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Intracellular Guanosine-5′-*O*-(2-thiodiphosphate) Alters the Dynamics of Receptor-Mediated Responses in Bullfrog Sympathetic Neurons

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SUMMARY

The mechanism by which intracellularly applied guanosine-5′-*O*-(2-thiodiphosphate) alters responses to chicken II luteinizing hormone-releasing hormone, muscarine, and substance P in bullfrog sympathetic neurons was examined. Whole-cell recordings were made from enzymatically dissociated single neurons. Guanosine-5′-*O*-(2-thiodiphosphate) was applied intracellularly by adding it to the pipette solution with fixed amounts of GTP. Guanosine-5′-*O*-(2-thiodiphosphate) did not affect the proportion of cells that responded to any of the agonists. Guanosine-5′-*O*-(2-thiodiphosphate) decreased the amplitude of the responses to submaximal concentrations of agonist. At maximal concentrations of agonist, guanosine-5′-*O*-(2-thiodiphosphate) did not decrease the response to the first application of agonist; however, with guanosine-5′-*O*-(2-thiodiphosphate) intracellularly, successive responses to maximal concentrations of agonist were decreased in amplitude and increased in time course. Intracellular guanosine-5′-*O*-(2-thiodiphosphate) did not accelerate the rate or magnitude of desensitization to substance P. A kinetic model of receptor-guanine nucleotide-binding protein (G protein) coupling predicts that a decrease in the available G protein pool should decrease both the magnitude and the time course of the build-up of active G proteins. The results are consistent with the hypothesis that guanosine-5′-*O*-(2-thiodiphosphate) binds tightly to G proteins, thereby effectively decreasing the available G protein pool with repeated agonist applications.

The neurons of the sympathetic ganglia of the bullfrog possess a time- and voltage-dependent potassium current termed I_M (1). I_M is an important regulator of cellular excitability and is known to be modulated by four types of membrane receptors in amphibian sympathetic neurons. Agonists at cholinergic muscarinic, LHRH, SP, and purinergic receptors all decrease I_M.

Several laboratories have provided evidence that the responses to these agonists involve coupling of the receptors to G proteins (2–4). When compounds that activate G proteins, such as guanosine-5′-*O*-(3-thiotriphosphate) or AlF_4, are applied intracellularly, they mimic the effect of agonists to decrease I_M and, when present during agonist application, result in an irreversible inhibition of I_M. Intracellular application of GDPβS decreases the ability of agonists to inhibit I_M.

Numerous other physiological studies have used intracellular application of GDPβS to study G protein-mediated phenomena (5–8). The ability of GDPβS to block the response to an agonist has been taken as support for the conclusion that the agonist works through a G protein. Recently, several studies have noted that the effectiveness of GDPβS to block agonist responses increases with successive agonist applications (4, 9, 10). We have previously shown that, when GDPβS and GTP are applied intracellularly in ratios ranging from 4:1 to 9:1 GDPβS/GTP, the magnitude of the inhibition of I_M in response to a maximal dose of cII-LHRH, muscarine, or SP is not significantly decreased. With successive applications of agonist, however, the response to that agonist is decreased (10).

These findings indicate that GDPβS affects receptor-coupled pathways in a "use-dependent" manner. It has generally been assumed that GDPβS acts specifically to inhibit activation of G proteins, but it is possible that GDPβS might have other actions. For example, similar results might have been observed if GDPβS acted at the receptor level or enhanced desensitization to agonists.

To further elucidate the mode of action of GDPβS on receptor-mediated inhibition of I_M, the effects of GDPβS on the responses to cII-LHRH, muscarine, and SP have been quanti-

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ABBREVIATIONS: I_M, M current; cII-LHRH, chicken II luteinizing hormone-releasing hormone; GDPβS, guanosine-5′-*O*-(2-thiodiphosphate); SP, substance P; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MEM, minimum essential medium; LHRH, luteinizing hormone-releasing hormone; G protein, guanine nucleotide-binding protein.

fied. The results have been compared with a theoretical model of receptor-G protein interactions.

Materials and Methods

Cell isolation. Bullfrogs (*Rana catesbeiana*) were anesthetized by hypothermia and killed by double pithing. The sympathetic chains were dissected bilaterally. Sympathetic ganglia were incubated for 90 min in dissociation medium with collagenase A (1 mg/ml) and trypsin (0.5 mg/ml) and then for 90 min in dissociation medium with collagenase B (1 mg/ml). The ganglia were then triturated 25–50 times with a long-shanked glass Pasteur pipette, to free single cells from the ganglia. Cells were stored in growth medium at 4°.

Solutions. The composition of solutions (in mM unless otherwise noted) was as follows. Intracellular (or recording electrode) solution was KCl, 120; $MgCl_2$, 2; EGTA, 5; HEPES, 10; ATP, 1.5; leupeptin, 0.00001; pH 6.8 (with KOH); plus various concentrations of guanine nucleotides as indicated below. Extracellular solution was NaCl, 118; KCl, 2.4; $CaCl_2$, 1.8; $MgCl_2$, 1.8; sodium pyruvate, 5; glucose, 5; HEPES, 10; tetrodotoxin, 0.0003; pH 7.4 (with NaOH). Dissociation medium was NaCl, 98; KCl, 2.4; NaH_2PO_4, 0.6; $MgCl_2$, 1.8; sodium pyruvate, 5; creatine, 5.7; glucose, 5; HEPES, 20; pH 7.4 (with NaOH); with 10 ml/liter medium 199, 100 units/ml penicillin, and 100 μg/ml streptomycin. Growth medium was NaCl, 118; KCl, 2.4; creatine, 5.7; glucose, 5; sodium pyruvate, 5; HEPES, 20; pH 7.4 (with NaOH); with 10 ml/liter 100× MEM vitamins, 100 units/ml penicillin, 100 μg/ml streptomycin, 20 ml/liter 50× MEM essential amino acids, 10 ml/liter 100× MEM nonessential amino acids, and 1 mg/ml bovine serum albumin.

