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SYNERGISTIC ACTIVATION BY SEROTONIN AND GTP ANALOGUE AND INHIBITION BY PHORBOL ESTER OF CYCLIC Ca^{2+} RISES IN HAMSTER EGGS

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SUMMARY

1. Synergistic activation of a GTP-binding protein (G protein) by external serotonin (5-hydroxytryptamine, 5-HT) and internally applied guanosine-5′-*O*-(3-thiotriphosphate (GTPγS) in hamster eggs was demonstrated by the facilitation of repetitive increases in cytoplasmic Ca^{2+} as measured by their associated hyperpolarizing responses (HRs) and by aequorin luminescence.

2. Rapid application of 70 nM-5-HT caused a single HR of 10–12 s duration and with a delay of 80 s. The critical concentration of 5-HT to cause an HR was 50 nM.

3. With 10 μM-5-HT four to six HRs were often elicited with a delay to the first HR of 8–30 s. HRs disappeared after prolonged or repeated application of 5-HT, indicating an apparent desensitization.

4. 5-HT-induced HRs were completely inhibited by the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (TPA) (100 nM). Conversely, the PKC inhibitor sphingosine (2 μM) enhanced the series of HRs by shortening the delay to the first HR (3–9 s) and by causing more HRs.

5. Ionophoretic injection of GTPγS into the egg usually produced a large HR with a delay of 120–240 s followed by a series of much smaller HRs. When 5-HT was applied within 1 min of injection of GTPγS, 70 nM-5-HT induced a number of large HRs and even 1 nM-5-HT could induce HR(s). In contrast, when 5-HT was applied after the size of GTPγS-induced HRs had declined, as much as 10 μM-5-HT could only elicit a single large HR. Thus, GTPγS apparently caused a sensitization and then a desensitization of the action of 5-HT.

6. GTPγS-induced Ca^{2+} transients were facilitated when injected in the presence of 5-HT concentrations as low as 0·1 nM. The time delay to the first HR was 65 s in 0·1 nM-5-HT or 4 s in 100 nM-5-HT whereas it was 170 s without 5-HT (mean values). The magnitude as well as frequency of HRs succeeding the first HR was enhanced by 5-HT at concentrations above 0·01 nM.

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7. TPA (100 nM) blocked the GTPγS-plus-5-HT-potentiated HRs after the first four to five HRs. Sphingosine (2 μM) augmented the series of HRs.

8. 5-HT_2 receptor antagonists, cyproheptadine (1 μM), spiperone (10 μM) and ketanserin (10 μM), inhibited the facilitation of GTPγS-induced HRs by 10 nM-5-HT and blocked HRs caused by 1 μM-5-HT alone.

9. It is concluded that G protein that stimulates phosphoinositide (PI) hydrolysis leading to inositol 1,4,5-trisphosphate production and repetitive Ca^{2+} transients is activated by a 5-HT-bound 5-HT_2 receptor in such a way that GTP–G protein binding is accelerated. On the other limb of PI signalling system, the activation of PKC seems to operate as part of a negative feedback loop which is partly responsible for desensitization in the agonist-induced repetitive Ca^{2+} transients.

INTRODUCTION

A variety of cells respond to agonists with oscillatory rises in the intracellular calcium ion concentration $[Ca^{2+}]_i$, as measured directly or inferred from indirect measurement of membrane potential or current (see review by Berridge, Cobbold & Cuthbertson, 1988). In many cases, oscillatory $[Ca^{2+}]_i$ rises are induced by agonists known to stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) such as serotonin in blowfly salivary gland (Rapp & Berridge, 1981), noradrenaline and vasopressin in hepatocytes (Woods, Cuthbertson & Cobbold, 1987*a*), histamine in HeLa cells (Sauvé, Simmoneau, Parent, Monette & Roy, 1987) and endothelial cells (Jacob, Merritt, Hallam & Rink, 1988) and acetylcholine in *Xenopus* oocytes (Kusano, Miledi & Stinnakre, 1982) and lacrimal gland cells (Evans & Marty, 1986). A possible mechanism for agonist-induced $[Ca^{2+}]_i$ oscillation is that agonist–receptor binding activates a G protein that stimulates PIP_2 phosphodiesterase, and a product of PIP_2 hydrolysis, inositol 1,4,5-trisphosphate (IP_3), causes repetitive releases of Ca^{2+} from intracellular stores (Berridge, Cobbold & Cuthbertson, 1988; Berridge & Galione, 1988; Rink & Jacob, 1989).

Fertilized mammalian eggs exibit repetitive Ca^{2+} rises, as inferred from Ca^{2+}-dependent hyperpolarizations in hamster eggs (Miyazaki & Igusa, 1981) or measured with Ca^{2+}-dependent luminescent protein aequorin in mouse eggs (Cuthbertson, Whittingham & Cobbold, 1981). In hamster eggs periodic Ca^{2+} transients have been demonstrated with Ca^{2+}-sensitive microelectrodes (Igusa & Miyazaki, 1986) and with aequorin (Miyazaki, Hashimoto, Yoshimoto, Kishimoto, Igusa & Hiramoto, 1986). A possible mechanism for the cyclic Ca^{2+} rises in fertilized hamster eggs has been proposed, based on a linkage of continuous Ca^{2+} influx to internal Ca^{2+} release (Igusa & Miyazaki, 1983). Repetitive Ca^{2+} transients are also induced in unfertilized hamster eggs by microinjecting the hydrolysis-resistant GTP analogue GTPγS (Miyazaki, 1988) or by continuous injection of IP_3 (Swann, Igusa & Miyazaki, 1989). Sperm-mediated Ca^{2+} rises as well as the GTPγS-induced Ca^{2+} transients are blocked by pre-injection of guanosine-5′-*O*-(2-thiodiphosphate) (GDPβS) (Miyazaki, 1988). These findings have led to the conclusion that repetitive Ca^{2+} rises in hamster eggs are mediated through the activation of a G protein that leads to cycles of IP_3-induced Ca^{2+} release (Miyazaki, 1989).

The hamster egg has some advantages for physiological analysis of cell signalling

that involves repetitive Ca^{2+} rises, because controlled microinjection of chemicals into the cell is feasible and because increases in $[Ca^{2+}]_i$ are easily monitored by simultaneous membrane potential recordings of the hyperpolarizations caused by a Ca^{2+}-activated K^+ conductance (Igusa & Miyazaki, 1986; Miyazaki, 1988). Recently, we have found that GTPγS-induced Ca^{2+} transients are inhibited by phorbol ester (Swann *et al.* 1989) and that 5-hydroxytryptamine (5-HT) causes repetitive hyperpolarizations (Katayama, Swann & Miyazaki, 1989). Thus, the hamster egg provides a useful model system to study how agonist–receptor interaction causes Ca^{2+} oscillations. In the present work we investigated the synergistic activation of a G protein by ligand (5-HT)–receptor binding and by an internally applied GTP analogue (GTPγS). Particular attention was focused on (1) measuring with high temporal resolution the facilitation of the reaction causing Ca^{2+} rises, and (2) analysing the effect of protein kinase C activity on the receptor–G protein-PIP_2 hydrolysis pathway.

METHODS

Eggs

Female golden hamsters were treated with an intraperitoneal injection of 20 i.u. pregnant mare's serum gonadotropin, followed 48 h later by 20 i.u. human chorionic gonadotropin (HCG) (Yanagimachi, 1969). The superovulated females were stunned and killed by dislocating the cervical vertebrae with a metal bar and mature eggs were collected from the oviducts 15·5 h after HCG injection. The surrounding cumulus cells were removed by treatment with 0·05% hyaluronidase (1–1·5 min at 22–24 °C) and the zona pellucida was dissolved with 0·07% trypsin (1 min) (Igusa & Miyazaki, 1983). Zona-free eggs were then transferred to a 0·4 ml drop of M2 medium (see below) in a 35 mm plastic Petri dish and covered with paraffin oil. The dish was pre-treated with poly-L-lysine (50 μg/ml) to make eggs stick to the bottom of the dish. The dish was mounted on a phase contrast inverted microscope stage heated to 31–33 °C.

