Regulation of the Inositol 1,4,5-Trisphosphate-activated Ca\(^{2+}\) Channel by Activation of G Proteins*

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Xin Xu, Weizhong Zeng, and Shmuel Muallem‡

From the Department of Physiology, University of Texas Southwestern Medical School, Dallas, Texas 75235

Streptolysin O-permeable pancreatic acini were used to study the regulation of the inositol 1,4,5-trisphosphate (IP\(_3\))-activated Ca\(^{2+}\) channel (IPACC) by agonists and antagonists. Measurements of the apparent affinity for IP\(_3\) (K\(_{\text{app}}\)IP\(_3\)) showed that the IPACC is dynamically controlled during cell stimulation and inhibition, i.e. agonists decreased and antagonists increased K\(_{\text{app}}\)IP\(_3\). K\(_{\text{app}}\)IP\(_3\) was also independently regulated by thimerosal, Ca\(^{2+}\) content of the stores, the incubation temperature, activation of protein kinases, and inhibition of protein phosphatases, but none of these mechanisms contributed to the regulation by agonists and antagonists. Incubating the cells with low concentration of GTP\(_S\) or AIF\(_3\) reproduced the effect of the agonist on K\(_{\text{app}}\)IP\(_3\). Moreover, low [GTP\(_S\)] allowed activation of the IPACC by agonists at basal levels of IP\(_3\) and markedly impaired channel inactivation by antagonists. Channel sensitization by GTP\(_S\) also restored the ability of thimerosal to mobilize Ca\(^{2+}\) from internal stores with no change in cellular IP\(_3\) levels. The combination of low [GTP\(_S\)] and thimerosal locked the channel in an open, antagonist-insensitive state. All modulatory effects of GTP\(_S\) are independent of phospholipase C activation and IP\(_3\) production. We propose that the dynamic regulation of the IPACC by a G protein-dependent mechanism can play a major role in triggering and maintaining Ca\(^{2+}\) oscillations at low agonist concentrations when minimal or no changes in IP\(_3\) level take place.

Ca\(^{2+}\) mobilizing agonists stimulate the production of inositol 1,4,5-trisphosphate (IP\(_3\)), which releases Ca\(^{2+}\) from intracellular stores located in the endoplasmic reticulum (ER) (1). Stimulation with high agonist concentration leads to a persistent activation of the IP\(_3\)-dependent Ca\(^{2+}\) channel, which results in a single transient change in free cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (2). On the other hand, at low agonist concentrations usually oscillations in [Ca\(^{2+}\)]\(_i\) are observed (1, 2). Only partial correlation exists between the type of the [Ca\(^{2+}\)]\(_i\) signal generated and IP\(_3\) production. In most cases, high agonist concentration increases IP\(_3\) to a supermaximal concentration, while at low agonist concentration it is difficult to demonstrate stimulated production of IP\(_3\) (1, 2). In addition, several agonists such as parathyroid hormone acting on osteoblasts (3, 4) or bradykinin acting on 3T6 fibroblasts (5) cause substantial Ca\(^{2+}\) release from intracellular stores with no apparent increase in IP\(_3\). However, basal levels of IP\(_3\), although varying widely among different cell types, are almost always higher than that needed for maximal Ca\(^{2+}\) release (1, 6–9).

A plausible explanation for Ca\(^{2+}\) release in the absence of IP\(_3\) production and in the face of high basal levels of IP\(_3\) is that low [agonists] trigger Ca\(^{2+}\) release by regulating the affinity of the IP\(_3\)-activated Ca\(^{2+}\) channel (IPACC) for IP\(_3\). Studies in several cellular systems and the isolated and reconstituted IPACC showed the presence of multiple mechanism of channel regulation. Besides activation by IP\(_3\), the channel can be regulated by Ca\(^{2+}\) in a bi- or triphasic manner (10–13). ATP can regulate the channel directly (10, 11, 14) or by an indirect mechanism (13, 15), which may involve phosphorylation/dephosphorylation reactions (13). Recent elegant work by Cameron et al. (16) showed the intimate association of the cerebellar IPACC with calciuacinurin. The channel's affinity to IP\(_3\) was regulated by the combined action of protein kinase C and calciuacinurin (16). The same system was used to show that protein kinase A reduces the apparent affinity of the IPACC to IP\(_3\) (14, 16). In hepatocytes, protein kinase A increased the affinity for IP\(_3\) in Ca\(^{2+}\) releasing IP\(_3\) concentrations. Indeed, rat liver, prestimulation with agonists was shown to modify the behavior and/or affinity for IP\(_3\) (26). More recently we showed that agonists can activate and antagonists inactivate the IPACC independent of IP\(_3\) metabolism (27, 28). How such a regulation is achieved, what mechanism the agonists use to modulate Ca\(^{2+}\) release and the antagonists to terminate the release is not known.

