Desensitization and resensitization of human platelets to 5-hydroxytryptamine at the level of signal transduction

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Desensitization of platelets to 5-hydroxytryptamine (5HT) (1 μ M), during active removal of the agonist by the platelet 5HT-uptake system, was studied at the level of signal transduction. Desensitization to 5HT was dose-dependent and homologous. Without occupation of the 5HT₂ receptor, neither an increase in cytosolic [Ca²⁺] (30 nM ionomycin), nor a separate or simultaneous activation of protein kinase C (by 10 μ M 1-oleoyl-2-acetyl-glycerol), could induce desensitization to 5HT (1 μ M). During the early phase of desensitization, the 5HT₂ receptor was coupled to phospholipase C, whereas during the late phase of desensitization this coupling was disconnected. However, after disappearance of the agonist, the coupling in the resting platelet recovered quickly, and was nearly complete (82 %) after 30 min. During this resensitization, the 5HT-inducibility of activation

of phospholipase C, of the increase in cytosolic [Ca²⁺] and of stimulation of protein kinase C were restored in parallel. The time course for resensitization of the 5HT-induced increase in cytosolic [Ca²⁺] was independent of the presence of extracellular Ca²⁺. It is concluded that, after dissociation of 5HT from the platelet 5HT₂-receptor, 5HT-induced responses rapidly resensitize. Because of its short duration and the parallelism in recovery between the different 'down-stream phospholipase C' intracellular transduction signals, it is considered that desensitization arises from a reversible change in the transduction mechanism at a step up to or including the activation of phospholipase C. Neither desensitization nor resensitization to 5HT is dependent on the presence of extracellular Ca²⁺.

INTRODUCTION

The physiological response of cells to biologically active compounds such as hormones and neurotransmitters tends to fade as a function of time [1]. This phenomenon, known as desensitization, is an essential mechanism in tight regulation of physiological processes, since desensitization, by limiting the responsiveness of cells to one or more external stimuli, enables quick adaptation of the cellular action in response to changing extracellular needs. However, in physiological situations in which cells respond repeatedly upon repeated exposure to the same or different agonists, desensitization must be reversible. The termination of desensitization is called resensitization [2,3].

At the molecular level, the intracellular mechanisms responsible for the physiological response to an external stimulus are controlled by a complex interplay between different receptor-induced intracellular messengers. Therefore, from a molecular point of view, desensitization might theoretically be mediated by every mechanism that decreases the amount of intracellular messengers or prevents them from interacting with their intracellular target.

Intracellular messengers are generated during a cascade of reactions initiated by an effector protein upon occupation of a receptor by its agonist. G-proteins are so-called transducers that form the coupling between receptor-occupation and activation of the effector [4]. This coupling can be measured in intact cells by monitoring the agonist-induced generation of intracellular messengers [5]. As long as an agonist can induce an immediate increase in intracellular messengers, its receptor must be considered to be coupled to an effector. For cellular responses that are evoked by G-protein-coupled receptors linked to phospholipase C as effector, not much is known at the molecular level about the mechanisms responsible for desensitization [3,6].

From energetic point of view, disconnection of the receptor-effector coupling by a change in the receptor molecule, in the G-protein or in the effector molecule, seems the most rational desensitization mechanism, since it blocks the initial step in the generation of all second messengers. However, alternative mechanisms that limit the intracellular action of one or more of the intracellular messengers, and thus contribute to attenuation of the physiological response, can not be excluded.