Most chemicals were obtained from Sigma Chemical or Fisher Scientific. Proteolytic enzymes, nucleotides, SP, and leupeptin were obtained from Boehringer-Mannheim, medium 199 and antibiotics from GIBCO, and cII-LHRH from Peninsula.

In most experiments with GTP, the concentration was 400 μM. When GDPβS was used, it was added to the pipette at a concentration of 320, 353, or 360 μM, as indicated, and GTP was added to provide a total guanine nucleotide concentration of 400 μM. Providing a fixed amount of GTP decreases the cell-to-cell variability in the effects of guanine nucleotides (6). To ensure that the effects observed were due to GDPβS and not due to the change in GTP, other experiments were conducted with 40 μM GTP.

Whole-cell recordings. All experiments were conducted at room temperature, ~20°. Whole-cell recordings (11) were made with electrodes of 0.25–1 MΩ filled with intracellular solution. The recordings were filtered at 1 kHz and stored on magnetic tape.

Measurement of I_M. During whole-cell recordings, the membrane potential was maintained at a holding potential of −30 mV. Every 8 sec, the voltage was stepped to −50 mV for 500 msec, followed by return to −30 mV. Upon a voltage step from −30 mV to −50 mV, the outward current decreases due to the time- and voltage-dependent closing of M channels (12). Upon return to −30 mV from −50 mV, the outward current returns exponentially (see Fig. 1A). The amplitude of this exponential relaxation of current is termed $I_M(-30)$ and provides a measure of I_M that is independent of changes in other currents or "leak" currents. Instantaneous and steady state current amplitudes at −30 mV and −50 mV were determined by computer.

Control of extracellular solutions. Drugs were applied by single-cell superfusion, as described previously (10). Briefly, the extracellular solution perfusing the cell of interest was controlled by positioning the cell in front of one of a series of glass capillary tubes, each connected to a reservoir containing a different extracellular solution. Moving a different tube to the cell changed the extracellular solution. This system allows accurate and rapid solution changes (see Fig. 4, *inset*) and does not require that the entire bath be flooded with drug. The bath was constantly perfused separately with control extracellular solution.

Because agonist exposure results in a time- and concentration-dependent desensitization of the inhibition of I_M (13–17), agonists were applied only long enough for the change in current to reach a steady state. An inward current is also activated in some cells by these agonists, particularly LHRH analogs. Because the inward current is more evident with longer drug applications (18), the contribution of this inward current was minimized by using brief agonist applications.

Dose-response curves. To assess the effects of submaximal concentrations of the agonists with GTP in the pipette, various concentrations of the agonists were applied to the cells in random order. To avoid desensitization, drug was applied only long enough for the response to peak. The cell was then superfused with control extracellular solution for at least 2 min, or until the current had apparently recovered from the previous agonist application, before proceeding to the next agonist application. Responses to maximal concentrations of agonists were obtained either after assessment at lower doses or in separate cells.

If a cell did not respond to submaximal doses of agonist, a maximal dose was applied to determine whether that cell was responsive to agonist. If the cell did not respond to a maximal concentration, it was categorized as unresponsive to that agonist and was not included in the dose-response analysis.

Time course measurements. To measure the time course of the response, the time taken for the decrease in outward current to go from 25% to 75% of its maximum amplitude, T_{25-75}, has been measured. As demonstrated in Fig. 4A, *inset*, the extracellular solution change had a T_{25-75} of about 175 msec. This is much faster than the T_{25-75} for inhibition of outward current after drug application. Thus, the time-dependent changes in outward current were not a consequence of the time course of the change in extracellular solution.

Statistics. Values are presented as mean ± standard error. Statistical comparisons were made by an unbalanced analysis of variance.

Results

Cellular properties. Most of the dissociated cells were spherical or ellipsoid. Some cells had 5–10-μm lengths of the axon attached to the soma, but most were without processes. Average soma diameter was 39 ± 1 μm (n = 74 cells). Whole-cell capacitance and series resistance were read from the dials of the amplifier after compensation of the transient in response to +5-mV, 3.3-msec pulses from a holding potential of −50 mV. Cell capacitance averaged 66 ± 1.8 pF (n = 69), and series resistance was 1.4 ± 0.1 MΩ (n = 69). The resting potential, measured within the first 2 min after the patch was broken, was −55 ± 2.3 mV (n = 69).

Dose-response curves. The effects of various concentrations of cII-LHRH, muscarine, and SP on $I_M(-30)$ and on the steady state outward current at −30 mV were measured as illustrated in Fig. 1A. The dose-response curves for inhibition of $I_M(-30)$ by cII-LHRH, muscarine, and SP with GTP in the pipette are shown in Fig. 1B. There was considerable variability in responsiveness around the middle of the dose-response curves. At higher concentrations the maximum degree of inhibition by each of the agonists was about 75%.

Responses to selected concentrations of cII-LHRH, muscarine, and SP with GDPβS in the pipette are also illustrated on Fig. 1B. Because the action of GDPβS is dependent on prior agonist applications (10), these data represent the percentage inhibition of $I_M(-30)$ observed in response to the first application of the agonist. The effects of GDPβS were tested at maximal concentrations of agonist and at concentrations near the middle of the dose-response curves. At submaximal concentrations of agonist, the responses were smaller in the presence of GDPβS. At maximal concentrations of agonist, however, intracellular GDPβS did not significantly affect the observed decrease in $I_M(-30)$ in response to the first agonist application. As quantified below, with successive agonist applications