Solutions and chemicals

A modified Krebs–Ringer solution (M2 medium) (Fulton & Whittingham, 1978) was used. The composition was (mM): NaCl, 94·6; KCl, 4·8; $CaCl_2$, 1·7; $MgSO_4$, 1·2; KH_2PO_4, 1·2; sodium lactate, 22; sodium pyruvate, 0·5; glucose, 5·6; $NaHCO_3$, 5·1; HEPES, 19·4, buffered at pH 7·4. Bovine serum albumin was added before use (4 mg/ml).

Serotonin (5-hydroxytryptamine, 5-HT) (Sigma), dopamine (3-hydroxytyramine) (Sigma), cyproheptadine (Sigma) and ketanserin (kindly provided by Kyowa Hakko Ltd, Tokyo) were dissolved in M2 medium, and spiperone (Jannsen), phorbol, 12-myristate 13-acetate (TPA) (Sigma) and sphingosine (Sigma) were dissolved in dimethyl sulphoxide. Stock solutions were stored at −70 or −20 °C, and they were diluted with M2 medium before use. The injection solution of guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) consisted of 20 mM-GTPγS (Boehringer Mannhein Biochemicals) and 20 mM-HEPES buffered at pH 7·0.

Electrophysiology and microinjection

A glass microelectrode filled with 4 M-potassium acetate was inserted into an egg. Electrode resistance ranged between 40 and 60 MΩ. In experiments with 5-HT application alone, constant current pulses of 0·1 nA and 0·3 s duration were applied every 5 s through the intracellular electrode by means of a bridge circuit, and a change in membrane conductance was monitored, based in the linear current–voltage relationship (Miyazaki & Igusa, 1982). Ca^{2+} transients were monitored on a pen recorder and an oscilloscope by the hyperpolarizing responses (HRs) caused by the Ca^{2+}-activated K^+ conductance (Igusa & Miyazaki, 1986).

For injecting GTPγS a second micropipette was inserted into the egg. Injection pipettes were broken at the tip and had resistances of 5–20 MΩ if filled with 3 M-KCl. GTPγS was injected ionophoretically with negative current pulses: 5 pulses of 5 nA and 1·5 s duration with an interval

of 0·5 s between pulses. Membrane conductance was monitored by injecting 0·5 nA pulses through the injection pipette at a desired period. All current injections were monitored by measuring the current passed to ground through a current–voltage converter.

Drug application

5-HT was rapidly applied to the experimental egg through a glass pipette: the tip of the pipette (inner diameter, *ca* 40 μm) was moved to about 150 μm away from the egg (egg diameter, 70 μm)

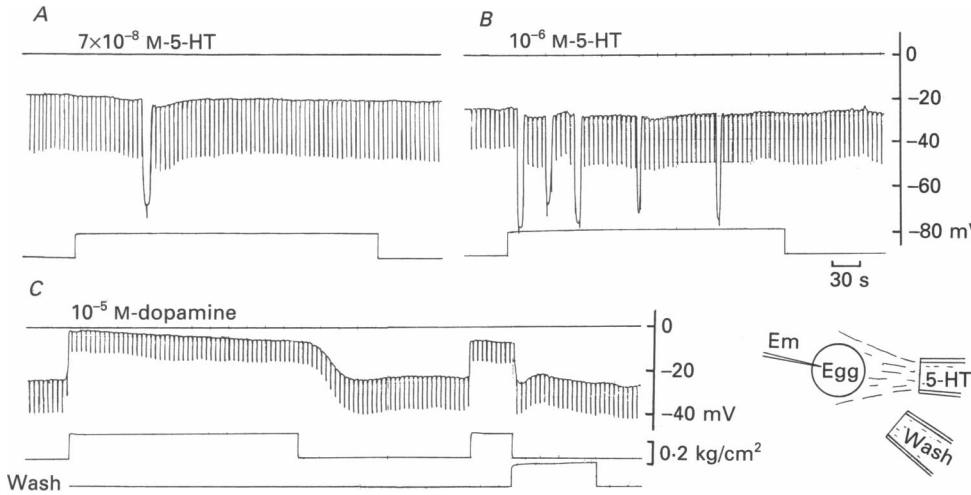

Fig. 1. Hyperpolarizing responses (HRs) induced by 5-HT (*A* and *B*) and depolarization caused by dopamine (*C*) in unfertilized hamster eggs. The solution containing the drug (indicated at the top of each record) was rapidly applied through a pipette by gas pressure, which is shown in the bottom trace. Inset illustrates the 'puffing method' (see Methods). Changes in membrane resistance were monitored by applying constant current pulses (see Methods). The scale for time and pressure is common in *A–C*.

and immediately the solution was ejected by gas pressure applied by an electronically controlled valve system (Medical System Corp., Great Neck, NY, USA) (see inset of Fig. 1). The solution was applied for 2–7 min under a constant pressure, the magnitude of which was converted to voltage and recorded simultaneously with the membrane potential. This 'puffing method' enabled us to instantaneously apply drugs at a desired concentration and to measure a time lag to each response. An evaluation of this method was shown by the application of dopamine (Fig. 1*C*): it induced a depolarization attaining a constant level within 1 s of turning the pressure on. As shown in Fig. 1*C*, the applied drug seemed to remain near the egg after turning the pressure off, but any possible residual effect could be removed by washing with a second pipette. When the effects of 5-HT antagonists, TPA or sphingosine were examined, eggs were placed in M2 medium containing these drugs before recording and they were puffed with the solution containing both 5-HT and these drugs during recording.

Experiments with aequorin

To investigate the spatial distribution of Ca^{2+} transients, aequorin was injected into the egg through a third micropipette 4–7 min before injection of GTPγS or application of 5-HT solution. The aequorin solution contained 9 mg/ml purified aequorin (gift from Dr Shimomura, Marine Biological Laboratory, Woods Hole), 100 μM-EGTA, 20 mM-KCl, and 7 mM-morpholino propanesulphonic acid (MOPS) buffered at pH 7·2. The solution was sucked up in the tip of a bevelled glass micropipette filled with silicon oil and then the tip was capped with salad oil. The aequorin solution (14–18 pl) was injected into an egg by pressure with a microinjector (Narishige, IM-4B,

Tokyo, Japan). Luminescence generated by the intracellular Ca^{2+}-aequorin reaction was visualized as light spots on the TV monitor screen using a supersensitive TV camera system (Hamamatsu Photonics, C1966-20, Hamamatsu, Japan) (for details of the photon-counting imaging see Miyazaki *et al.* 1986). Total light spots during a Ca^{2+} transient were accumulated by means of an image processor and then photographed.