5-Hydroxytryptamine (5HT), a weak platelet agonist, induces in platelets an increase in cytosolic [Ca²⁺] ([Ca²⁺]_{cyt}) that coincides with shape change, followed by (reversible) aggregation [7]. At the level of signal transduction, activation of the 5HT₂ receptor triggers, most probably via a G-protein, the phospholipase C-mediated hydrolysis of PtdIns(4,5)P₂ to yield Ins(1,4,5)P₃ and 1,2-diacylglycerol (DAG), which is subsequently phosphorylated to phosphatidic acid (PA) [8,9]. When platelets are prelabelled with [³²P]P_i, addition of the amine results in a marked increase in ³²P incorporation into PA [8,9]. Increased incorporation of ³²P is also found in a 47 kDa and a 20 kDa protein, which are substrates of protein kinase C and myosin light-chain kinase (MLCK) respectively [10]. 5HT also induces a release of Ca²⁺ from intracellular stores, mediated by InsP₃ [11]. Thus the intracellular messengers that can be detected upon platelet activation by 5HT are: InsP₃ (or other inositol phosphates), DAG (by its phosphorylation to PA), [Ca²⁺]_{cyt}, phosphorylated 47 kDa protein and phosphorylated 20 kDa protein. We focused in this work mainly on the 5HT-induced increase in [Ca²⁺]_{cyt} to describe the desensitization-resensitization process of platelets to 5HT at the level of signal transduction. In platelets, desensitization to a large variety of agonists has been studied [12–15]. Evidence for the involvement of two different pathways of desensitization is presented, one mediated by protein kinase C and another that is independent of this enzyme [14]. The latter is

Abbreviations used: [Ca²⁺]_{cyt}, cytosolic free Ca²⁺ concentration; OAG, 1-oleoyl-2-acetyl-glycerol; Ca²⁺_{ex}, extracellular Ca²⁺; PA, phosphatidic acid; 5HT, 5-hydroxytryptamine; DAG, 1,2-diacylglycerol; PRP, platelet-rich plasma; MLCK, myosin light-chain kinase; VP, vasopressin; H7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride.

suggested to be completely independent from activation of phospholipase C and to operate much more quickly for weak than for strong agonists.

In vivo, many mediators exert their function at sites where their concentration decreases due to the presence of avid uptake systems or degradation by specific enzymes, especially at locations where repeated stimulation occurs, e.g. in synaptic clefts [16]. The extracellular removal of agonists might be expected to influence the kinetics of their desensitization–resensitization process *in vivo*. 5HT is efficiently taken up by human platelets [17]. The platelet 5HT₂ receptor thus offers the interesting opportunity to study the kinetics of desensitization and subsequent resensitization under the control of an agonist-removing mechanism, exactly as they occur *in vivo*. As far as we know, investigations on resensitization of human platelets have never been reported.

MATERIALS AND METHODS

Human venous blood and platelet-rich plasma (PRP) were obtained as described previously [8].

Preparation of ³²P-labelled platelets, measurement of PA formation and SDS/PAGE to estimate phosphorylation of 47 kDa and 20 kDa proteins were performed exactly as described elsewhere [10].

For [Ca²⁺]_{cyt} measurements, PRP was incubated with 2 μ M Fura 2 AM at room temperature for 12 min. After the loading procedure, platelets were isolated by gel filtration on Sepharose CL-2B in 125 mM NaCl/2.7 mM KCl/1 mM MgSO₄/0.5 mM Na₂HPO₄/2 mM NaHCO₃/25 mM Hepes/10 mM glucose/0.1 mM EGTA/0.1 % BSA, pH 7.4. If platelets were also labelled with ³²P, Na₂HPO₄ was omitted and labelling with [³²P]P_i preceded loading with Fura 2. The platelet concentration was adjusted to 2.2 $\times 10^8$ /ml, 1.2 μ mol/ml CaCl₂ was added, and platelets were incubated for at least 15 min at room temperature before the experiment was started. The platelet suspension was kept at room temperature until the experiment started by incubation of batches for 5 min at 37 °C before the first compound or agonist was added. All subsequent additions were done at 37 °C. Thus all fluorescence readings (excitation at 340 nm; emission at 510 nm) were also recorded at 37 °C. Changes in the fluorescence were measured on 1.1 ml samples and calibrated to changes in [Ca²⁺]_{cyt} (K_D = 224 nM) after correction for dye leakage as described elsewhere [18,19].

The 5HT concentration in platelet suspensions was quantified by HPLC. Briefly, 500 μ l of platelet suspension was mixed with 50 μ l of 4M HClO₄ containing hydroxyindolecarboxylic acid (4 μ M). After centrifugation (20 min, 10000 g), 40 μ l samples of acidified supernatants were injected directly into the HPLC apparatus (Varian HPLC model 5060), equipped with a micro-computer-controlled reciprocating single-piston pump system; electrochemical detection (BAS model 4B, Bioanalytical systems) with a glassy carbon electrode was performed as described elsewhere [20].

