsP$_3$ and suggest that InsP$_3$ may serve a physiological role in the chick heart. (Circulation Research 1988;62:299–305)
**Materials and Methods**

**Cell Preparation**

Hearts were excised from 13-day-old chick embryos, sliced open, and rinsed in Krebs-Henseleit medium buffered with 20 mM HEPES. The tissue was minced in the same buffer and dissociated for 40 minutes at 37°C in a Ca++-Mg++-free buffer containing 0.25% trypsin. Tissue fragments were washed in buffer containing 1% bovine serum albumin, and cells were dispersed by trituration. This suspension was filtered through nylon mesh, and cells were washed and collected by centrifugation. The dispersed cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum and 1% Fungibact and plated overnight in 35-mm² tissue culture plates. To label the phosphoinositides for measurement of [3H]InsP formation, 1 μCi/ml [3H]inositol was included in the medium. This concentration was increased to 20-40 μCi/ml when [3H]InsP₁ and [3H]InsP₂ were also quantified. Approximately 7×10⁶ viable cells/heart were obtained from each cell preparation. Viability was 85-96% as determined by trypan blue exclusion.

**Assay Conditions**

Following overnight incubation at 37°C in a 95% air-5% CO₂ atmosphere, cell monolayers were washed with DMEM buffered with 20 mM HEPES, and the culture plates were incubated on a tray in a water bath at 37°C for 10 minutes. For assays conducted with nonpermeabilized cells, HEPES-buffered DMEM was replaced with 0.75 ml of the same medium containing 10 mM LiCl and the appropriate drugs. For assays requiring permeabilized cells, the HEPES-buffered DMEM medium was replaced with an intracellular buffer containing KCl 110 mM, NaCl 10 mM, KH₂PO₄ 1 mM, HEPES 20 mM, MgCl₂ 4 mM, EGTA 1 mM, Na₃ATP 3 mM, creatine phosphate 8 mM, and creatine kinase 6 U/ml (pH 7.0). Saponin (50-100 μg/ml) was added to the buffer, and the cells were incubated at 37°C for 5 minutes. The permeabilized cells were then washed 3 times with the same intracellular buffer without saponin, and 0.75 ml of fresh intracellular buffer containing 10 mM LiCl and the appropriate drug was added. Except where noted, both nonpermeabilized and permeabilized cells were incubated with drugs and lithium chloride for 20–30 minutes. Following the drug incubation, the medium from permeabilized cells was transferred to a tube containing 0.25 ml 40% trichloroacetic acid (TCA) (final TCA concentration, 10%). Approximately 80% of the [3H]InsP₁ and [3H]InsP₂ and 40% of the [3H]InsP₃ formed in saponin-treated cells stimulated with guanosine-5′-O-(3-thiotriphosphate) (GTPγS) was released into the medium. The remainder was associated with the cell fraction. Since the amount of [3H]InsP remaining in the cell fraction was not increased by hormone or GTPγS (suggesting that these cells were unresponsive), only the medium was analyzed in most permeabilized cell experiments. Following drug incubations with nonpermeabilized cells, the medium was aspirated and discarded since an insignificant amount of labeled product is found in this fraction. Ten percent TCA was added to the cells remaining on the plates, and cells were scraped into 1 ml total volume.

**Separation of [3H]inositol Phosphates**

The TCA cell extract and acidified medium were extracted 5 times with 4 volumes of H₂O-saturated ether. The [3H]inositol phosphates were separated by anion exchange column chromatography following the procedure described previously that was based on that reported by Berridge et al. Modifications in the column separation procedure of Batty et al. were used to separate InsP₁, and InsP₂ for some of the more recent experiments. In the modified procedure, following application of the sample, the column was washed with 8 ml H₂O. InsP₂ was eluted with 8 ml of 5 mM sodium tetraborate: 150 mM sodium formate, and the column was washed with an additional 32 ml of the same solution. InsP₃ was eluted with 8 ml of 100 mM formic acid (FA): 300 mM ammonium formate (AF) followed by an 8-ml wash with this same buffer. InsP₄ was eluted with 8 ml of 100 mM FA: 750 mM AF followed by an 8-ml wash with this same buffer. InsP₄ was eluted with 8 ml of 100 mM FA: 1 M AF. Radioactivity in all of the eluted fractions was quantified by liquid scintillation counting.