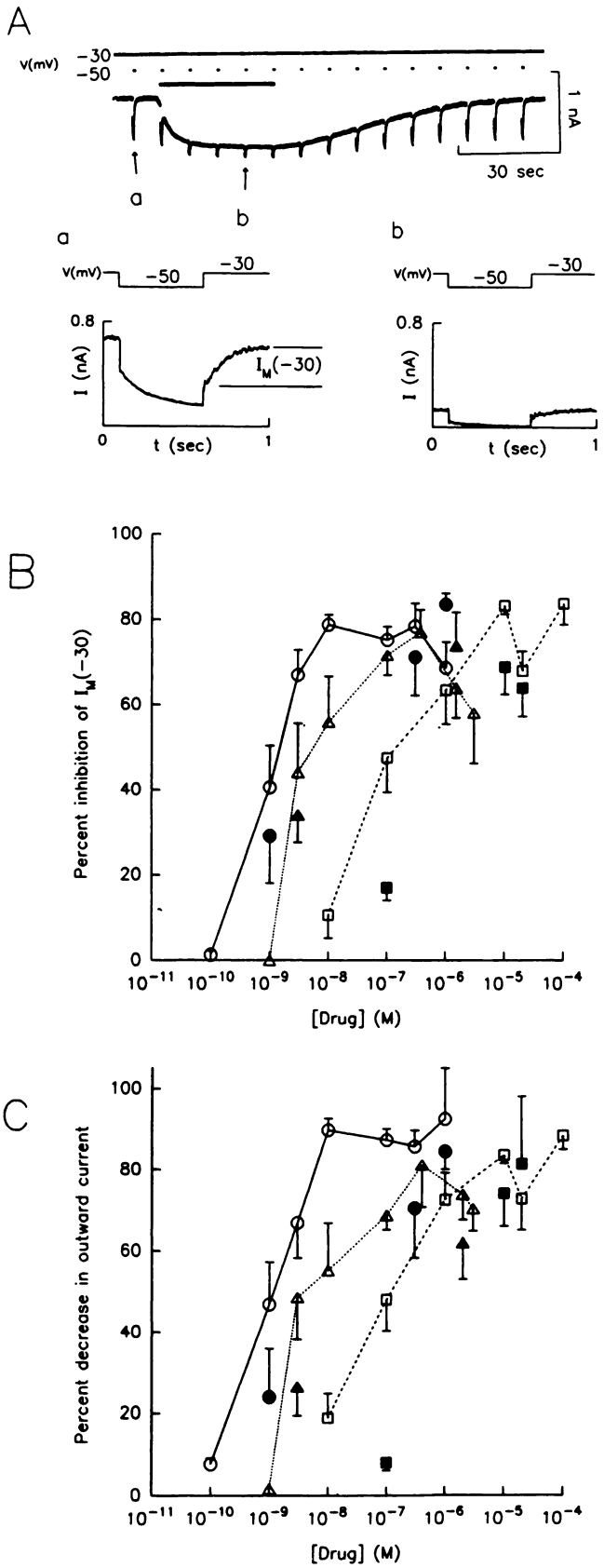

Fig. 1. Current tracings and dose-response curves for cII-LHRH, muscarine, and SP. A, *Top*, sample voltage and current tracings are shown on a slow time scale. The membrane potential was held at −30 mV and stepped to −50 mV for 500 msec every 8 sec, as indicated by *tracing V(mV)*. Muscarine was applied by single-cell superfusion for the period indicated by the *bar above the current trace*. Note the decrease in the size of the outward current at −30 mV and of the current changes during the voltage steps after muscarine application. Upon removal of the muscarine and return to control extracellular solution, the current returns. *Bottom*, the current is shown on an expanded time scale for voltage steps before (*a*) and during (*b*) muscarine application. *a*, Measurement of $I_M(-30)$. B, The effects of various concentrations of cII-LHRH (○, GTP; ●, GDPβS), muscarine (□, GTP; ■, GDPβS), and SP (△, GTP; ▲, GDPβS) were measured as the percentage of decrease in $I_M(-30)$. ○, □, △, 400 μM GTP in the recording electrode; ●, ■, ▲, 360 μM GDPβS and 40 μM GTP in the recording electrode. *n*, 5–12 for each *point*. *Bars*, standard error. For clarity, the points for SP at 3×10^{-7} M and at 10^{-6} M have been offset slightly on the concentration axis. C, Effects of various concentrations of cII-LHRH (○, GTP; ●, GDPβS), muscarine (□, GTP; ■, GDPβS), and SP (△, GTP; ▲, GDPβS) on the level of outward current at −30 mV, in the same cells as in A. The points for SP at 3×10^{-7} M and at 10^{-6} M have been offset slightly on the concentration axis.

TABLE 1

Proportion of neurons that responded to cII-LHRH, muscarine, or SP with and without GDPβS intracellularly

For the control cells, 400 μM GTP was included in the pipette. For the cells with GDPβS, the [GDPβS] was between 320 and 360 μM with [GTP] = 40 to 80 μM in the pipette.

Agonist	Control		GDPβS	
	n	%	*n*	%
cII-LHRH	81 of 90	90	117 of 131	89
Muscarine	109 of 138	79	173 of 204	85
SP	149 of 222	67	131 of 164	75

GDPβS did alter the response to maximal concentrations of agonist.

To study the kinetics of the responses to the agonists, the change in steady state current at −30 mV was also measured. The dose response curves for inhibition of this current by cII-LHRH, muscarine, and SP are shown in Fig. 1C. Note that the shape of these curves and the effects of GDPβS on the amplitude of this current are similar to the dose-response curves for inhibition of $I_M(-30)$ shown in Fig. 1B. This is consistent with I_M being the predominant steady current at −30 mV (19).

Proportion of responsive cells. Every bullfrog sympathetic neuron does not show an inhibition of I_M in response to every agonist. To see whether GDPβS altered the ability of the cells to respond to agonist, the percentage of cells that responded to each agonist was determined in the presence and absence of intracellular GDPβS. Responsiveness was determined at cII-LHRH concentrations greater than 5 nM, muscarine concentrations greater than 750 nM, and SP concentrations greater than 50 nM. These concentrations of agonist produce, on average, >60% inhibition of I_M (Fig. 1, B and C) with GTP in the pipette. GDPβS did not decrease the proportion of cells that responded to any of the agonists (Table 1).