For the assessment of platelet shape change [21], citrated (0.131 % trisodium citrate, 2H₂O) PRP (3 $\times 10^8$ platelets/ml) was diluted 1:3 with Krebs–Henseleit buffer without Ca²⁺, at pH 7.4, containing 5 mM glucose and 13 mM sodium citrate, and was stored at 37 °C. Platelet shape-change reactions were recorded photometrically in this buffer-diluted PRP: 0.45 ml of diluted PRP (10⁸ platelets/ml; plasma 30 %, v/v) was incubated (5 min at 37 °C) before addition of 5HT to the stirred sample.

5HT was obtained from Janssen Chimica (Beerse, Belgium), adrenaline from Sigma (Basel, Switzerland) and vasopressin (VP) from Calbiochem (La Jolla, CA, U.S.A.). They were all

dissolved in 0.9 % NaCl. Ionomycin and 1-oleoyl-2-acetylgllycerol (OAG) were also purchased from Calbiochem, ketanserin was from the Janssen Research Foundation (Beerse, Belgium) and Fura 2 AM was from Molecular Probes (Eugene, OR, U.S.A.). H7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride] was from Fluka (Buchs, Switzerland). These compounds were all dissolved in dimethyl sulphoxide. BSA (pure grade) was obtained from Serva (Heidelberg, Germany). Sepharose CL2B came from Pharmacia (Uppsala, Sweden).

RESULTS

The concentration of 5HT in the extracellular medium decreases as a function of time

Since our aim was to characterize the desensitization–resensitization process of platelets to 5HT during active 5HT uptake by these cells, we first investigated the effect of platelet 5HT uptake on the concentration of 5HT in the extracellular medium. The data presented in Table 1 show that 1 μ M 5HT, which is a near-maximally effective concentration [8] for platelet activation, decreased to undetectable levels within 7.5 min, when added to a suspension of 2.2 $\times 10^8$ platelets/ml. Owing to this avid uptake of 5HT, receptor-binding studies on intact platelets to measure the occupation of the 5HT₂ receptor during the desensitization process as it occurs *in vivo* are impossible (such studies involve the use of radiolabelled 5HT and measurement of binding of the label to the intact platelets). Therefore, occupation of 5HT₂ receptors in intact platelets can only be estimated based on the kinetics of disappearance of 5HT from the medium (Table 1) and the dissociation rate of 5HT from this receptor, measured on platelet membranes. Dissociation of 5HT from platelet 5HT₂ receptors is complete within 5 min after the agonist has been washed off (results not shown; J. E. Leysen, personal communication, according to reference [22]).

Taken together, these data suggest that, 7.5 min after the addition of 10⁻⁶ M 5HT to platelets under our experimental conditions, the amine had almost completely disappeared from the extracellular medium and that most probably receptor occupation was not maximal.

The protein kinase C inhibitor H7 has previously been used by others [11] to study the role of protein kinase C in desensitization of human platelets to 5HT without the presence of the 5HT-uptake system being taken into account. Therefore, we verified whether H7 influences the platelet 5HT-uptake system. Table 1

Table 1 Platelet 5HT uptake as a function of time, and the effect of H7

Platelets isolated by gel filtration (2.2 $\times 10^8$ platelets/ml, 37 °C) were stimulated with 1 μ M 5HT at zero time in the absence or presence of H7 (60 μ M, 5 min preincubation). At indicated times, samples were taken for determination of the 5HT concentration as described in the Materials and methods section. The results shown are means $\pm$ S.E.M. ($n = 4$); ND, not detectable.

Time (min)	5HT concn. (μ M)	
	Control	+ H7
0.5	0.88 $\pm$ 0.03	0.98 $\pm$ 0.02
1	0.59 $\pm$ 0.03	0.95 $\pm$ 0.01
2	0.19 $\pm$ 0.02	0.88 $\pm$ 0.03
5	0.03 $\pm$ 0.01	0.78 $\pm$ 0.02
7.5	ND	0.56 $\pm$ 0.03
15	ND	0.45 $\pm$ 0.03

Figure 1 Early desensitization of human platelets to 5HT: effect of different compounds on the 5HT-induced mobilization of $[Ca^{2+}]_{cyt}$

Fura-2-loaded platelets were stimulated with 5HT, and 30 s later different compounds were added at the concentrations indicated (Epi, adrenaline). The protein kinase C inhibitor H7 was added 5 min before stimulation with 5HT, to allow penetration into the cytosol. The tracings shown are representative of four different platelet preparations.

demonstrates that 5HT uptake was markedly attenuated in the presence of H7 (60 μ M).