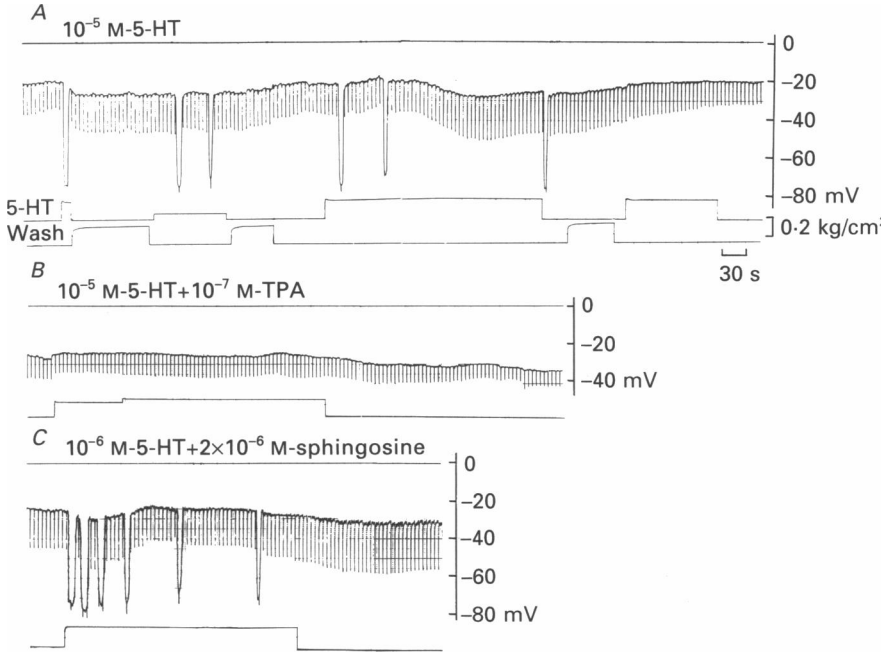

Fig. 2. *A*, repeated application of 10 μM-5-HT followed by a washing solution from a second pipette. *B* and *C*, inhibition of 5-HT-induced HRs by 100 nM-TPA (*B*) and facilitation of 5-HT-induced HRs by 2 μM-sphingosine (*C*). TPA or sphingosine was contained in both the bathing medium and the perfusion medium.

RESULTS

5-HT-induced Ca^{2+} transients

Rapid application of 70 nM-5-HT caused an HR with a substantial delay, as shown in Fig. 1*A*. The delay time, defined as the interval between the start of 5-HT application (the moment of turning pressure on) and the start of HR, was 80 ± 11 s (mean $\pm$ S.D., $n = 9$) for 70 nM-5-HT. Each HR appeared in an all-or-none fashion, having an approximately constant shape: about 50 mV in amplitude and 10–12 s in duration. Each HR was also associated with a decrease in the membrane resistance (a conductance increase). A small hyperpolarization followed HRs, but this was associated with a slight decrease in the membrane conductance (Fig. 1*A*). No significant depolarization was observed. Investigations using aequorin indicated that the 5-HT-induced HR corresponded to a transient increase of intracellular Ca^{2+} in the entire egg (not shown). With 70 nM-5-HT four out of five eggs (4/5 eggs) showed a single HR. One HR occurred in 1/4 eggs at 50 nM but no eggs responded at 10 nM

(0/4 eggs). Thus, the critical concentration of 5-HT to cause HRs was 50 nM. Another amine, dopamine, at more than 1 μM caused a sustained depolarization associated with a conductance increase (Fig. 1*C*), indicating that the 5-HT-induced Ca^{2+} transient is not a non-specific response to amines.

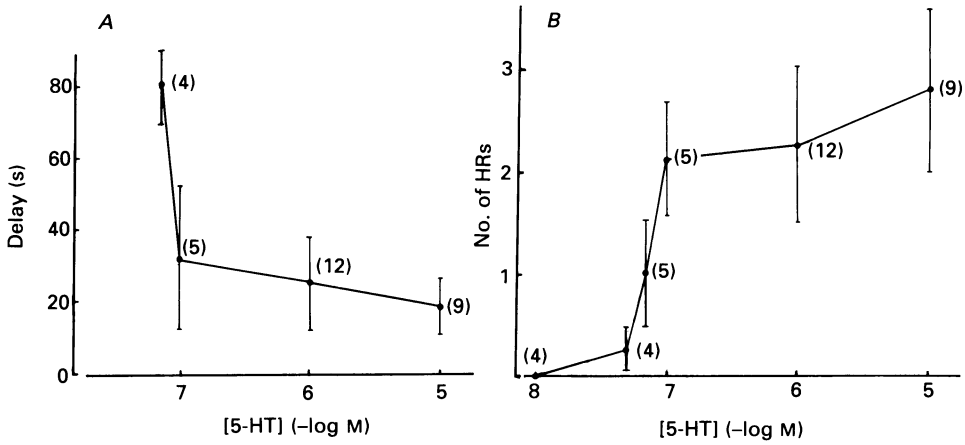

Fig. 3. The dose dependence of 5-HT-induced HRs. The delay time to the first HR (*A*) and the number of induced HRs (mean ± S.D.) (*B*) are plotted as a function of 5-HT concentration. The number of eggs examined is indicated in parenthesis. HR(s) occurred in 4/5 eggs at 70 nM and 1/4 eggs at 50 nM-5-HT, so that the mean ± S.D. of the number of HRs was calculated in all eggs examined but that of the delay time was calculated in only the four eggs that responded to 70 nM-5-HT.

At higher concentrations, 5-HT produced multiple HRs (Fig. 1*B*). The delay to the first response was remarkably shortened and it was 19 ± 8 s ($n = 9$) for 10 μM-5-HT (Fig. 2*A*). The minimum value was 8 s. The time lag to induce Ca^{2+} transients is significant, considering that the delay of dopamine-induced depolarization was 1 s or less (Fig. 1*C*). Figure 3 illustrates the dose dependence of the delay time and number of HRs. One or two HRs succeeded the first HR at an interval of about 30 s (Fig. 1*B*). A total four to six HRs were often generated by 10 μM-5-HT. Later HRs tended to be smaller in magnitude and shorter in duration and they occurred at longer intervals, eventually disappearing during prolonged application of 5-HT.

It is possible that 5-HT acts only as an initial trigger of a series of HRs. In Fig. 2*A* a short application of 10 μM-5-HT was followed by a washing solution from a second pipette. It was shown that the continuous presence of 5-HT is required for generating HRs. Figure 2*A* also shows that with repeated applications of 5-HT, there is longer delay before each HR and that eventually 5-HT fails to induce HRs. Disappearance of HRs upon prolonged or repeated application of 5-HT indicates an apparent desensitization in the 5-HT-induced Ca^{2+} transients.

The induction of Ca^{2+} transients by 10 μM-5-HT was completely inhibited by the PKC activator TPA (Fig. 2*B*). Eggs were incubated in 100 nM-TPA for about 10 min before application of 5-HT. No HRs occurred in all six eggs examined. Conversely, the pKC inhibitor sphingosine enhanced the 5-HT-induced Ca^{2+} transients as shown in Fig. 2*C*. The delay time to the first HR induced by 1 μM-5-HT in the presence of

2 μM-sphingosine was 5 ± 2 s (mean $\pm$ S.D., $n = 7$) whereas it was 26 ± 13 s ($n = 12$) for 1 μM-5-HT alone. The minimum value was 3 s. The number of induced HRs in sphingosine-treated eggs was $4{\cdot}1 \pm 1{\cdot}2$ whereas it was $2{\cdot}3 \pm 1{\cdot}5$ for 1 μM-5-HT alone. Three to four HRs appeared in the early period of 5-HT application at intervals of