In the present studies we used agonist/antagonist responsive, streptolysin O (SLO)-permeabilized pancreatic acini to demonstrate that in the same cells the IPACC can be regulated by multiple and independent mechanisms. More importantly, a G protein-dependent mechanism is used by agonists to activate the channel at basal levels of IP\(_3\). Such activation dramatically impairs the ability of the antagonist in inactivating the IPACC. Once activated by the G protein-dependent mechanism, the channel can be locked in the open state by TMS. These findings point to the possible mechanism by which agonists can initiate Ca\(^{2+}\) release and Ca\(^{2+}\) oscillations with minimal changes in IP\(_3\) concentrations.
EXPERIMENTAL PROCEDURES

Preparation of Pancreatic Acini—Acini were prepared from a rat pancreas using standard collagenase digestion (27). In brief, the pancreas was removed, minced, and incubated for 5–6 min at 37 °C in a solution composed of (in mM) 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 Heps (pH 7.4 with NaOH), 10 glucose, 10 pyruvate, 0.1% bovine serum albumin, 0.02% soybean trypsin inhibitor (solution A) and supplemented with 165 μg/ml collagenase P. After digestion, the acini were collected and washed by centrifugation and kept in solution A on ice until use.

Measurement of Ca²⁺ Uptake and Release in Permeabilized Cells—This procedure was identical with that described before (27, 28). About 100 mg acini were washed twice with a solution containing 145 mM KCl and 10 mM Heps (pH 7.4 with NaOH) and once with the same solution that was treated with Chelex 100. The acini were transferred to a fluorimeter cuvette containing a warm (37 °C) incubation medium composed of the Chelex-treated solution, 3 mM ATP, 5 mM MgCl₂, 10 mM creatine phosphate, 5 units/ml creatine kinase, 10 μM antymycin A, 10 μM oligomycin, 0.02% soybean trypsin inhibitor, 1 μM Fluo 3, and 0.4 unit/ml SLO. Fluo 3 fluorescence was recorded at an excitation wavelength of 488 nm and emission wavelength of 530 nm. Calibration of Fluo 3 signals was as described before (27, 28) using a Kd of 370 nM at 37 °C. In several experiments, IP₃-mediated Ca²⁺ release was measured at 0 °C. For these experiments, the Kd for Fluo 3 was measured to be 215 nm, using EGTA to buffer and set the different [Ca²⁺]. The Kd values for EGTA at 0 °C were taken from Martell and Smith (29).

Measurement of IP₃—Acini were washed and incubated in permeabilization medium as for measurement of Ca²⁺ uptake and release. After a 1.5-min incubation at 37 °C, if required, TMS or U73122 was added, and, after a 2.5-min incubation, the cells were stimulated with agonists. At the indicated times, 100-μl samples were transferred to 100 μl of solution containing 15% perchloric acid, mixed and kept on ice for about 20 min to allow complete protein precipitation. After a 2-min centrifugation at 10,000 × g, the supernatants were transferred to clean tubes. Standards of IP₃ were prepared in permeabilization medium and processed in a similar manner. Perchloric acid was precipitated, and IP₃ was extracted by the addition of 0.2 ml of tri-n-acetylamine and 0.2 ml of freon. The samples were mixed vigorously, centrifuged for 30 s at 10,000 × g to separate the phases, and 15–25 μl of the upper layer was used for mass measurement of IP₃, by a standard radioligand binding assay (13).

RESULTS

Regulation of K_app for IP₃—The maintained intact signaling system in the SLO-permeabilized cells allowed examination of the acute effect of agonists and antagonists on the IP₃-activated Ca²⁺ channel (IPACC). Fig. 1 shows the properties and demonstrates the advantages of this experimental system. Pancreatic acinar cells are fully permeabilized to small molecules within 10–15 s of exposure to the concentration of SLO used and reduced [Ca²⁺] in the incubation medium to the 50–80 nm range within 2 min of incubation at 37 °C. Addition of increasing concentrations of IP₃ resulted in discrete events of [Ca²⁺] increase, typical of the quantal behavior of Ca²⁺ release (30). Plotting the increases in medium [Ca²⁺] as a function of IP₃ concentration showed a saturation kinetic with an apparent affinity for IP₃ (K_app P₂) of 0.43 ± 0.02 μM (Fig. 1a, Table I) and a Hill coefficient of 1.42 ± 0.026 (n = 33). Stimulating the cells with low concentrations of the muscarinic agonist, carbachol, caused small Ca²⁺ release. Titration of Ca²⁺ release in these cells showed that carbachol stimulation decreases the K_app P₂ by about 2.53-fold to 0.17 μM (Fig. 1b, Table I), without changing the Hill coefficient. On the other hand, stimulation by maximal [carbachol] and inactivation by the antagonist atropine was followed by a 1.42-fold increase in the K_app P₂ to about 0.61 ± 0.02 μM (Fig. 1c, Table I), again without an apparent change in the Hill coefficient. Thus, agonist stimulation increases whereas antagonist inhibition decreases the apparent affinity of the IPACC to its ligand IP₃. These changes in K_app P₂ are independent of the total amount of Ca²⁺ release since the release was measured at the same Ca²⁺ buffering conditions.