Based on the ability of 5HT to induce the production of intracellular messengers at different stages after an initial stimulation with this agonist, the desensitization–resensitization process of platelets to 5HT as a function of time could be divided into three periods: (1) an early phase of desensitization, followed by (2) a late phase of desensitization and (3) a resensitization phase.

Early desensitization phase

In Figure 1 the 5HT-induced increase in $[Ca^{2+}]_{cyt}$ is used to illustrate the first period of desensitization of platelets to this agonist. 5HT (1 μ M) induced an immediate increase in $[Ca^{2+}]_{cyt}$ (Figure 1a). However, this intracellular signal was transient and returned to basal levels within 2 min after addition of the agonist. Since a second stimulation with 5HT 30 s after the first one (Figure 1a) could not inhibit the decrease, removal of the agonist via the 5HT-uptake system was not rate-determining in this process. We suppose that, at this stage, the mobilization of Ca^{2+} induced by the activation of phospholipase C still coupled to 5HT₂ receptors was counteracted and dominated by Ca^{2+} -removing mechanisms such as Ca^{2+} efflux and Ca^{2+} resequestration into intracellular stores. This suggestion is supported by the fact that elimination of the Ca^{2+} -generating mechanism by removal of 5HT from the 5HT₂ receptor after the addition of ketanserin, a selective 5HT₂-receptor antagonist [8], resulted in an immediate fall in the 5HT-induced Ca^{2+} response (Figure 1b), which also indicates that at least a part of the 5HT₂ receptor molecules was coupled to phospholipase C during the initial period of desensitization.

This conclusion was confirmed by the effect of adrenaline.

Figure 2 Late desensitization of human platelets to 5HT: parallelism between different platelet responses

Platelets were stimulated first (left panels) with 5HT (1 μ M; A) or solvent (0.9% NaCl; B), and 7.5 min later (right panels) 5HT (1 μ M; A and B) was given again. Preparation of platelets and measurement of platelet shape change, $[Ca^{2+}]_{cyt}$ and PA formation as a function of time were performed as described in the Materials and methods section. The three platelet responses shown were not simultaneously measured on the same platelet preparation, but they are each representative of at least five different platelet preparations from different donors.

Adrenaline (3 μ M), which did not induce an increase in $[Ca^{2+}]_{cyt}$ alone (Figure 1d), amplified the response of platelets to 5HT (Figure 1d). The adrenaline-induced increased activation of phospholipase C during the response to 5HT can be dose-dependently inhibited by ketanserin [23], which strongly indicates that adrenaline can only generate second messengers via phospholipase C that is already coupled to occupied receptors. The adrenaline-induced amplification of the 5HT response (Figure 1c) 30 s after stimulation with 5HT can thus be interpreted as enhanced coupling of the 5HT₂ receptor to phospholipase C, and is a second indication that 5HT₂ receptors were still coupled to phospholipase C during this period.

It has been repeatedly suggested that protein kinase C induces early desensitization by inhibiting receptor-coupled phospholipase C activation in a negative-feedback mechanism for receptor-mediated Ca^{2+} signalling [11,13,14,21]. In this context, inhibition of the early desensitization phase of human platelets to 5HT with H7 has been described by others, using 0.3 mM H7 and 10 μ M 5HT [11]. Although H7 has been shown to be maximally effective at 60 μ M and to inhibit the phosphorylation of the 47 kDa protein by protein kinase C in human platelets by 40% at this concentration [24], we did not find any effect of this inhibitor (60 μ M) on the 5HT-induced mobilization of Ca^{2+}

Figure 3 Desensitization to 5HT₂ is homologous

Fura 2-loaded platelets were stimulated with either 5HT (1 μ M) or VP (30 nM) and $[Ca^{2+}]_{cyt}$ was monitored as changes in fluorescence, as described in the Materials and methods section. The tracings shown are representative of four different platelet preparations.

during the early phase of desensitization (Figure 1e). Higher concentrations of H7 even inhibited the 5HT-induced mobilization of Ca^{2+} (results not shown).