To avoid confounding the results with nonresponding cells, the data presented below include only those cells that exhibited a response to the agonist under study. Although GDPβS did not decrease the initial responses to maximal concentrations of agonist (Fig. 1) or the proportion of cells that responded to each agonist (Table 1), it did affect the magnitude and time course of the responses to repeated applications of maximal concentrations of agonist. The progressive nature of this effect may provide some insight into the mechanism of action of GDPβS.

Effects of GDPβS with repeated agonist applications. In the presence of GTP, consecutive agonist applications resulted in decreases in current that were similar in both ampli-

tude and time course. Fig. 2 shows three successive responses to cII-LHRH with 400 μM GTP in the recording electrode. During the first application of cII-LHRH (Fig. 2A), the outward current decreased by 83% and $I_M(-30)$ by 87%. The second application (Fig. 2B) decreased the outward current by 86% and $I_M(-30)$ by 86%. The third (Fig. 2C) decreased the outward current by 82% and $I_M(-30)$ by 85%.

To compare the time course of the responses to cII-LHRH with GTP in the pipette, three tracings have been normalized and are shown in Fig. 4A. With GTP in the cell, successive responses to cII-LHRH had similar time courses.

With GDPβS in the pipette, two changes were noted with successive agonist applications, i.e., the time course of the response was slowed and the amplitude was decreased. Fig. 3 shows three successive responses to cII-LHRH with GDPβS in the pipette. During the first cII-LHRH application (Fig. 3A) the outward current decreased by 76% and $I_M(-30)$ by 67%. During the second cII-LHRH application (Fig. 3B) the outward current decreased by 68% and $I_M(-30)$ by 57%. The outward current dropped by only 45% and $I_M(-30)$ by 33% during the third cII-LHRH application (Fig. 3C).

Whereas GDPβS decreased the amplitude of the responses, it increased the time constant of the responses. Normalization of consecutive tracings (Fig. 4B) revealed a significant slowing of the successive responses to cII-LHRH when GDPβS was included in the recording electrode. In this cell the T_{25-75} increased from 5.8 sec (Fig. 4B, *trace a*) to 10.4 sec (Fig. 4B, *trace b*) to 14.8 sec (Fig. 4B, *trace c*).

To quantify the effects of the agonists, the amplitudes of $I_M(-30)$ before agonist application, at the peak of inhibition by agonist, and after washout of agonist were measured. The percentage of inhibition of $I_M(-30)$ for each application was calculated. The T_{25-75} for the inhibition of the outward current

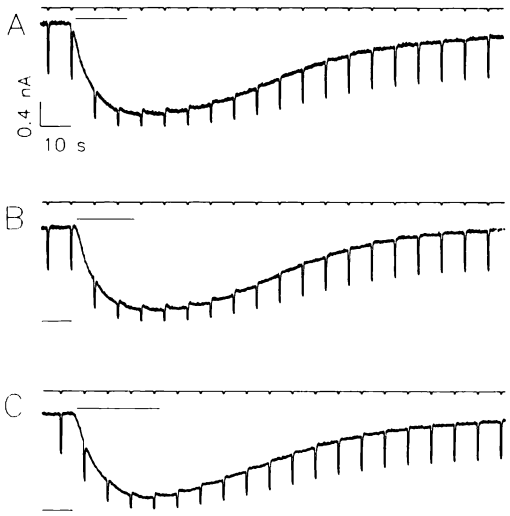

Fig. 2. Three consecutive responses to cII-LHRH with GTP (400 μM) in the pipette. *Top tracing of each panel*, membrane potential held at −30 mV and stepped to −50 mV for 500 msec every 8 sec. *Bottom tracing of each panel*, resultant membrane current. *Bar above the current trace*, cII-LHRH (300 nM) application. *Bar below the current trace*, 0 current level. A, The first application of cII-LHRH was applied 270 sec after the patch was broken and resulted in a decrease of outward current from 1750 pA to 290 pA and of $I_M(-30)$ from 510 pA to 65 pA. B, The second application, applied at 460 sec, decreased the outward current from 1620 to 230 pA and $I_M(-30)$ from 360 to 50 pA. C, The third cII-LHRH application, at 670 sec, decreased the outward current from 1650 to 290 pA and $I_M(-30)$ from 325 to 50 pA.

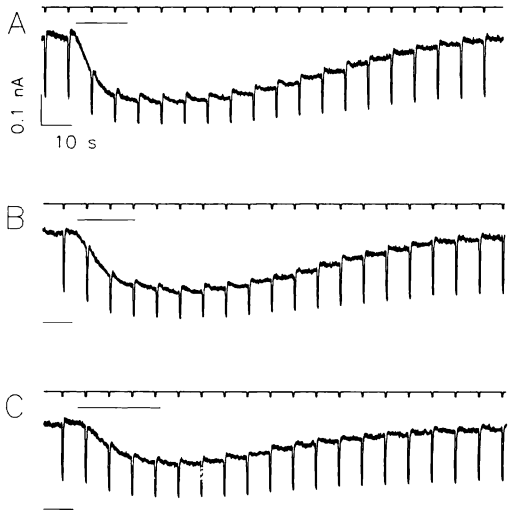

Fig. 3. Three consecutive responses to cII-LHRH with GDPβS (360 μM) and GTP (40 μM) in the pipette. *Top tracing of each panel*, membrane potential held at −30 mV and stepped to −50 mV for 500 msec every 8 sec as indicated. *Bottom tracing of each panel*, membrane current. *Bar above the current trace*, cII-LHRH (300 nM) application. *Bar below the current trace*, 0 current level. A, The first application of cII-LHRH was applied 260 sec after the patch was broken and resulted in a decrease of outward current from 305 pA to 75 pA and of $I_M(-30)$ from 150 pA to 50 pA. B, The second application, applied at 460 sec, decreased the outward current from 300 pA to 95 pA and $I_M(-30)$ from 150 pA to 65 pA. C, The third cII-LHRH application, at 850 sec, decreased the outward current from 265 pA to 145 pA and $I_M(-30)$ from 120 pA to 80 pA. The response to cII-LHRH was smaller and slower with each successive application.

at −30 mV was also measured. Results from a number of cells with cII-LHRH are presented in Fig. 5.