To study the possible mechanism by which the agonist and antagonist regulate the K_app P₂, we characterized the effect of different compounds reported to affect IP₃-mediated Ca²⁺ release and the relationship between them. Examples of individual experiments are shown in Figs. 2 and 3, and the combined results are summarized in Table I. Treating the cells with relatively low concentrations of GTPγS, which by itself caused no or minimal Ca²⁺ release, decreased K_app P₂ in a concentration- and time-dependent manner (Fig. 2b, Table I). Incubation with 10 μM GTPγS for 30 s increased the affinity for IP₃ by about 2.4-fold. Thimerosal (TMS) was shown to induce [Ca²⁺] oscillations in pancreatic acini (21) and increase the affinity for IP₃ in several cell types (19–24). Fig. 2c shows that unlike the case in intact cells, TMS (up to 500 μM) alone was unable to cause Ca²⁺ release in permeable cells. However, as little as 100 μM TMS decreased K_app P₂ by about 4-fold (Table I). Figs. 2 and Table I show the results with 100 μM TMS since this concentration was sufficient to maximally modify the effects of the agonist and GTPγS on IPACC (see below).

The effect of Ca²⁺ content in the IP₃-mobilizable Ca²⁺ pool on IPACC has been studied extensively in several cell types (31–38). Fig. 2d shows that overloading the IP₃-sensitive pool with Ca²⁺ caused small, but significant increase in the affinity to IP₃ (see also Table I). Similar K_app P₂ was measured after one (not shown), two (Fig. 2d), and four pulses of 5 μM Ca²⁺ (not shown). Finally, the temperature of the incubation medium had a profound effect on K_app P₂ but without changing the quantal nature of Ca²⁺ release. Thus, Fig. 2e shows that after 2.5 min of cell permeabilization and Ca²⁺ loading at 37 °C and a subsequent 3-min incubation at 0 °C, increasing concentrations of IP₃ induce discrete and finite Ca²⁺ release events. The release was much slower than that at 37 °C, which allowed a clear resolution of each Ca²⁺ release event. In separate experiments, Ca²⁺ release at submaximal [IP₃] was followed up to 15


Table I

Apparent affinity for IP₃ in activating Ca²⁺ release

| Conditions | K<sub>app</sub>IP₃ (µM) |
|------------|-----------------------|
| Control   | 0.43 ± 0.02 (n = 33) |
| 10 µM carbachol | 0.17 ± 0.006 (n = 8) p < 0.01<sup>a</sup> |
| 5 µM GTPγS   | 0.28 ± 0.012 (n = 15) p < 0.01<sup>a</sup> |
| 10 µM GTPγS  | 0.18 ± 0.008 (n = 4) p < 0.01<sup>a</sup> |
| 100 µM TMS   | 0.11 ± 0.004 (n = 15) p < 0.01<sup>a</sup> |
| Ca²⁺-loaded  | 0.34 ± 0.02 (n = 3) p < 0.05<sup>a</sup> |
| 0°C         | 0.052 ± 0.004 (n = 3) p < 0.01<sup>a</sup> |
| Ca²⁺-loaded + carbachol (10 µM) | 0.12 ± 0.004 (n = 3) p < 0.05<sup>b</sup> |
| 0°C + carbachol | 0.14 ± 0.002 (n = 3) p < 0.05<sup>1</sup> |
| Ca²⁺-loaded + GTPγS (5 µM) | 0.20 ± 0.013 (n = 3) p < 0.05<sup>b</sup> |
| 0°C + GTPγS  | 0.26 ± 0.001 (n = 3) p < 0.05<sup>b</sup> |
| Ca²⁺-loaded + TMS (100 µM) | 0.081 ± 0.003 (n = 3) p < 0.05<sup>b</sup> |
| 0°C + TMS    | 0.013 ± 0.001 (n = 3) p < 0.05<sup>b</sup> |
| Okadaic acid (0.12 µM) | 0.45 ± 0.02 (n = 5) NS<sup>c</sup> |
| Okadaic acid then GTPγS (10 µM) | 0.36 ± 0.03 (n = 4) p < 0.01<sup>d</sup> |
| Okadaic acid then carbachol (10 µM) | 0.36 ± 0.04 (n = 3) p < 0.01<sup>e</sup> |
| H7 (0.5 mM)  | 0.43 ± 0.03 (n = 6) NS<sup>a</sup> |
| H7 then GTPγS (5 µM) | 0.13 ± 0.01 (n = 4) p < 0.01<sup>d</sup> |
| H7 then carbachol (10 µM) | 0.11 ± 0.01 (n = 3) p < 0.05<sup>c</sup> |
| 2 mM carbachol then 100 µM atropine | 0.61 ± 0.02 (n = 8) p < 0.02<sup>c</sup> |
| Carbachol/atropine then 10 µM GTPγS | 0.62 ± 0.04 (n = 3) NS<sup>a</sup> |
| Carbachol/atropine then 100 µM TMS | 0.17 ± 0.02 (n = 3) p < 0.01<sup>f</sup> |

<sup>a</sup> p values relative to control.
<sup>b</sup> Relative to Ca²⁺ load.
<sup>c</sup> Relative to incubation at 0°C.
<sup>d</sup> Relative to same concentration of GTPγS.
<sup>e</sup> Relative to 10 µM carbachol.
<sup>f</sup> Relative to 2 mM carbachol then 100 µM atropine.