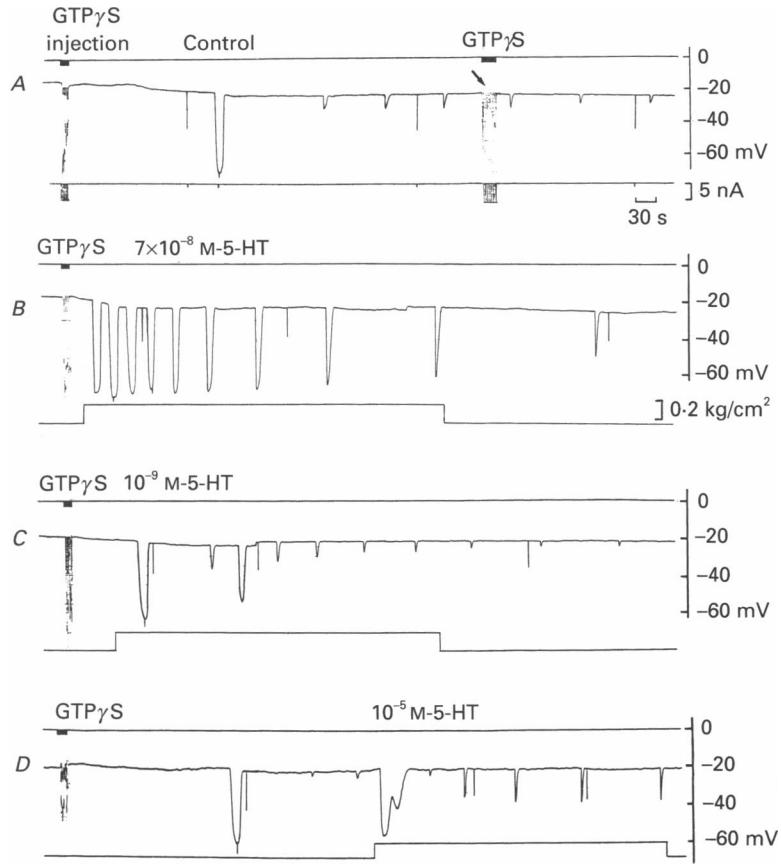

Fig. 4. Ionophoretic injection of GTPγS into an unfertilized hamster egg (*A*) and injection of GTPγS followed by the application of 5-HT (*B–D*). Full explanations are in the text. In *A*, the bottom trace shows current pulses for GTPγS injection (5 nA) and small pulses for monitoring the membrane resistance (0·5 nA). The arrow in *A* indicates additional injection of GTPγS. The trace of current pulses is omitted in *B–D* and in subsequent figures as well.

15–30 s and later HRs occurred with increasing intervals (Fig. 2*C*). These data suggest that PKC has an inhibitory effect on the 5-HT-induced Ca^{2+} transients.

Application of 5-HT after injection of GTPγS

Figure 4*A* shows HRs induced by microinjection of GTPγS into an egg with five pulses of 5 nA (see Methods). Usually, a large HR of about 40 mV in amplitude and 12 s duration was first generated with a delay of 120–240 s (170 ± 49 s, $n = 8$). The delay time for the GTPγS-induced response was defined as the interval between the

end of injection pulses and the start of the first HR. Then a series of much smaller HRs appeared after a pause of 2–3 min following the first HR, whilst the membrane resistance was little altered (Fig. 4*A*). Additional injection (arrow in Fig. 4*A*) did not compensate for the decline in the series of HRs, indicating an apparent densitization in GTPγS-induced Ca^{2+} transients. The small HRs occurred at fairly constant intervals of 45–90 s for 10–20 min. This pattern of HRs was obtained in 8/14 eggs. Another six eggs showed no HRs. With twenty injection pulses of 5 nA, 80% of eggs show a large HR followed by a series of much smaller HRs, 10% of eggs show only a large HR and 10% of eggs show no HRs (Swann *et al.* 1989). Therefore, we considered that the present method of five pulses of 5 nA is close to the critical injection current of GTPγS for inducing HRs.

In Fig. 4*B* and *C* 5-HT was applied 15 s (*B*) or 54 s (*C*) after the end of GTPγS injection pulses in the period before any large HR appears. Seventy nanomolar 5-HT, which is nearly the critical concentration for inducing an HR (Figs 1*A* and 3), dramatically produced a series of large HRs with a delay of 12 s after the start of 5-HT application (Fig. 4*B*). Six large HRs with a round top were generated at intervals of 20–40 s until a decline in the HR series began to occur about 4 min after GTPγS injection. Under these conditions even 1 nM-5-HT could evoke a large HR with a delay of 30 s (84 s after injection of GTPγS), although HRs declined rapidly (Fig. 4*C*). In this case a middle-sized HR occurred between smaller HRs. Thus, internal GTPγS apparently potentiated the action of 5-HT.

In Fig. 4*D* 5-HT was applied after a large HR and small HRs appeared, which is the period after desensitization of GTPγS-induced HRs has developed. Ten micromolar 5-HT, which normally produces four to six HRs (Figs 2*A* and 3*B*), evoked only one large HR followed by a smaller HR in fusion (Fig. 4*D*). Fused HRs were produced by 70 nM-5-HT as well (not shown). Subsequent HRs declined remarkably, although they occurred more frequently with greater amplitude in comparison with those caused by GTPγS alone. Thus, the effects of 5-HT were reduced when the GTPγS-induced response had densensitized.

Injection of GTPγS after application of 5-HT

In the following experiments eggs were placed in medium containing 5-HT, and GTPγS was injected 5–20 min later. As shown in Fig. 5, GTPγS-induced HRs were facilitated when injected in the presence of 5-HT at subthreshold concentrations for 5-HT-induced HRs. The facilitated HRs, however, always declined with time. Since HRs were variable in size, shape or duration, it was difficult to evaluate the nature of the Ca^{2+} transients from HRs alone. Therefore, the distribution and magnitude of intracellular Ca^{2+} rises were investigated with aequorin luminescence in relation to each HR (Figs 5*D* and 6). Large HRs with a round top (1 and 2 in Fig. 5*D*) corresponded to increases of intracellular Ca^{2+} in the entire egg (1 and 2 in Fig. 6) with the Ca^{2+} rise starting in wide area, not at a localized point. A propagating Ca^{2+} rise, as is seen upon fertilization of hamster eggs (Miyazaki *et al.* 1986), was not observed. Middle-sized HRs with a sharp top which were seen during the decline in an HR series (3, 4 and 5 in Fig. 5*D*) correlated with Ca^{2+} rises in a diffuse region of the egg (3, 4 and 5 in Fig. 6). The number of light spots or intensity of aequorin luminescence decreased in parallel with the decline in the size of HR. The HR series

reached a fairly constant level consisting of small HRs. These small HRs (6 and 8 in Fig. 5*D*) were due to smaller or partial increase in Ca^{2+} (6 and 8 in Fig. 6). All HRs were in phase with the increase in aequorin luminescence. Even if an HR was only a few millivolts (8 in Fig. 5*D*), a Ca^{2+} rise was easily recognized in a part of the egg (8 in Fig. 6) by comparing the light spots during an HR with those taken during the pause between small HRs (7 in Fig. 5*D* and Fig. 6). Thus, HRs were confirmed to be good indicator of Ca^{2+} transients.