Fig. 5A shows the change in the magnitude of I_M just before agonist application and at the peak of inhibition by agonist. There was a slow decrease in the amplitude of I_M with time, but the degree of this run-down was not different with GTP or GDPβS intracellularly. The current at the peak of inhibition by agonist remained at a similar level in response to successive agonist applications with GTP in the pipette. With GDPβS in the cell, there was not as much inhibition of the current with successive agonist applications.

"Over-recovery" of I_M has been observed occasionally after agonist application. Over-recovery is observed as an increase of I_M over the predrug level after washout of agonist. The amplitude of I_M after washout of agonist is plotted in Fig. 5B. There was, on average, not a significant amount of over-recovery or a significant difference between the degree of recovery observed with either GTP or GDPβS in the pipette. We frequently observe over-recovery when drugs are applied for longer times or when the intracellular solution contains lower concentrations of calcium buffer.

Based on the data in Fig. 5A, the percentage of inhibition of $I_M(-30)$ with successive agonist applications has been calculated and is plotted in Fig. 5C. With GTP in the pipette, consecutive applications of agonist resulted in similar degrees of inhibition of $I_M(-30)$. GDPβS intracellularly decreased the ability of the agonist to inhibit $I_M(-30)$ with successive agonist applications.

To study the effects of GDPβS on the kinetics of the responses, the effects of the agonists on the rate of decrease of the holding current at −30 mV have been measured (Fig. 5D).

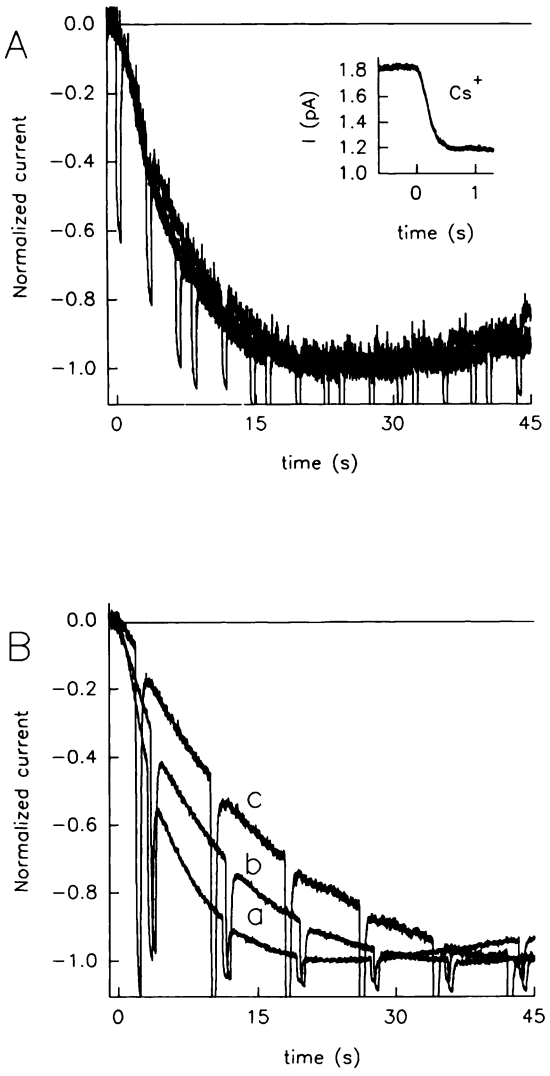

Fig. 4. Comparison of successive responses to cII-LHRH with GTP or GDPβS applied intracellularly. A, The amplitudes of three current tracings have been normalized with respect to outward current amplitude. The base-line current at −30 mV before drug application was set to 0 and the peak inhibition to −1. GTP was present at 400 μM in the recording electrode. The time courses of the responses are comparable. *Inset*, change in outward current at −30 mV in response to extracellular application of Cs^+. The extracellular solution was changed from one containing 2.4 mM KCl and 118 mM NaCl to one containing 20 mM CsCl and 103 mM NaCl. The T_{25-75} of the single-cell superfusion system was 175 msec, much faster than the time course of the responses to cII-LHRH. B, Three successive responses (*traces a*, *b*, and *c*) to cII-LHRH (300 nM) with 360 μM GDPβS and 40 μM GTP in the recording electrode have been normalized. This clearly illustrates the slowing of the time course of the responses to cII-LHRH in the presence of intracellular GDPβS.

The decrease in outward current correlates well with the decrease in $I_M(-30)$ (Fig. 1, B and C). With GTP in the cell, the T_{25-75} increased slightly with consecutive agonist applications. Larger increases in the T_{25-75} were observed with GDPβS in the pipette.

To test the possibility that GDPβS might have different effects on the responses to different agonists, similar experiments were conducted with muscarine and SP as agonists. The response to muscarine (10 μM) was examined in 15 cells with GDPβS intracellularly, and the response to SP (1 μM) was measured in seven cells with GDPβS intracellularly. The effects of GDPβS on the responses to muscarine and SP were the same as the effects of GDPβS on the responses to cII-LHRH.