Samples prepared for high-performance liquid chromatography (HPLC) analysis of the isomers of InsP₁ and InsP₂ were labeled overnight with 40 μCi/ml [3H]inositol and incubated for 2.5 minutes in 20 mM HEPES-buffered Krebs-Henseleit medium with 10 mM LiCl ± 100 μM carbachol. The reactions were stopped with 10% TCA, and the samples were ether extracted. Samples were injected onto a Whatman Partisil 10-SAX column (Whatman, Clifton, New Jersey) using a Hewlett-Packard HP 1090 HPLC (Hewlett-Packard, Waldbronn, FRG). The different inositol phosphates were eluted by a 26-minute gradient of 1.0 M AF with 0.7 M phosphoric acid (pH 3.7) that was started after running the column in 100% deionized water for 6 minutes at 1.25 ml/min. Fractions were collected every 0.25 minutes, and radioactivity in each fraction was determined by scintillation counting. The positions of [3H]Ins-1,4,5-P₃ and [3H]InsP₄ were verified by comparison with authentic [3H] standards (New England Nuclear, Boston, Massachusetts). The relative positions of Ins-1,4,5-P₃, Ins-1,3,4-P₃, and InsP₄ were consistent with those described by others.

**Binding to Muscarinic Receptors**

Cell monolayers cultured as described above were washed with HEPES-buffered DMEM and incubated in this medium for 10–20 minutes at 37°C. For saturation binding assays, the medium was replaced with 5 ml HEPES-buffered Krebs-Henseleit medium containing 0.01–5.0 nM [3H]quinuclidinyl benzilate ([3H]QNB) ± 10 μM atropine (to define nonspecific binding). After 75 minutes, the QNB was removed, plates were washed 3 times, and 1N NaOH was added. The following day, the sodium hydroxide extract was...
transferred to scintillation vials, the plates rinsed with 1N HCl, and the rinse added to the vials for liquid scintillation counting. The nonspecific binding was less than 10% under these conditions.

Materials

Fertilized white leghorn chicken eggs (McIntyre Poultry and Eggs, San Diego, California) were incubated at 39°C in a Leahy model 80 incubator (Higginsville, Missouri). Carbamylcholine (carbachol) chloride, dl-isoproterenol HCl, guanosine diphosphate (GDP), guanosine 5'-triphosphate (GTP), 5'-guanylylimidodiphosphate (GppNHp), 5'-adenylylimidodiphosphate (AppNHp), adenosine 5'-triphosphate (ATP), phosphocreatine (disodium salt), and creatine phosphokinase (from rabbit muscle, type I) were from Sigma, St. Louis, Missouri. Guanosine-5'-O-(3-thiotriphosphate) (GTPyS) was from Boehringer Mannheim, Indianapolis, Indiana. Trypsin 1-300 was from ICN Biomedicals, Cleveland, Ohio. [3H]Inositol (10-20 Ci/mmol) and [3H]QNB (33-38 Ci/mmol) were from New England Nuclear, Boston, Massachusetts. Ag1-X8 anion exchange resin (100-200 mesh, formate form) was from Bio-Rad Laboratories, Richmond, California.

Statistics

Data were analyzed using the unpaired Student's t test. The p values were obtained using a one-tailed test. Data are expressed as mean ± SEM.

Results

We previously reported that muscarinic receptor stimulation leads to [3H]InsP accumulation in intact chick heart cells, with 1 mM carbachol eliciting a maximal response. These data indicated that a phosphoinositide-specific phospholipase C was activated but did not indicate whether the polyphosphoinositides [phosphatidylinositol monophosphate (PIP) and PIP2] or only PI was hydrolyzed in response to receptor stimulation. In this paper, we show that [3H]InsP; and [3H]InsP2 (the inositol phosphate products of the polyphosphoinositides) are also formed in response to muscarinic stimulation (Figure 1). Both [3H]InsP; and [3H]InsP2 are significantly elevated (p<0.05) within 10 seconds. In contrast, the accumulation of [3H]InsP occurs only after an initial lag of 30 seconds and thereafter increases linearly for at least 30 minutes.

The relation between these time courses suggests that muscarinic stimulation initially activates a polyphosphoinositide-specific phospholipase C resulting in the formation of InsP3. The time courses suggest that the InsP3 formed is subsequently metabolized to InsP2 and InsP. We have also measured the accumulation of the inositol phosphates in the absence of lithium and have found that the relation of the time courses of their accumulation is essentially the same (data not shown). The phosphorylation of Ins-1,4,5-P3 to InsP4 and its subsequent dephosphorylation to Ins-1,3,4-P3 also apparently occur in embryonic chick heart cells as accumulation of both isomers of InsP3, as well as of InsP4, is seen at short time intervals following carbachol treatment (Figure 2).
Table 1. Release of Lactate Dehydrogenase From Chick Heart Cells During Different Permeabilization Conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (min)</th>
<th>Total cell LDH released (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saponin (µg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>&lt;1</td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>31</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
<td>48</td>
</tr>
<tr>
<td>200</td>
<td>5</td>
<td>58</td>
</tr>
<tr>
<td>200</td>
<td>10</td>
<td>61</td>
</tr>
<tr>
<td>200</td>
<td>20</td>
<td>93</td>
</tr>
</tbody>
</table>

LDH, lactate dehydrogenase.