Fig. 2. Effect of GTPγS, TMS, Ca²⁺ load, and temperature on K<sub>app</sub>IP₃. After completion of Ca²⁺ uptake and stabilization of medium (Ca²⁺) at about 55 nM (a), the cells were treated with 5 µM GTPγS (b) or 100 µM TMS (c). In experiment d, where indicated, two additions of 5 µM CaCl₂ to the incubation medium were made. In experiment e, the acini were first incubated for 2.5 min at 37°C, then transferred to a thermostated cuvette and incubated for 3 min at 0°C before the first addition of IP₃. The trace shows the last part of the incubation at 0°C under control conditions. After each treatment, the K<sub>app</sub>IP₃ was measured by incremental addition of IP₃. In experiments a–d, additions of IP₃ were as those shown in trace a. The numbers in traces e indicate the concentrations of IP₃ used for this titration. The results of all similar experiments are summarized in Table I.

Fig. 3. Additivity in the regulation of K<sub>app</sub>IP₃. Experimental protocols were similar to those described in the legend to Fig. 2. In experiments a and b, the stores were overloaded by two additions of 5 µM CaCl₂. For clarity, the loading traces are shown only for experiment a. After stabilization of [Ca²⁺], the cells were treated with 5 µM GTPγS (a) or 100 µM TMS (b) before addition of IP₃. The numbers in trace a indicate the concentrations of IP₃ used for experiments a and b. In experiments c and d, the acini were incubated for 2 or 1.5 min at 37°C before addition of 5 µM GTPγS (c, GTPγS-treated) or 100 µM TMS (d, TMS-treated). After an additional 0.5- or 1-min incubation at 37°C, respectively, the cells were cooled and K<sub>app</sub>IP₃ was measured by the addition of the IP₃ concentrations indicated by the numbers in trace c. The results of all similar experiments are summarized in Table I.
controls. Next, 11740 carbachol (TMS, to regulate or prevented the effect of GTP kinases and phosphatase inhibitors tested reduced similarity between their effects on GTP 

However, their value for the present studies is in showing the questions as to the physiological significance of these findings. The effect of submaximal [GTP-S] on the incubation medium to increase the 

Table I lists the effect of the most effective compounds, okadaic acid and H7. Treatment with 0.12 μM okadaic acid largely prevented the effect of 30 s of treatment with 10 μM GTP-γS. H7 at 0.5 mM, a concentration sufficient to inhibit most protein kinases (38), had no effect in unstimulated cells but it increased the effect of submaximal [GTP-S]. The lack of selectivity in the effect of kinase and phosphatase activators/inhibitors raises questions as to the physiological significance of these findings. However, their value for the present studies is in showing the similarity between their effects on GTP-γS and carbachol, which were different from those of TMS, Ca2+ load, and low temperature.

Regulation of K<sub>app</sub>IP<sub>3</sub> Is Independent of PLC Activation—At this stage of the study, the possibility arose that the effects of carbachol and GTP-γS on K<sub>app</sub>IP<sub>3</sub> were simply due to partial modulation of PLC and a persistent, small global or local increase in [IP<sub>3</sub>]. Three types of experiments were performed to exclude this possibility. The first is shown in Fig. 4, a–c, in which U73122 was used to inhibit PLC. U73122 did not reduce the effect of either carbachol (Fig 4b), GTP-γS (Fig 4c), or TMS (not shown) on K<sub>app</sub>IP<sub>3</sub>, although it inhibited the small Ca<sup>2+</sup> release evoked by low concentrations of carbachol. Table II shows that stimulation with 10 μM carbachol had a small effect on IP<sub>3</sub>, whereas U73122 slightly reduced the basal level of IP<sub>3</sub>. In the presence of U73122, IP<sub>3</sub> level was not changed by carbachol or GTP-γS. The second evidence is provided by the finding that prestimulation with low [GTP-γS] had a marked effect on K<sub>app</sub>IP<sub>3</sub> with minimal or no effect on IP<sub>3</sub> levels, Ca<sup>2+</sup> release, or the size of the IP<sub>3</sub>-mobilizable Ca<sup>2+</sup> pool.