An analysis of variance was conducted to determine the statistical significance of these findings. The analysis was run with the presence or absence of intracellular GDPβS as a between-subjects factor and agonist application number as a within-subjects factor (or repeated measure). The statistical significance of the effects of GDPβS alone and of application number alone and the significance of the interaction between GDPβS and application number were examined. The results of the analysis were similar for each of the agonists and can be summarized as follows. The level of $I_M(-30)$ before agonist application (Fig. 5A) and the level of $I_M(-30)$ after washout of agonist (Fig. 5B) displayed a significant run-down with application number. These parameters, which reflect the basal level of I_M, were not significantly affected by GDPβS. In no case did GDPβS alone show a significant effect on any of the measured parameters in the absence of an interaction with application number; the significant effects of GDPβS depended on successive agonist applications. There were significant interactions between GDPβS and application number on $I_M(-30)$ at the peak of agonist effect (Fig. 5A), the percentage of inhibition of $I_M(-30)$ (Fig. 5C), and the T_{25-75} for inhibition of outward current (Fig. 5D). Thus, we conclude that the effects of GDPβS on responses to cII-LHRH, muscarine, or SP are 1) to decrease the ability of successive agonist applications to inhibit I_M and 2) to slow the time course of successive responses.

The manipulation of the intracellular constituents depends upon diffusion of the electrode contents into the cell. To be sure that adequate time was allowed between breaking the membrane patch and applying the agonists, we examined the effect of GDPβS on the response to cII-LHRH in three cells about 14 min after breaking the patch. The results obtained in these three cells were the same as the results obtained from cells exposed to agonist at earlier times (Fig. 5).

In the experiments with GDPβS, the [GTP] was lowered to maintain a total guanine nucleotide concentration of 400 μM. To assure that the effects described were due to the addition of GDPβS and not due to the decrease in [GTP], additional controls were conducted with 40 μM GTP in the pipette for each of the agonists. An analysis of variance comparing the cells with 40 μM GTP and the cells with 400 μM GTP found no significant effects of lowering the GTP concentration to 40 μM.

Does GDPβS affect desensitization? Although homologous desensitization to agonists occurs in these cells, the controls with GTP indicate that the progressive decrease in I_M with consecutive agonist applications cannot be accounted for by the degree of desensitization normally observed. But, the results might be explained by an enhancement of desensitization with GDPβS intracellularly. Desensitization to SP was studied with GDPβS in the cell (Fig. 6). Desensitization was examined in five cells with GDPβS intracellularly (three with 360 μM GDPβS and 40 μM GTP and two with 320 μM GDPβS and 80 μM GTP). During a sustained application of SP (1 μM), the inhibition of I_M desensitized to 85 ± 7% of the predrug level at a rate of 0.93 ± 0.2%/sec. In cells containing 400 μM GTP, the average degree of desensitization was 100 ± 7% and the rate was 1.3 ± 0.2%/sec (17). So, the effects of GDPβS cannot be explained as an enhancement of desensitization.

Kinetic modeling. To aid in the interpretation of our findings, we have utilized a kinetic model of receptor-G protein

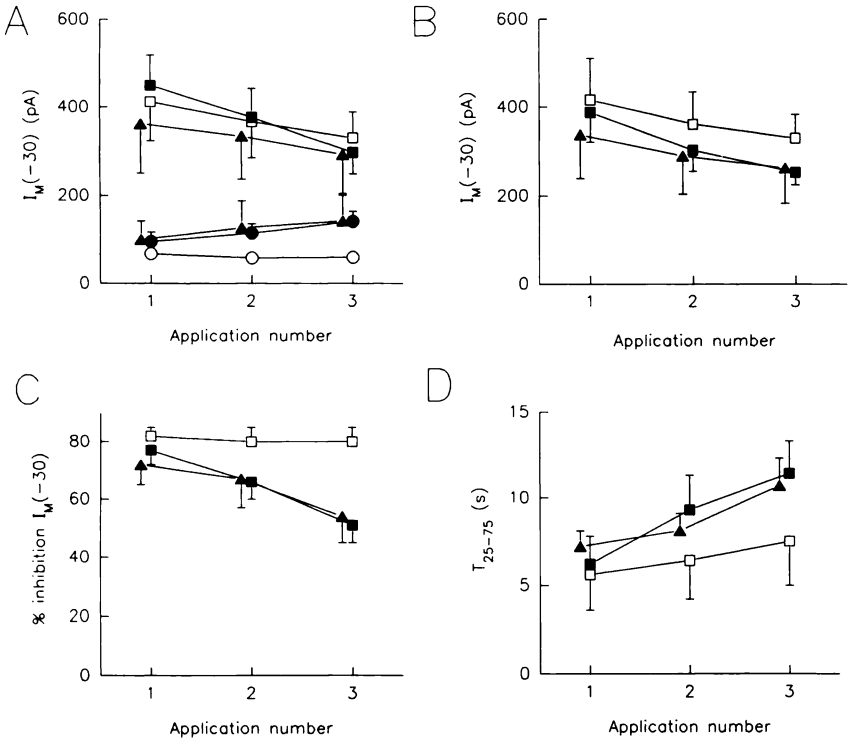

Fig. 5. Comparison of the magnitudes and time courses of successive responses to cII-LHRH (300 nM) with GTP or GDPβS intracellularly. □, ○, GTP (400 μM) (n = 9). ■, ●, ▲, GDPβS (360 or 353 μM with 40 or 47 μM GTP). For eight cells with GDPβS (■, ●), the first cII-LHRH application began, on average, 180 sec, the second 350 sec, and the third 535 sec after the patch was broken. For three other cells with GDPβS (▲), the first cII-LHRH application began 820 sec, the second 1025 sec, and the third 1215 sec after the patch was broken. For clarity, the *triangles* have been offset slightly to the left. *Bars*, standard error. A, Magnitude of $I_M(-30)$ just before (□ GTP; ■, ▲, GDPβS) and at the peak of the response (○, GTP; ●, ▲, GDPβS) after successive cII-LHRH applications. B, Level of $I_M(-30)$ (□, GTP; ■, ▲, GDPβS) upon recovery from each of the three consecutive cII-LHRH applications. C, Percentage of inhibition of $I_M(-30)$ (□, GTP; ■, ▲, GDPβS) for each application. D, Change in T_{25-75} (□, GTP; ■, ▲, GDPβS) with consecutive cII-LHRH applications.