To determine whether a guanine nucleotide-binding protein couples receptor stimulation to PI hydrolysis in the heart, we developed a saponin-permeabilized cell preparation that would allow external manipulation of the intracellular GTP concentration. Several concentrations of saponin and variable time periods for saponin treatment were tested to determine the optimal conditions for permeabilization. A concentration of 100 µg/ml saponin for 5 minutes was selected because following this treatment most cells remain attached to the culture dishes and are permeable to trypan blue, while only 12% of the total cell lactate dehydrogenase is released into the medium (Table 1), indicating that the cells remain impermeable to large intracellular proteins.

Addition of the nonhydrolyzable GTP analogue GTPyS to permeabilized chick heart cells markedly increases the accumulation of [3H]InsP, [3H]InsP₂, and [3H]InsP₃ (Figure 3). The response to GTPyS occurs in a dose-dependent manner with a half-maximal effect at 1 µM and a maximal response with 100 µM (Figure 4). In contrast, GTPyS is ineffective in promoting PI hydrolysis in the intact cell except when high concentrations (above 30 µM) are used. GppNHp is also effective in promoting the accumulation of [3H]InsP in the permeabilized cell, while AppNHp is not (Figure 5). Over the course of our experiments, GTP and GDP gave variable responses and did not consistently stimulate the accumulation of [3H]InsP. The stimulation of [3H]InsP accumulation occasionally seen in response to GDP probably results from the conversion of GDP to GTP by the ATP-regenerating system included in the intracellular buffer.

Carbachol also stimulates PI hydrolysis in the permeabilized chick heart cell (Figure 6). This indicates that some of the endogenous GTP remains. The stimulation is, however, less than half that seen in the intact cell. One explanation could be that the saponin treatment causes a loss of muscarinic receptors. However, when [3H]QNB binding sites on intact and permeabilized cells were compared, receptor densities of 223 and 226 fmol/mg protein and Kᵥ values of 117 and 96 pM were obtained indicating that there is no change in receptor density or affinity for QNB. A more likely explanation is that the poorer response is due to loss of endogenous guanine nucleotides necessary for...
Discussion

As we have discussed previously, muscarinic agonists increase phosphoinositide hydrolysis in the heart although muscarinic receptor stimulation is not generally thought to mediate excitatory responses (secretion and contraction) associated with calcium mobilization.22,23 Knowing whether InsP$_3$ is formed in the chick heart cell is essential to understanding the role of PI turnover in mediating cardiac muscarinic responses. If InsP$_3$ is formed, the phosphoinositide response might play a modulatory role in the mobilization of calcium in the heart. If InsP$_3$ is not formed, PI turnover may function exclusively through the formation of diglyceride and activation of protein kinase C.

The experiments shown here clearly demonstrate that InsP$_3$ is increased in carbachol-stimulated embryonic chick heart cells. In addition, they document that [H]InsP$_3$ is significantly increased at the earliest time point that we measured (9 seconds). These data suggest that InsP$_3$ is the primary product of the hormone-stimulated phospholipase and that it is the breakdown of PIP$_2$ that is stimulated. The rapid formation of InsP$_3$ is consistent with a physiological function for this second messenger in mediating responses to carbachol. The slower formation of InsP$_4$, and particularly of InsP$_5$, would reflect, at least in part, the dephosphorylation of InsP$_3$ to these products, a pattern typically seen in preparations in which InsP$_3$ increases rapidly. The production of InsP$_4$, InsP$_5$, and InsP$_6$ has been demonstrated also in rat ventricle in response to both muscarinic and $\alpha$-adrenergic-receptor stimulation.23

The observation that InsP$_3$ is formed does not necessarily imply calcium mobilization since isomers of InsP$_3$ with little activity are known to exist. The 1,4,5-isomer of InsP$_3$ has been shown to have the most potent biological activity in releasing calcium, while the 1,3,4-isomer has considerably less.24 It was, therefore, important to establish that the InsP$_3$ formed in the chick heart cell was the active isomer. The data shown in Figure 2 establish that there is significant accumulation of the 1,4,5-isomer of InsP$_3$, at an early time following the addition of carbachol. There is also at this time a significant amount of label in the 1,3,4-isomer and its precursor, InsP$_1$ (presumably inositol 1,3,4,5-P$_4$, although there may also be isomers of InsP$_3$). The formation of InsP$_3$ and the 1,3,4-isomer suggests that a significant amount of the active 1,4,5-isomer is metabolized through a phosphorylation-dephosphorylation cycle. The importance of this pathway has not yet been established, though a role for InsP$_3$ in the regulation of cellular Ca$^{2+}$ at the level of the plasma membrane has been described.25