The third and most convincing evidence against stimulation of PLC by carbachol or GTP-γS as the cause of the change in K<sub>app</sub>IP<sub>3</sub> is shown in Fig. 4, d and e. In these experiments, 25 μg/ml IP<sub>3</sub> competitive inhibitor heparin (39, 40) were added to the incubation medium to increase the K<sub>app</sub>IP<sub>3</sub> under control conditions from 0.43 μM (Table I) to 1.67 μM (Fig. 4d). This should dilute the effect of any IP<sub>3</sub> generated by GTP-γS or carbachol stimulation by about 4-fold (1.67/0.43) to virtually eliminate the effect of the agents on K<sub>app</sub>IP<sub>3</sub>. Fig. 4e shows that this is not the case. Pretreatment with GTP-γS in the presence of 25 μg/ml heparin had the same effect on K<sub>app</sub>IP<sub>3</sub> as in control cells. Similar results were obtained with heparin concentrations between 5 and 50 μg/ml with cells stimulated with 10 μM carbachol (not shown). Hence, together the three protocols indicate that carbachol and GTP-γS modified K<sub>app</sub>IP<sub>3</sub> independent of PLC stimulations.

G Protein-dependent Regulation of IPACC—The finding that GTP-γS can modulate K<sub>app</sub>IP<sub>3</sub> independent of PLC activation suggested that activation of a G protein(s) by GTP-γS was sufficient to modulate interaction of IPACC with its ligand. Further support for this notion was obtained by testing the effect of AlF<sub>3</sub>, which activates mainly heterotrimeric but not small G proteins (41). All the effects of GTP-γS presented above and in subsequent figures could be initiated by 1 mM NaF + 0.2 μM AlCl<sub>3</sub> (not shown). 10 mM NaF + 2 μM AlCl<sub>3</sub> induced strong Ca<sup>2+</sup> release comparable with that observed with 100 μM GTP-γS (see below) or 2 mM carbachol.

More dramatic evidence for an effect of G protein(s) activation on the activity of the IPACC was obtained when the effect of a low concentration of GTP-γS on the response to carbachol, atropine, and TMS was studied. Fig. 5a shows that exposure of control or stimulated cells to 100 μM TMS in the absence of GTP-γS augmented the effect of low concentrations of carbachol on Ca<sup>2+</sup> release (Fig. 5, b and c), without affecting IP<sub>3</sub> levels during the first 20 s of cell stimulation (Table II). Performing complete concentration dependence of both IP<sub>3</sub> production and Ca<sup>2+</sup> release showed that pretreatment with 100 μM TMS had no effect on IP<sub>3</sub> production while increasing the apparent affinity for carbachol-mediated Ca<sup>2+</sup> release by about 6.3-fold.

**Fig. 4. Effect of carbachol and GTP-γS on K<sub>app</sub>IP<sub>3</sub> is independent of PLC.** Acini incubated at 37°C were exposed to 10 μM U73122 (a–c) or 25 μg/ml heparin (d and e) before stimulation with 10 μM carbachol (b) or 5 μM GTP-γS (c and e). a and d are the respective controls. Next, K<sub>app</sub>IP<sub>3</sub> was measured by incremental additions of the indicated [IP<sub>3</sub>]. Note the high [IP<sub>3</sub>] used in experiments d and e.

**Table II**

| Conditions                  | Carbachol  |
|-----------------------------|------------|
|                             | 0 μM       | 10 μM | 2000 μM |
| Control (n = 24)            | 4.1 ± 0.16 | 8.3 ± 0.44 | 63.1 ± 2.8 |
| TMS (100 μM) (n = 5)        | 3.8 ± 0.26 | 7.8 ± 0.38 | 56.4 ± 2.7 |
| GTP-γS (2 μM) (n = 6)       | 4.3 ± 0.28 | 11.2 ± 1.4 | 64.3 ± 2.6 |
| GTP-γS (5 μM) (n = 8)       | 5.1 ± 0.31 | 3.8 ± 0.22 | 31.8 ± 1.9 |
| U73122 (10 μM) (n = 3)      | 3.7 ± 0.24 |        |        |
| U73122 + GTP-γS (5 μM) (n = 3) | 4.1 ± 0.26 |        |        |
On the other hand, GTPγS profoundly modified the effect of carbachol. Treating the cells with as little as 2 μM GTPγS for 30 s was sufficient to cause maximal Ca2+ release by 10 μM carbachol (Fig. 5d).

The effect of GTPγS was independent of PLC activation became even more evident when the effect of 2 μM GTPγS on the dose response to carbachol and atropine was measured. Fig. 6 shows that under control conditions the concentration dependence curves for carbachol stimulation of IP3 production and Ca2+ release were identical. Half-maximal stimulation (EC50) of Ca2+ release was at 210 ± 13 μM (n = 8), and the EC50 for IP3 production was 236 ± 27 μM (n = 4). In the presence of 2 μM GTPγS, the EC50 for IP3 production was reduced by about 2.9-fold to 82 ± 11 μM (n = 4), whereas the EC50 for Ca2+ release was reduced by 90-fold to 2.35 ± 0.13 μM (n = 8).