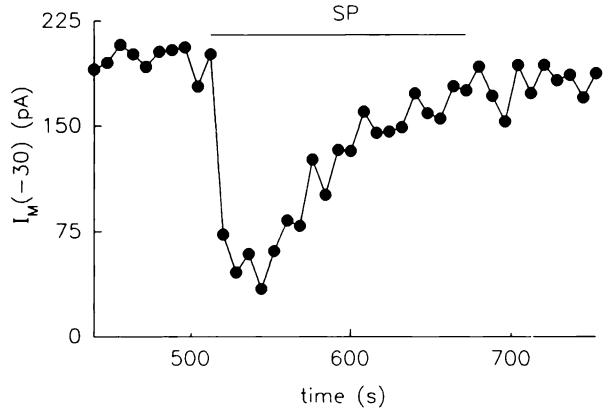

Fig. 6. Desensitization to SP with GDPβS intracellularly. SP (1 μM) application was begun about 205 sec after the patch was broken and continued for 160 sec. The pipette contained 360 μM GDPβS and 40 μM GTP. In the persistent presence of SP, the decrease in current waned.

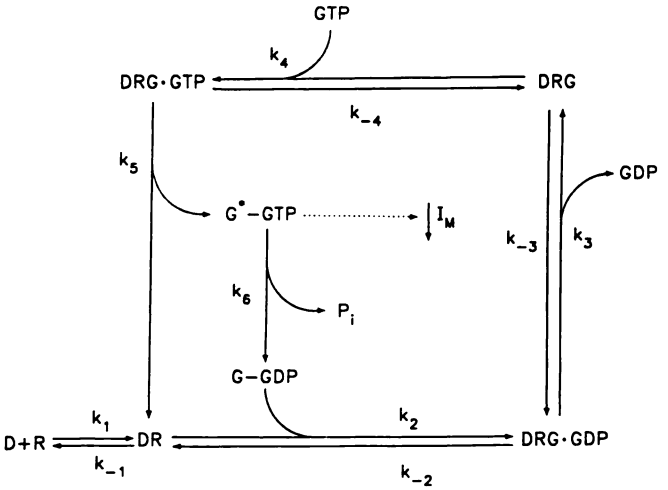

Fig. 7. Schematic diagram of the pathways described by the model of Thomsen and co-workers (20, 21). *D*, Drug; *R*, receptor; *G-GDP*, GDP-bound G protein; *G-GTP*, GTP-bound G protein; *G*-GTP*, active form of G protein. The degree of inhibition of I_M should be positively correlated with the amount of G*-GTP formed, although whether G*-GTP acts directly on the M channel or through some other pathway is unknown. Rate constants (21) were set to $k_1 = 5 \times 10^6$ M^{-1} sec^{-1}, $k_{-1} = 0.5$ sec^{-1}, $k_2 = 1 \times 10^8$ M^{-1} sec^{-1}, $k_{-2} = 0.1$ sec^{-1}, $k_3 = 5.0$ sec^{-1}, $k_{-3} = 1 \times 10^{-4}$ sec^{-1}, $k_4 = 5 \times 10^6$ M^{-1} sec^{-1}, $k_{-4} = 1.0$ sec^{-1}, $k_5 = 0.1$ sec^{-1}, and $k_6 = 2$ sec^{-1}. Starting concentrations were [D] = 10 μM, [GDP] = 10 μM, and [GTP] = 10 μM.

interactions recently developed by Thomsen and co-workers (20, 21). The model incorporates the pathways outlined in Fig. 7. The differential equations describing the model are presented in Ref. 20.

The utility of the model was to reveal how changes in the components would be expected to affect the magnitude and time course of the accumulation of active G proteins, designated G*-GTP in the model. The primary components of the model are drug, receptor, and G protein. Given the data described above, we presumed that GDPβS was having some cumulative effect, from application to application, on one of these components. Because the amount of drug was controlled, we were interested in determining whether the effects of GDPβS could be accounted for by changes in the number of available G proteins or might be accounted for by changes in receptors from one application to the next. The concentrations of drug, GDP, and GTP that we have used in the model are essentially in excess, and reasonable increases do not significantly alter the results from the model.

First, the effects of changing the amount of available G protein on the kinetics of the responses were examined. Simulations were run with different starting concentrations of G-GDP, which is equivalent to decreasing the G protein pool. Decreasing the amount of available G proteins decreases both the rate at which G*-GTP is formed (Fig. 8A) and the amount of G*-GTP formed (Fig. 8B).