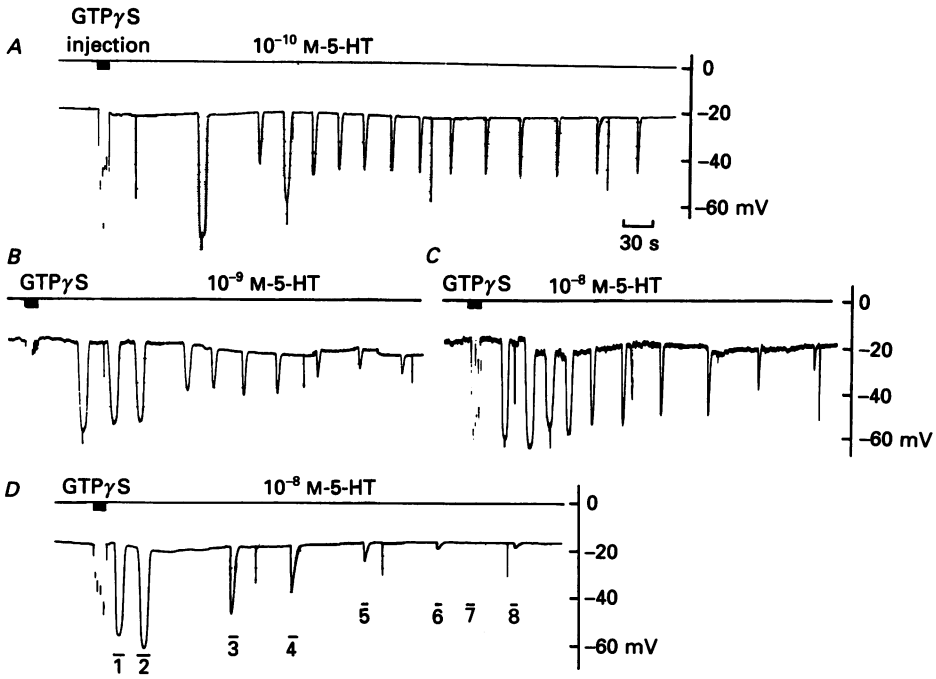

Fig. 5. Facilitation of GTPγS-induced HRs by 5-HT. GTPγS was injected in the continuous presence of 5-HT. In *D*, the egg was injected with aequorin, and each horizontal bar indicates the time for accumulation of aequorin luminescence shown in Fig. 6. The number of large HRs tended to be smaller in aequorin-injected eggs (compare *D* with *C*).

Facilitation of Ca^{2+} transients

The facilitation of GTPγS-induced Ca^{2+} transients by 5-HT was analysed by studying HRs. The probability of induction of HRs with five injection pulses of GTPγS was 6/7 in the presence of 10 pM-5-HT or 7/7 in 100 pM-5-HT in contrast to 8/14 in 5-HT-free medium. Dose-dependent facilitation by 5-HT (Figs 5 and 8*A*) was seen in: (1) shortening of the delay to the first HR, (2) increase in the number of large and middle-sized HRs, and (3) increase in the frequency of HRs. The following parameters were taken for the evaluation of facilitation: (1) delay – the time between the end of injection pulses and the start of the first HR; (2) number of large HRs – HRs which were more than 90% of the first HR in size and had round tops were

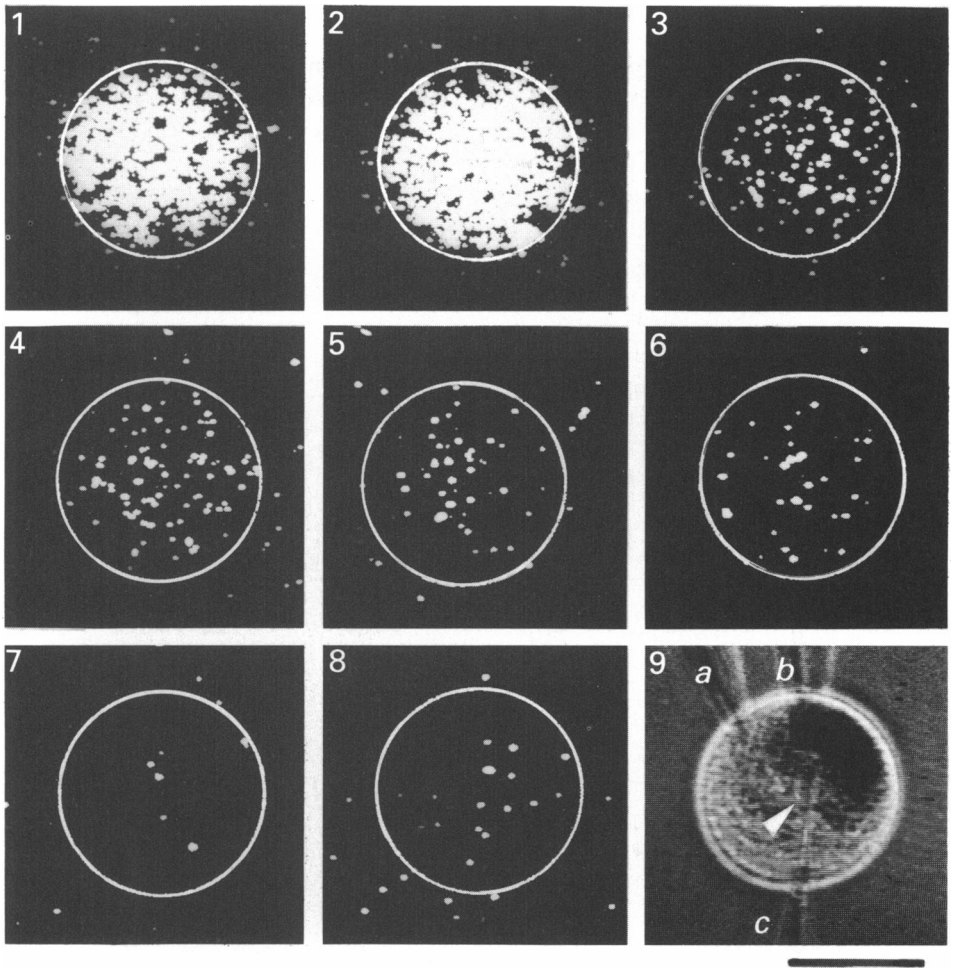

Fig. 6. Spatial distribution of aequorin luminesce during each Ca^{2+} transient upon injection of GTPγS in the presence of 10 nM-5-HT, recorded in the egg for Fig. 5*D*. All light spots on the TV monitor screen were accumulated during the period indicated by the bar with corresponding numbers in Fig. 5*D*. Photograph 7 shows background light level between two Ca^{2+} transients, accumulated for a time comparable to those of 6 and 8. The contour of the egg was traced with white ink, based on the bright-field image. Photograph 9 shows the egg in bright field with three micropipettes: *a*, recording electrode; *b*, injection pipette for GTPγS (the tip is indicated by the white arrow); *c*, injection pipette for aequorin. Scale bar, 50 μm.

defined as large HRs (corresponding to a Ca^{2+} rise in the entire egg); (3) number of HRs/5 min – the number of HRs which occurred within 5 min of the onset of the first HR was counted (including the first HR), irrespective of their size.

The dose dependence of the facilitation is demonstrated in Fig. 7. The critical effective concentration of 5-HT lay between 10 and 100 pM. The most remarkable change was seen in the delay time (Fig. 7*A*). The change was significant in 100 pM-5-HT (65 s in average, $n = 6$) and even more pronounced in 10 nM-5-HT (26 s,

$n = 12$). In 100 nM or more 5-HT, which was suprathreshold concentration for 5-HT-induced HRs, the first HR started during injection of GTPγS in 10/19 eggs (delay time was counted as zero), although averaged values ranged between 2·5 and 7·5 s. The number of large HRs increased with increasing the 5-HT concentration (Fig. 7*B*).