The effect of low [GTPγS] on signal termination by atropine is shown in Fig. 7. In the absence of GTPγS, atropine inhibited the IPACC of cells stimulated with 2 mM carbachol (measured from the rate of [Ca2+]i reduction in carbachol-stimulated cells, see Fig. 1 and Ref. 27) with an IC50 of 0.33 ± 0.02 μM (n = 5). Under the same conditions, atropine accelerated IP3 hydrolysis (measured from the rate of IP3 reduction relative to continuously stimulated cells as in Fig. 11 below and Ref. 27) with an IC50 of 0.21 ± 0.03 μM (n = 3). Including 2 μM GTPγS in the incubation medium shifted the IC50 for IP3 production 4-fold, to 0.83 ± 0.09 μM (n = 3), while the IC50 for inhibition of Ca2+ release was increased about 2600-fold to 860 ± 65 μM (n = 5). The fact that atropine inhibited PLC activation in the presence of GTPγS shows clearly that the effect of GTPγS was independent of PLC activation, since binding of nonhydrolyzable GTP analogues to the α subunit of G proteins, including Gαq (41, 42) and Gαq (43), irreversibly stimulates the α subunits and prevents inhibition by antagonists.

Stabilization of IPACC in an Active State—In previous studies, we reported that antagonists inactivate the IPACC independent of IP3 metabolism (27, 28). In Fig. 1 and Table I, we showed that termination of carbachol stimulation with atropine significantly increased Kapp-P3. To better understand the mechanism of channel inactivation, we first tested the effect of atropine on the various mechanisms shown to affect Kapp-P3. Fig. 8 illustrates the effect of carbachol stimulation and atropine inhibition of the ability of GTPγS and TMS to modify Kapp-P3. Similar experiments were performed to test the effect of Ca2+ load and low temperature. Carbachol stimulation followed by atropine inhibition did not prevent the reduction in Kapp-P3 caused by TMS (Fig. 8c), Ca2+ load, or low temperature (not shown), while completely preventing the effect of GTPγS (Fig. 8b).

Incubating the cells with atropine alone without carbachol stimulation had no effect on the ability of GTPγS to reduce Kapp-P3 (not shown). Table I shows that after carbachol and atropine treatment the Kapp-P3 was 0.61 μM. It remained the same (0.62 μM) after a 1.5-min incubation with 10 μM GTPγS. This value should be compared to the Kapp-P3 of 0.18 μM measured in control cells incubated with 10 μM GTPγS for 30 s (Table I). These experiments indicate that, in permeabilized cells, once the IPACC was inactivated by atropine, the inactivation could not be reversed by activation of G proteins.

Because the effect of carbachol was modified by both GTPγS and TMS, to understand how they may modify channel activity we tested the effect of TMS on the modulation of IPACC by GTPγS. Interestingly, GTPγS markedly sensitized the effect of TMS to cause maximal discharge of the Ca2+ stores. This is illustrated in Fig. 9. Fig. 9a shows that addition of 100 μM TMS to cells treated with 5 μM GTPγS induced rapid and maximal Ca2+ release. Similar results were obtained when GTPγS was added to TMS-treated cells, but the time course of Ca2+ release was significantly slower. Accordingly, as all other effects of GTPγS, the effect shown in Fig. 9 was time- (not shown) and concentration-dependent (Fig. 9c). In the absence of TMS, high
concentrations of GTPγS could release Ca\(^{2+}\), which was probably due to stimulation of PLC to generate IP\(_3\). TMS actually partially inhibited the production of IP\(_3\) generated by all concentrations of GTPγS while sensitizing activation of Ca\(^{2+}\) release by GTPγS. It is important to note that despite the absence of an increase in IP\(_3\) the effect of TMS and GTPγS on Ca\(^{2+}\) release was still inhibited by heparin (Fig. 9c). This would suggest that TMS + GTPγS sensitized the IPACC to a level that maximal and rapid Ca\(^{2+}\) release was observed at the level of IP\(_3\) present in unstimulated cells.

In the next stage, we tested the effect of channel sensitization by GTPγS and TMS on the inactivation induced by atropine. Fig. 10a shows that 1 mM atropine completely inactivated channels activated by 10 \(\mu\)M carbachol and 2 \(\mu\)M GTPγS. However, treating the cells with 100 \(\mu\)M TMS prior to stimulation with carbachol and GTPγS completely prevented channel inactivation by atropine. The small reduction in medium [Ca\(^{2+}\)] due to atropine probably reflects the activity of channels that were not accessed by GTPγS. The channels were permanently stabilized in an active state since medium [Ca\(^{2+}\)] remained elevated for at least 15 min with no sign of decline. To show that TMS and GTPγS did not inhibit the SERCA pumps and that the maintained high level of medium [Ca\(^{2+}\)] was due to stabilizing the IPACC in an active state, the channel was inhibited by heparin. Addition of heparin resulted in channel inactivation and, consequently, rapid Ca\(^{2+}\) uptake into the IP3-sensitive pool at a rate comparable to that measured in Fig. 10a after addition of atropine.