Whether the rapid formation of Ins-1,4,5-P$_3$, following muscarinic receptor stimulation affects cellular responses remains to be determined. There is evidence that InsP$_3$ releases calcium from cardiac sarcoplasmic reticulum, albeit slowly.26 It has also been suggested that the positive inotropic response to the $\alpha$-receptor agonist phenylephrine may be related to phosphoinositide hydrolysis.27 Furthermore, muscarinic agonists can depolarize and increase contractility (in a catecholamine-independent manner) in mammalian and avian myocardium.28-31 Under appropriate conditions, a positive inotropic response may correlate with the PI hydrolysis stimulated by carbachol.28,29

The use of a permeabilized heart cell preparation allows one the advantage of being able to manipulate the intracellular concentration of guanine nucleotides and other physiological substances and yet maintain, to some extent, the functional integrity of the cardiomyocyte. Short exposure of cells to a detergent such as saponin has been shown to cause the formation of pores in the plasma membrane while leaving intact the membranes of the organelles.32 Digitonin treatment of adult rat heart cells also produces cells that retain typical morphology when ATP is included.34 Use of the appropriate level of detergent in the permeabilization procedure results in cells that allow small molecules (e.g., trypan blue and inositol phosphates) to cross the plasma membrane but that do not allow free passage of large intracellular proteins. This is illustrated by the inability of the chick heart cells to exclude trypan blue following permeabilization although they retain approximately 90% of their intracellular lactate dehydrogenase. Also, the observed effect of exogenous guanine nucleotides on PI turnover in the permeabilized, but not the intact, cell suggests that exogenous nucleotides can enter cells that have been treated with saponin. A loss of phosphatases from the permeabilized cell (or limited access of the phosphatases to the inositol polyphosphates that have diffused into the extracellular medium) may also occur since there is proportionally more inositol bisphosphate and trisphosphate (relative to inositol monophosphate) in the permeabilized, as compared with the nonpermeabilized, cell (cf., Figures 1 and 3).

In the permeabilized cell preparation, there is marked stimulation of inositol phosphate accumulation by exogenous guanine nucleotides. The stimulation
occurs over a concentration range similar to that observed in other systems. The hypothesis that the effect of GTPyS is due to the interaction with a GTP-binding protein is supported by the observation that other guanine nucleotides are also effective in promoting PI hydrolysis in the permeabilized cell, while AppNHp is not. We have previously shown in the intact chick heart cell that pertussis toxin blocks muscarinic receptor–mediated inhibition of adenylate cyclase but does not block muscarinic stimulation of PI hydrolysis. Pertussis toxin pretreatment also fails to block GTPyS-stimulated InsP formation in the permeabilized cell (data not shown). The putative G-protein shown here to regulate phospholipase C in chick heart cells would, therefore, appear to be a protein other than G or G, both of which can be ribosylated by pertussis toxin. We are currently examining the properties of this G-protein and its relation to the G-proteins regulating adenylate cyclase and K+ channels in the chick heart.

In summary, we have examined phosphoinositide metabolism in the chick heart cell and have shown that the active isomer of InsP, inositol 1,4,5-P, is formed in response to carbachol with a time course appropriate for it to be of physiological significance. The formation of InsP, provides evidence that muscarinic stimulation in the heart activates a polyphosphoinositide-specific phospholipase C and suggests the possibility that calcium metabolism may be altered through changes in PI metabolism. A guanine nucleotide–binding protein appears to be involved in the activation of the myocardial phosphoinositide-specific phospholipase C since, in the permeabilized cell and in the absence of hormone, guanine nucleotides are capable of stimulating PIP hydrolysis. These data suggest that cardiac phosphoinositide hydrolysis and the consequent formation of InsP, may be regulated not only by muscarinic and α-adrenergic–receptor stimulation but also at the level of a guanine nucleotide–binding protein.

Acknowledgment

We thank Barbara Thompson for carrying out the HPLC analysis of the inositol phosphate isomers.

References

5. Irvine RF, Anggard EE, Letcher AJ, Downes CP: Metabolism of inositol 1,4,5-trisphosphate and inositol 1,3,4-trisphosphate in rat parotid glands. Biochem J 1985;229:505–511
25. Irvine RF, Moor RM: Micro-injection of inositol 1,3,4,5-tetrasphosphate activates sea urchin eggs by a mechanism dependent on external Ca2+. Biochem J 1986;240:917–920
26. Fabiato A: Inositol (1,4,5)-trisphosphate-induced release of Ca2+ from the sarcoplasmic reticulum of skinned cardiac cells (abstract). Biophys J 1986;49:190a

Key Words • guanine nucleotide • inositol trisphosphate • muscarinic receptor • heart • phospholipase C
Guanine nucleotide-dependent inositol trisphosphate formation in chick heart cells.
L G Jones, D Goldstein and J H Brown

doi: 10.1161/01.RES.62.2.299

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/62/2/299

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/