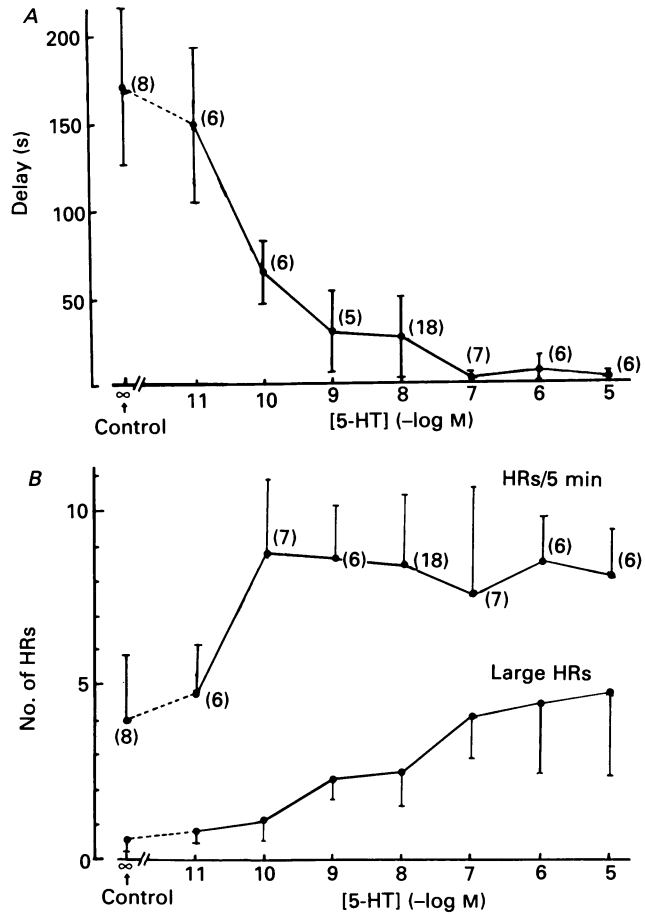

Fig. 7. The dose dependence of the facilitation of GTPγS-induced Ca^{2+} transients by 5-HT. The delay time to the first HR (*A*) and the number of large HRs and number of HRs in the first 5 min (*B*) were plotted as a function of 5-HT concentration. These parameters of HRs are explained in the text. For 0 and 10 pM-5-HT concentrations, the mean ± S.D. was calculated in the eggs that showed HRs. All eggs responded with HRs with more than 10 pM-5-HT, but accurate measurement of the delay time was missed in a few eggs in *A*, which were discarded. Numbers in parentheses are numbers of eggs examined.

The number of HRs in the first 5 min in the HR series increased from 4·0 ± 2·2 (mean ± S.D., $n = 8$) without 5-HT to 8·7 ± 2·1 ($n = 7$) in 100 nM-5-HT (Fig. 7*B*). The frequency of HRs appeared to be dose independent at higher 5-HT concentrations. The interval at which Ca^{2+} transients can repeatedly occur seems to be limited. Despite the similar frequency in HRs, a dose dependence was also seen in the

declining phase of the series of HRs in that the number of middle-sized HRs as well as large HRs was greater at higher 5-HT concentrations.

Effects of TPA and sphingosine

Figure 8*B* shows GTPγS-induced HRs in the presence of 100 nM-TPA together with 100 nM-5-HT. Compared with the control response in 100 nM-5-HT (Fig. 8*A*),

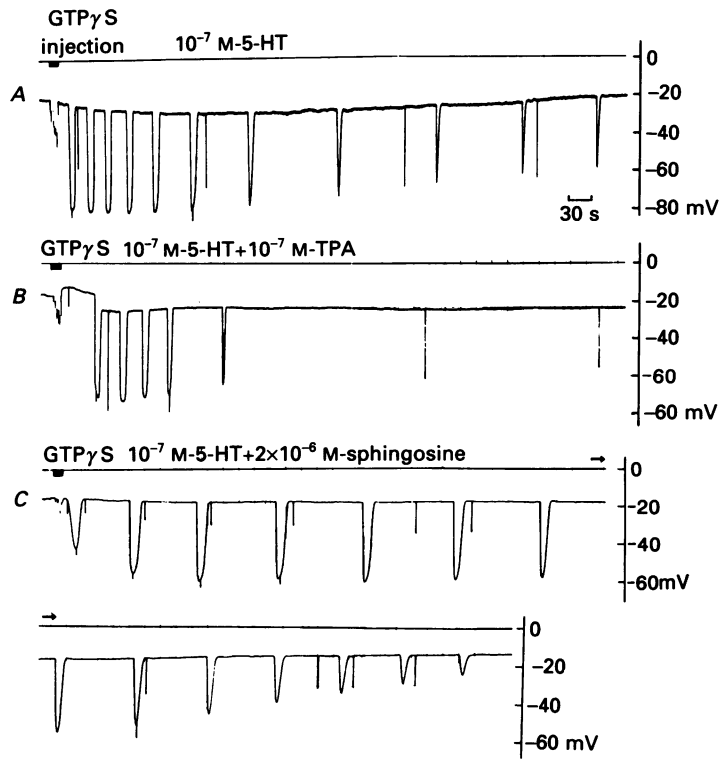

Fig. 8. GTPγS-induced HRs in the presence of 100 nM-5-HT (*A*), 100 nM-5-HT + 100 nM-TPA (*B*) and 100 nM-5-HT + 2 μM-sphingosine (*C*). In *C*, the upper record continues to the lower record.

the first HR occurred with a long delay (76 ± 45 s, $n = 4$ compared to the control 4 ± 5 s, $n = 7$). Early large HRs were similarly induced, but after the first four to five HRs further HRs were blocked in 5/5 eggs (see Fig. 8*B*). In the presence of 2 μM-sphingosine and 100 nM-5-HT, the number of large HRs increased from 4·1 ± 1·2 ($n = 7$, without sphingosine) to 5·9 ± 1·4 ($n = 11$), but the number of HRs in the first 5 min decreased (from 7·6 ± 3·2, $n = 7$ to 5·0 ± 1·8, $n = 11$). As shown in Fig. 8*C*, large HRs having a slightly longer duration (12–16 s) than usual HRs were repeated with a longer interval of 100–120 s. This series of HRs is similar to sperm-mediated HRs (Igusa & Miyazaki, 1983). However, even these GTPγS-induced HRs declined in amplitude within 10 min.

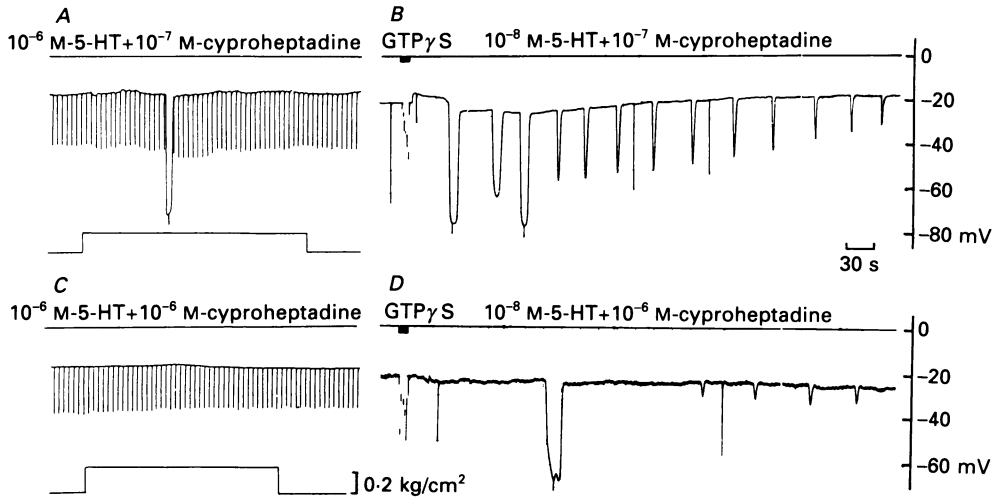

Fig. 9. Inhibitory effects of cyproheptadine on 5-HT-induced HRs (*A* and *C*) or on GTPγS-plus-5-HT-potentiated HRs (*B* and *D*). Cyproheptadine was contained in both the bathing medium and the perfusion medium in *A* and *C* and contained in the bathing medium together with 5-HT in *B* and *D*. Records were obtained from different eggs.