As indicated above, TMS alone (not shown) or GTPγS alone (Fig. 10a and Fig. 7), although reducing the affinity for atropine, never prevented channel inactivation by atropine. That activation of G proteins by GTPγS and channel sensitization by TMS was required to prevent channel inactivation is further emphasized in the experiments shown in Fig. 10, c and d. As we showed before (28), stimulation of Ca\(^{2+}\) release with 100 \(\mu\)M GTPγS did not prevent channel inactivation by atropine (Fig. 10c), even though IP3 levels under these conditions were very high (28). On the other hand, treating the cells with 2 \(\mu\)M (not shown) or 100 \(\mu\)M GTPγS (Fig. 10d) and 100 \(\mu\)M TMS in the absence of agonist stimulation was sufficient to prevent channel inactivation by atropine.

An important control for the experiments in Fig. 10 is to show that TMS did not prevent the hydrolysis of IP\(_3\) initiated by atropine. The results of such experiments are shown in Fig. 11. Even in the presence of 100 \(\mu\)M carbachol and 5 \(\mu\)M GTPγS, TMS accelerated, rather than inhibited, the hydrolysis of IP\(_3\). After 2 min of exposure to atropine, IP\(_3\) was reduced to basal levels, while the channel was fully activated (Fig. 10b).

**DISCUSSION**

A long standing question in understanding agonist-evoked [Ca\(^{2+}\)] oscillations is how low concentrations of agonists induce oscillations without an apparent or only small change in
Modulation of the IP3-activated Ca2+ channel (IPACC) can be potentially important in view of the high basal IP3 levels in most cells (1, 2, 6–9). Although several regulatory mechanisms of IPACC have been reported (10–26), the relationship between them and their role in agonist-dependent regulation of Ca2+ release is not known. The present study suggests the existence of multiple and independent mechanisms for regulation of IPACC, prominent among which is regulation by G protein activation. The latter is used by agonists and antagonists to modulate the KappIP3 in a reciprocal manner and thus facilitate Ca2+ release during agonist stimulation and impair the release during antagonist inhibition. Such a mechanism can contribute to the cyclical activation and inactivation of the IPACC during Ca2+ oscillations (44). Below we discuss the evidence in support of these findings.

Comparing the effect of many agents and conditions in the same cell type and experimental system showed that KappIP3 can be modulated by several independent mechanisms (Table I). Thus, additive effects were found between TMS, Ca2+ load, low temperature, and the agonists. The important implication of these findings is that although the IPACC can be regulated by various mechanisms, none of them appear to contribute to the regulation by agonists. Of course, regulation by Ca2+ load can have important physiological significance in that when the stores are loaded, they are primed for release by small additional change in KappIP3. It is likely that in empty stores the IPACC has the lowest affinity for IP3, which will facilitate channel inactivation at the termination of cell stimulation. Nonetheless, the regulation of KappIP3 by Ca2+ content in the ER is relatively modest and occurs by a mechanism different from that used by agonists.

Interestingly, despite the fact that variations in KappIP3 between compartmentalized Ca2+ pools account in large part for quantal Ca2+ release (28), none of the modulators of KappIP3 changed the quantal properties of Ca2+ release. This includes low temperature (Figs. 2 and 3). We particularly studied the effect of low temperature in detail since a previous report suggested that quantal Ca2+ release becomes continuous at low temperature, and this process is reversible (25). However, we failed to convert the quantal to a continuous Ca2+ release by short or long incubation at 25 or 0 °C or by any other modulator of KappIP3. In the earlier studies by Kindman and Meyer (25), one concentration of IP3 was used to demonstrate submaximal Ca2+ release at 37 °C and maximal release at 0 °C, without considering the effect of the temperature on KappIP3. Such an effect as demonstrated in the present study (Table I) can account well for the differences between the two studies. Our results of maintained quantal behavior under all conditions suggest that all modulators of KappIP3, including agonists, affect all IPACC equally rather than equalize KappP3 of channels of different compartments.

The present studies show that KappIP3 is dynamically controlled. Low concentrations of agonists, which minimally activate PLC, markedly reduced KappIP3 of the IPACC. Moreover, termination of cell stimulation with antagonists increased KappIP3 to a level above that measured in control cells. The antagonist was effective only if the cells were first stimulated with carbachol. The antagonist had no effect in control cells, and, when added before GTPγS, it did not prevent the GTPγS-dependent reduction in KappIP3. The combined effects of the agonist and antagonist indicate that the KappIP3 of the IPACC is dynamically controlled during cell stimulation/inhibition cycles. One advantage of such a dynamic control is that small changes in IP3 levels can lead to maximal opening or closing of the channel. This will be translated into a high cooperativity for interaction of IP3 with the IPACC and in channel activation/inactivation.