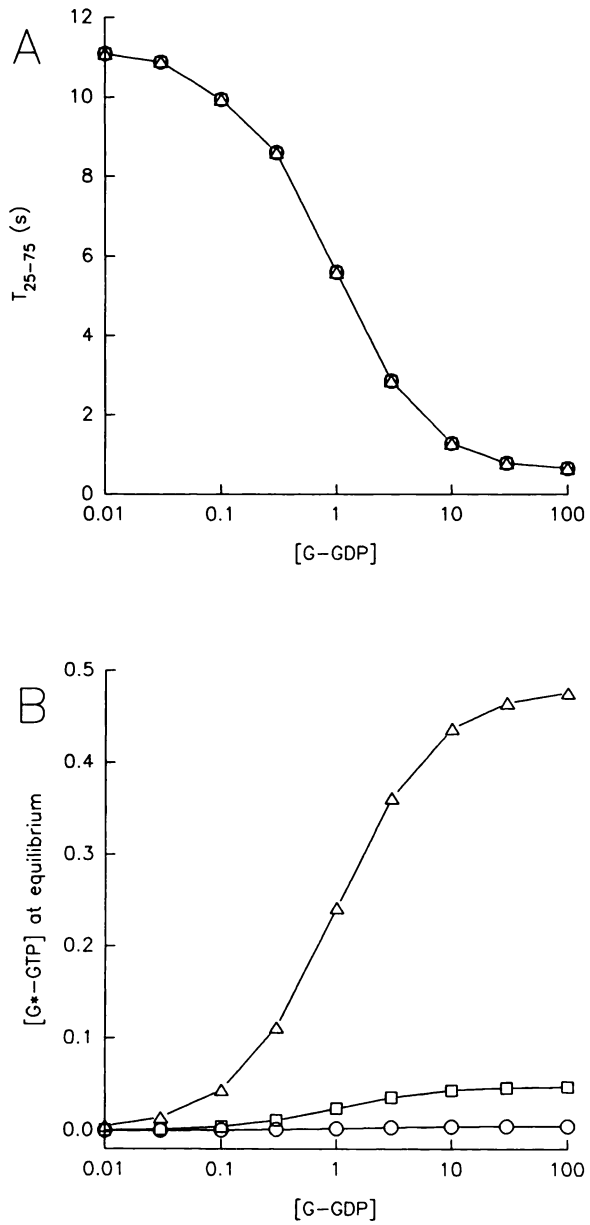

Fig. 8. Changes in the kinetics of the accumulation of G*-GTP in response to changes in the amount of available G proteins or the amount of available receptors, as predicted by the model of Fig. 7. The amount of available G-GDP was varied between 10 pM and 100 nM for receptor concentrations of 0.1 pM (○), 1 pM, (□), and 10 pM (△). A, Effects of changing the concentrations of G-GDP and R on the time course of accumulation of G*-GTP. The time for the concentration of G*-GTP to go from 25 to 75% of its equilibrium value was calculated. Note that all three curves are superimposed. B, Effects of changing concentrations of G-GDP and R on the equilibrium concentration of G*-GTP (in pM). Increasing either G-GDP or R results in an increased production of G*-GTP.

Second, the effect of reducing receptor number was simulated by decreasing the starting value of R. Decreasing the number of receptors decreases the amount of G*-GTP formed (Fig. 8B) but does not affect the time course of the increase (Fig. 8A).

Discussion

The dose-response curves for SP and cII-LHRH shown here are comparable to those reported for SP in bullfrog intact sympathetic ganglia (14) and for cII-LHRH in bullfrog dissociated sympathetic neurons (22). The muscarine effects at 10^{-6} M and above are similar to those reported in rat intact or dissociated superior cervical ganglion cells (23) and bullfrog intact sympathetic neurons (14), but the bullfrog dissociated neurons are slightly more responsive at lower concentrations of muscarine.

Previous studies have shown that GDPβS inhibits G protein-dependent phenomena (2–9). Our results appear to be consistent with other studies involving I_M. Most of these studies have simply added GDPβS to the pipette and seen decreased responses to agonists. If one assumes an endogenous GTP level of 25–50 μM (6), the actual ratios of GDPβS to GTP in these other experiments are comparable to the ratios used here. Previous studies have shown that intracellular GDPβS decreases the ability of ATP, LHRH, muscarine, and SP to inhibit I_M (2–4, 8).

The effects of GDPβS on the dose-response curves provide support for the concept that, at maximal doses of agonist, more G proteins are activated than are needed to produce maximal effects (24, 25). At maximal doses of agonist, inhibition of G proteins by GDPβS is not great enough to reduce the amount of active G protein below the level required to produce a maximal inhibition of I_M. The change in activated G proteins is thus invisible to our physiological measure. In the middle of the dose-response curves, changes in the number of activated G proteins should be, and were, evident as a decreased responsiveness with GDPβS.

A progressively increasing effect of GDPβS like that seen here might also be observed if the diffusion of GDPβS into the cell was the rate-limiting factor in the effectiveness of GDPβS. This alternative is unlikely for several reasons. First, relatively large electrodes were used for the recordings, which should enhance the diffusion of GDPβS from the pipette into the cell. Second, the possibility of slow diffusion was tested empirically. The results after 3 min were the same as if 14 min had been allowed after the patch was broken. Third, even if the diffusion of GDPβS was not complete, the ratio of GDPβS to GTP should be constant. Finally, from previous experiments, it is possible to get a decrease in successive responses to one agonist in the presence of GDPβS, yet subsequently observe large responses to the other agonists (10). Thus, the critical factor for the use-dependent effects of GDPβS appears to be receptor activation and not diffusion of GDPβS into the cell.

The kinetic model used here was developed to describe α_2-adrenergic modulation of adenylate cyclase. Based on present knowledge, it seems reasonable to assume that similar components, i.e., drug, receptor, and G protein, react to produce an active G protein species that can decrease I_M in sympathetic neurons. It is not as certain whether the rate constants that govern these processes in sympathetic neurons are similar. Furthermore, it has not been established whether G*-GTP acts directly on the M channel or through some other process. Given these latter two uncertainties, the results from the modeling cannot be considered to be quantitatively accurate; however, the relative changes in the kinetics and magnitude of the accumulation of G*-GTP should be reasonable. If we accept the validity of the model within these limits, we are provided with several insights regarding the receptor-mediated inhibition of I_M in sympathetic neurons and regarding the mechanism of action of GDPβS.

The available data do not support an action of GDPβS at either the receptor or the M channel. As the model shows, an action purely at the receptor level would decrease the magnitude of the effect of the agonist but would not change the time course of the response to a maximal dose of agonist. Additionally, GDPβS does not enhance receptor desensitization. If GDPβS acted at the M channel, it should affect successive agonist responses independently of the receptor activated. Previous studies have shown that GDPβS can exert a certain amount of receptor selectivity. For example, it is possible to decrease the effectiveness of successive applications of SP by putting GDPβS in the cell, yet still obtain a normal response to cII-LHRH (10). If GDPβS acted at the M channel, the responses to all agonists would be blocked to the same degree.

If GDPβS binds irreversibly to activated G proteins in competition with GTP and decreases the available G protein pool, both a slower and a smaller response is predicted by the model. This is consistent with the experimental observations. Certainly, more complicated explanations can be given. For example, GDPβS may be affecting the kinetics of the reactions, GDPβS may be altering the coupling between the G protein and some subsequent step, or GDPβS may be acting at multiple points in the pathway.

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