TABLE 1. GTPγS-induced HRs in the presence of 5-HT and its antagonists

	Delay (s)	Large HRs	HRs/5 min	No. of eggs
GTPγS alone	170±49	0·6±0·5	4·0±·1·9	8
GTPγS + 10 nM-5-HT	26±23	2·6±1·1	8·4±2·0	18
+ cyproheptadine				
0·1 μM	44±28	2·2±1·1	9·0±2·2	5
1 μM	132±24	0·8±0·5	4·3±0·5	4
+ spiperone				
1 μM	32±21	2·5±1·4	7·9±2·2	8
10 μM	212±45	0·8±0·5	2·0±0·0	4
+ ketanserin				
1 μM	76±36	2·0±0·7	8·0±1·4	5
10 μM	164±67	1·0±0·0	3·0±1·2	5

Measurement and calculation of three parameters are the same as in Fig. 7. Values are means ± S.D.

Inhibition by 5-HT antagonists

Figure 9*A* shows the response to 1 μM-5-HT plus 0·1 μM-cyproheptadine. A partial inhibition is recognized when Fig. 9*A* is compared with Fig. 1*B*. A complete block of 5-HT-induced HRs was observed in 3/4 eggs with 1 μM-cyproheptadine (Fig. 9*C*). In similar experiments, HRs occurred in 4/5 eggs for 1 μM-spiperone and in 5/5 eggs for 1 μM-ketanserin, but HRs were completely inhibited in 4/4 eggs for 10 μM-spiperone and in 3/4 eggs for 10 μM-ketanserin.

Figure 9*B* shows GTPγS-induced HRs in 0·1 μM-cyproheptadine together with 10 nM-5-HT. The responses were similar to those in Fig. 5*C*. With 1 μM-cyproheptadine the facilitatory effect of 5-HT on GTPγS-induced HRs was precluded

(Fig. 9*D*): the responses were similar to those induced by GTPγS alone (Fig. 4*A*). Inhibitory effects of 5-HT antagonists are quantitatively presented in Table 1. Complete inhibition to 10 nM-5-HT was obtained with 1 μM-cyproheptadine, 10 μM-spiperone and 10 μM-ketanserin.

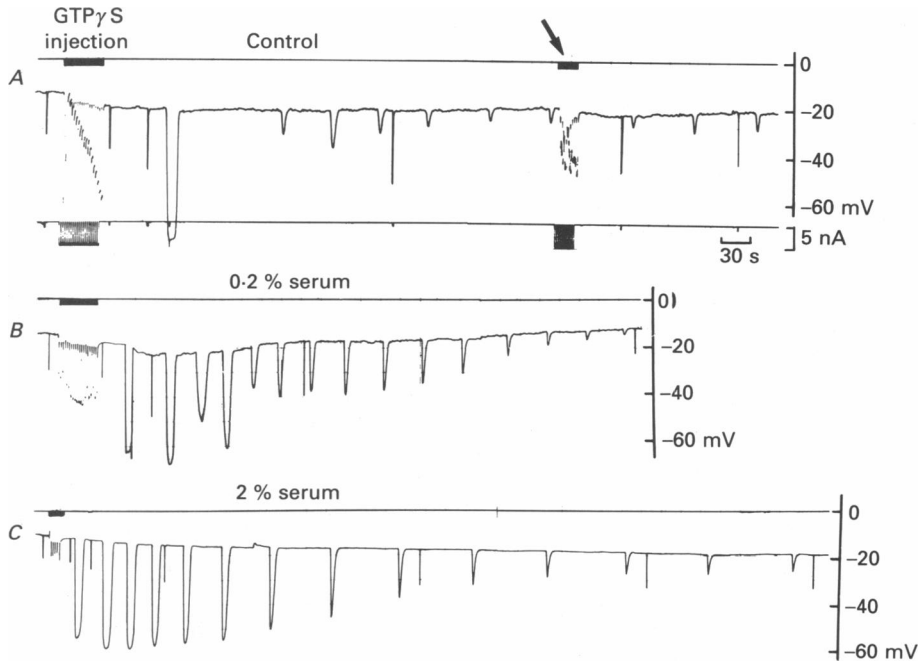

Fig. 10. Facilitation of GTPγS-induced HRs by human serum. GTPγS was injected with twenty current pulses in *A* and *B* or five pulses in *C*. Additional injection of GTPγS is indicated by the arrow in *A*.

Effect of serum

Our original reason for looking at the effect of 5-HT upon hamster eggs was because we found that heat-treated (56 °C, 45 min) human serum, which contains 5-HT, was effective in facilitating the GTPγS-induced Ca^{2+} transients. Figures 10*A* and *B* demonstrate that injection of GTPγS with twenty pulses produces more large HRs with a shorter delay in the presence of 0·2% serum. The facilitation by 2% serum was more pronounced in HRs induced by five injection pulses of GTPγS (Fig. 10*C*): the delay time was 25 ± 21 s ($n = 13$), the number of large HRs was $4{\cdot}9 \pm 1{\cdot}3$ and the number of HRs in the first 5 min of the HR series was $9{\cdot}1 \pm 1{\cdot}4$.

DISCUSSION

5-HT as an agonist for Ca^{2+} transients

Various cells respond to neurotransmitters and hormones with oscillatory increases in $[Ca^{2+}]_i$ (Berridge *et al.* 1988; Berridge & Galione, 1988), although the functional significance of the oscillation is still unknown. Models of Ca^{2+} oscillators have been proposed in relation to the phosphoinositide (PI) signalling pathway involving IP_3-induced Ca^{2+} release and Ca^{2+}-induced Ca^{2+} release (Berridge & Galione, 1988).

At fertilization in the hamster egg, a fused sperm is the stimulus for repetitive Ca^{2+} transients possibly by way of a G protein and PI signalling pathway (Miyazaki, 1988). The G protein that mediates Ca^{2+} transients in the hamster egg is pertussis toxin- and cholera toxin-insensitive (Miyazaki, 1988). A 'sperm receptor' has not yet been identified in the egg plasma membrane and so the mechanism of activating a G protein is only speculative (Miyazaki, 1989). In the present study 5-HT was found to be an agonist for receptor-mediated Ca^{2+} transients in the unfertilized hamster egg. 5-HT has been found to cause electrical changes reflecting Ca^{2+} oscillations in blowfly salivary gland cells (Rapp & Berridge, 1981) and intestinal 407 cells (Yada, Oiki, Ueda & Okada, 1986). 5-HT-induced currents have been observed in *Xenopus* oocytes transfected with rat brain mRNA, consisting of Ca^{2+}-dependent oscillatory currents and a Ca^{2+}-independent smooth current (Parker, Gundersen & Miledi, 1985). Of 5-HT receptor subtypes, 5-HT_{1C} and 5-HT_2 receptors are able to mediate PI turnover (Peroutka, 1988). In hamster eggs the 5-HT_2 receptor probably mediates Ca^{2+} transients, because HRs were inhibited by cyproheptadine, spiperone and ketanserin which are 5-HT_2 receptor antagonists. Using 5-HT we analysed receptor-mediated activation of a G protein and its desensitization in the hamster egg.

Synergistic activation of G protein by 5-HT and GTPγS

The present study demonstrated with high temporal resolution the time course of 5-HT-dependent Ca^{2+} rises. A remarkable feature in 5-HT-induced Ca^{2+} transients is the substantial time lag to induce the response. The delay time ranged between 8 and 90 s, depending on the 5-HT concentration. It is reasonable to consider that this time lag is mainly due to the delay in G protein–GTP binding, as described below.