Probably the most interesting finding of the present studies is that agonists and antagonists appear to modulate KappIP3 by a mechanism dependent on activation of G proteins. The first indication of this was the similarity between the effect of the agonist and preincubation with GTPγS on KappIP3. Both affected KappIP3 in cells treated with TMS, high Ca2+ load or incubated at low temperature. Both effects were inhibited similarly by activators of protein kinases, inhibitors of protein phosphatases, and augmented by inhibitors of protein kinases. Inhibition of agonist-activated cells by atropine to increase KappIP3 inhibited the effect of GTPγS, but not of any other agent or treatment. Together, these observations strongly suggest that agonist stimulation and GTPγS reduced the KappIP3 by the same mechanism. It is possible that the agonists and GTPγS activated heterotrimeric, rather than small G proteins, since all effects of GTPγS could be reproduced with low concentrations of AlF3. The effect of GTPγS described in the present study is different from the previously described modification of the size of the IP3-sensitive Ca2+ pool by GTP (45–47). GTPγS inhibited the GTP-induced expansion of the Ca2+ pool, whereas GTPγS increased the KappIP3 and millimolar concentrations of GTP were required to mimic the effect of low concentrations of GTPγS and AlF3.

That G proteins are involved in the effect of the agonist/antagonist and their full impact becomes more evident when the effect of their activation on agonist/antagonist-dependent changes in Ca2+ release and IP3 levels are considered. Even in the absence of preincubation, low concentrations of GTPγS (0.2–2 μM) markedly increased the potency of the agonist and decreased the potency of the antagonist in affecting Ca2+ release. Thus, when irreversible activation of G proteins was allowed by the presence of GTPγS, the agonists exceedingly sensitized the IPACC to trigger Ca2+ release at basal IP3. Measurement of IP3 levels showed that GTPγS had a small effect on IP3 production during agonist stimulation and did not
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prevent initiation of IP₃ hydrolysis by the antagonist. The latter excludes the possibility that the effects of GTP•S, or for that matter the agonist and the antagonist, on Ca²⁺ release was dependent on PLC activity (41-43). In fact, in cells stimulated with carbachol and GTP•S, 10–100 µM atropine completely inactivated PLC without having any inactivation effect on the IPACC (Fig. 7). These experiments, therefore, indicate that agonists use G protein activation to reduce Kₘ₂₃ and stabilize the channel in an open state. Once the channel is activated, complete inactivation of G proteins by the antagonist beyond (or different from?) that required to modulate PLC activity, is needed for channel inactivation. Indeed, preliminary experiments showed that muscarinic, bombesin, and cholera toxinβ-gector antagonists were between 100- and 1000-fold more effective in preventing cell stimulation when added before the respective agonist than in reversing agonist effects when added to stimulated cells.²

Further insight into the regulation of IPACC by G proteins was obtained when the effect of TMS on GTP•S-, agonist-, and antagonist-dependent Ca²⁺ release was studied. TMS and low GTP•S caused maximal Ca²⁺ release at basal IP₃ concentration. It is interesting that in intact cells 100 µM TMS caused significant Ca²⁺ release (21 and data not shown), whereas in permeabilized cells up to 500 µM TMS did not release Ca²⁺ but only decreased Kₘ₁₂₃. Incubating the cells with at least 0.2 µM GTP•S was sufficient to restore the ability of TMS to cause Ca²⁺ release with no change in IP₃ levels. Together, these observations indicate that: (a) TMS and GTP•S modulate the IPACC by different mechanisms, (b) the IPACC is regulated by G proteins in permeabilized and probably in intact cells, and (c) TMS appears to modulate the interaction of the G protein-dependent mechanism with the IPACC to stabilize the channel in an open state. Indeed, treating the cells with TMS and GTP•S, with or without agonist, completely prevents channel inactivation by the antagonist. This effect was absolutely dependent on the combined action of TMS and GTP•S to the extent that incubating the cells with very high GTP•S (100 µM) did not prevent the effect of atropine, while stimulation with 2 µM GTP•S and 100 µM TMS locked the channel in an antagonist-insensitive state. It is important to note that TMS increased, rather than decreased, the rate of IP₃ hydrolysis under all conditions, including incubation with GTP•S.

The implication of the studies with TMS, GTP•S, agonist, and antagonist is that all point to the involvement of G proteins in the regulation of Kₘ₂₃ by agonist stimulation and antagonist inhibition. They also point to the broad extent of such regulation. Such a regulatory mechanism can be very attractive in explaining how agonists can stimulate Ca²⁺ oscillations in the absence of stimulated IP₃ production. Our results suggest that minimal activation of G proteins (low GTP•S, ALF•γ or agonist concentrations), which is not sufficient to appreciably activate PLC, is sufficient to markedly decrease the Kₘ₂₃ and trigger large Ca²⁺ release at basal [IP₃]. This regulation is a dynamic process as revealed by the antagonist-induced increase in Kₘ₂₃ beyond that found in resting cells. It is well established in many cell systems that Ca²⁺ oscillations occur only at low agonist concentration, when usually no change in IP₃ is observed. It is easy to see how the dynamic regulation of Kₘ₂₃ described here can contribute or even dominate the mechanism by which agonists signal Ca²⁺ oscillations. Understanding how activation of G proteins modulate Kₘ₂₃ is essential to evaluate its role in Ca²⁺ oscillation in particular

² X. Xu and S. Muallem, unpublished observations.