It is a general understanding that ligand–receptor binding stimulates dissociation of G protein–GDP and binding of G protein–GTP, presumably by a transition of the guanine nucleotide-binding site from a 'closed' state to an 'open' state that allows nucleotide exchange. The resulting G protein–GTP activates effector enzymes such as adenylate cyclase and phospholipase C (Gilman, 1987). In the present paper we have demonstrated synergistic activation of a G protein by ligand–receptor binding and GTPγS. Induction of Ca^{2+} transients by 5-HT at a relatively low concentration (70 nM) was remarkably accelerated when GTPγS had been injected. Even a subthreshold dose of 5-HT (1 nM) in intact eggs could induce Ca^{2+} rises when combined with GTPγS injection (Fig. 4*B* and *C*). Thus, internal GTPγS apparently facilitates the activation of a G protein by 5-HT–receptor complex. Potentiation of agonist-induced current by GTPγS has been shown in dialysed secretory cells (Evans & Marty, 1986; Maruyama, 1988).

Internally applied GTPγS causes repetitive Ca^{2+} rises or Ca^{2+}-dependent membrane currents in several types of secretory cells (Llano & Marty, 1987; Penner & Neher, 1988; Maruyama, 1988). Injection of GTPγS or GTP into hamster eggs induces Ca^{2+} transients but a long time lag after injection is always observed (Miyazaki, 1988) (120–240 s in the present experiments). This is probably due to low rate constant in the binding between G proteins and GTPγS or GTP, since a remarkable facilitation was observed in GTPγS-induced Ca^{2+} transients when GTPγS was injected in the presence of an agonist 5-HT. With 5-HT the time lag could be reduced to 1/40th or less, and Ca^{2+} transients were enhanced in frequency

as well as in magnitude. The minimum effective concentration of 5-HT was about 1/1000 of that for inducing a Ca^{2+} rise by 5-HT alone.

These results clearly show that there is a synergism between 5-HT and GTPγS in activating G proteins. The synergistic action of agonists and GTPγS has been demonstrated in accumulation of IP_3 in pancreatic acinar cells (Merritt, Taylor, Rubin & Putney, 1986). The most straightforward explanation of this synergism between 5-HT and GTPγS is that 5-HT–receptor interaction causes more GTPγS to react with the G protein more rapidly hence more IP_3 is produced sooner. This could account for more frequent large HRs associated with the shortening of the delay time. Even 5-HT at low concentrations can cause HRs, probably because most of ligand–receptor–G protein complexes are bound by elevated GTPγS so that even a small number of ligand–receptor–G protein complexes are enough to stimulate PI hydrolysis leading to IP_3 production and Ca^{2+} release. However, considering the extremely low concentration of effective 5-HT for facilitation, additional effect(s) may be involved. In the absence of 5-HT the injected GTPγS will bind to many cellular G proteins, some of which could have some inhibitory effects. G proteins may, for example, cause diacylglycerol production from phosphatidylcholine and hence PKC activation without generating IP_3 (Loffelholz, 1989). This will inhibit Ca^{2+} transients in hamster eggs because of the negative feedback effect of PKC (see next section).

Negative feedback and desensitization

In many somatic cells, activating PKC with phorbol ester TPA inhibits agonist-induced PIP_2 break-down (Berridge, 1987). There is evidence that PKC exerts an inhibitory action on the pathway leading from receptor activation to IP_3 production in exocrine cells (Llano & Marty, 1987; Maruyama, 1989) and in hepatocytes (Woods, Cuthbertson & Cobbold, 1987*b*). A previous paper has shown evidence for an inhibitory effect of PKC on G protein-mediated Ca^{2+} transients in hamster eggs (Swann *et al.* 1989): GTPγS-induced HRs are inhibited by TPA and synthetic diacylglycerol and enhanced by sphingosine whilst IP_3-induced HRs are not affected. Therefore, it was suggested that the most plausible target of PKC is the putative G protein. In the present study 100 nM-TPA completely blocked HRs induced by 5-HT at the maximum concentration used (10 μM). Co-activation by 100 nM-5-HT and GTPγS injection overcame the inhibition of 100 nM-TPA in the initial four to five HRs, but later HRs were blocked. These findings support the existence of a negative feedback loop via PKC activity. In other cells, acetylcholine-induced current is also inhibited by TPA in lacrimal cells (Llano & Marty, 1987) and pancreatic acinar cells (Maruyama, 1989). However, GTPγS-induced responses are inhibited by TPA in the former as in hamster eggs whilst the responses are not affected by TPA in the latter, suggesting a difference in the target of PKC.

The Ca^{2+} transients induced by either 5-HT or GTPγS or both always show an apparent desensitization. In the series of HRs induced by GTPγS plus 5-HT, declining HRs (i.e. middle-sized HRs) had a sharp tip, appearing to be cut off. The desensitization is partly due to the inhibition by PKC, because the decline in the series of HRs was attenuated by sphingosine. It is noteworthy that sphingosine caused a clear shortening of the time lag in 5-HT-induced repetitive Ca^{2+} transients

of up to 3 s (Fig. 2*C*). This finding suggests that PKC-mediated inhibition pre-exists or precedes the G protein-mediated Ca^{2+} rises. A tonic inhibition by PKC may exist in hamster eggs as suggested in mouse 3T3 cells (Brown, Blakeley, Hamon, Laurie & Corps, 1987).

The desensitization seems to take place mainly at G protein–GTPγS binding rather than ligand–receptor–G protein binding, because (1) GTPγS injection can produce facilitated HRs even after a long incubation time (5–20 min) in 5-HT medium; (2) 5-HT can produce HR(s) at low concentrations if applied soon after GTPγS injection (before the action of GTPγS has not been desensitized yet), but 5-HT stimulation even with high concentrations is immediately desensitized when 5-HT is applied after GTPγS-induced HRs has undergone desensitization (Fig. 4). The desensitization could be due to the PKC directly phosphorylating the G protein controlling PI turnover (Sagi-Eisenberg, 1989). However, we cannot rule out the possibility that PKC also phosphorylates and inhibits the signalling function of the 5-HT receptor, as is the case in some types of cellular receptors (Cochet, Gill, Meidenhelder, Cooper & Hunter, 1984; Lynch, Charest, Bocckino, Exton & Blackmore, 1985).

In the present experiments, the most favourable condition for repetitive HRs was GTPγS injection in the presence of 5-HT and sphingosine. A series of large HRs with longer duration was produced with a little longer intervals, similar to sperm-mediated HRs. The reason for this paradoxical decrease in HR frequency is unknown. Even in this favourable condition Ca^{2+} transients are susceptible to desensitization: sperm-mediated HRs cannot be completely mimicked. Fertilizing sperm seem to stimulate G protein-mediated IP_3 production in a way that precludes feedback inhibition by PKC, as suggested previously (Swann *et al.* 1989).

Biological significance of 5-HT

5-HT is an inducer of oocyte maturation in the surf clam *Spisula* (Hirai, Kishimoto, Kadam, Kanatani & Koide, 1988). 5-HT is also contained in sea urchin embryos and its content varies rhythmically during development in time with the cell cycle and early embryogenesis (Renaud, Parisi, Capasso & De Prisco, 1983). However, to our knowledge, there are no previous reports of the effects of 5-HT on mammalian eggs.

We found that GTPγS-induced HRs are facilitated by human serum which is used for making hamster spermatozoa undergo 'capacitation' to be capable of interacting with eggs (Yanagimachi, 1970). Since human serum contains about 560 nM-5-HT (calculated from the value in Erspamer, 1954), the 0·2% serum used in Fig. 10*B* contains about 1 nM-5-HT, which is enough to facilitate GTPγS-induced Ca^{2+} rises. In further studies, it is important to investigate what substance(s) from the fertilizing sperm activates the G protein in the egg plasma membrane and what factors modulate the signal transduction of sperm–egg interaction.

Note added in proof. Miledi & Parker (1989) have recently reported that application of rabbit serum to *Xenopus* oocytes elicits an oscillatory chloride current with a dose-dependent delay and that the response is mediated through a PI–Ca^{2+} signalling pathway and is similar to currents evoked by acetylcholine and 5-HT.

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