Late desensitization phase

After the early desensitization phase, during which 5HT₂ receptors remain coupled to phospholipase C, 5HT-stimulated platelets enter a second desensitization phase, in which this coupling is completely disconnected. Although 5HT could no longer be detected in the extracellular medium (Table 1) 7.5 min after the first stimulation with 5HT, desensitization was still apparent at that time point. Figure 2 illustrates that, during this desensitization stage, 7.5 min after the first addition of 5HT (1 μ M), complete desensitization of platelet shape-change to 5HT is explained by total inability of this agonist to generate DAG, as measured by PA formation, or $InsP_3$ as measured by mobilization of Ca^{2+} . Obviously, coupling between 5HT₂ receptors and their effector, phospholipase C, was blocked. Accordingly, adrenaline (3 μ M) given 7.5 min after addition of 5HT (1 μ M) could no longer activate phospholipase C (results not shown) during late desensitization, in contrast with its effect during early desensitization (Figures 1c and 1d). Further characterization of the disconnection of the signal-transducing system

during the late phase of desensitization to 5HT was performed by measurement of agonist-induced mobilization of Ca^{2+} .

Receptor-ligand-induced desensitization is divided into two categories: homologous or agonist-specific desensitization, in which only the subsequent response to the desensitizing ligand is attenuated, and heterologous or agonist-non-specific desensitization, in which exposure to one agonist attenuates the responses induced by activation of other receptor types. Occupation of the 5HT₂ receptor induces desensitization to 5HT, in an homologous way. This is demonstrated by the experiment shown in Figure 3, which was performed in the absence of extracellular Ca^{2+} (Ca^{2+}_{ex}) (2 mM EGTA added before the first stimulation). Stimulation with VP (30 nM) of platelets, desensitized to 5HT (1 μ M), raised $[Ca^{2+}]_{cyt}$ to the same level (Figure 3a) as that observed after addition of the same dose of VP to untreated platelets (Figure 3b). Likewise, previous stimulation of platelets with 1 μ M 5HT had no effect on the response after 7.5 min to thrombin (0.02 unit/ml) or platelet-activating factor (10 nM) (results not shown). Conversely, stimulation of platelets with VP (30 nM), before the addition of 5HT (1 μ M), desensitized platelets to this VP, but not to 5HT (Figure 3b).

The desensitization of 5HT-stimulated mobilization of Ca^{2+} is dose-dependent. This is demonstrated in Figure 4. At 7.5 min after the first addition of different concentrations of 5HT in the presence of 1 mM Ca^{2+}_{ex} (Figure 4a), the platelets were activated by a second, constant, concentration (1 μ M). The amplitude of the elevation of $[Ca^{2+}]_{cyt}$ induced by the second addition progressively decreased as the initial concentration was raised. In the absence of Ca^{2+}_{ex} (2 mM EGTA added 5 min before the first addition of 5HT), we obtained the same results with slightly decreased peak levels of $[Ca^{2+}]_{cyt}$ (Figure 4b).

Essentially, all molecular events that take place during the signal transduction of the first stimulation can be considered to be potential candidates for having a regulatory role in desensitization. Therefore we investigated whether desensitization to 5HT could be induced by activation of protein kinase C and/or Ca^{2+} mobilization, which are the two main branches of the signal-transduction system after activation of phospholipase C [12].

To induce an increase in $[Ca^{2+}]_{cyt}$ and activation of protein kinase C without activation of the complete signal-transduction

Figure 4 Desensitization to 5HT₂ is dose-dependent

Fura 2-loaded platelets were stimulated with different concentrations of 5HT, as indicated, in (a) the presence or (b) the absence (2 M EGTA added) of 1 mM Ca^{2+}_{ex} . They were stimulated again with a maximal dose of 5HT 7.5 min later. The tracings shown are representative of six different platelet preparations.

Figure 5 Effect of increased $[Ca^{2+}]_{cyt}$ and activation of protein kinase C without receptor occupation on 5HT-induced mobilization of Ca^{2+}

Fura 2-loaded platelets were first triggered with 5HT ($1 \mu M$) (a), ionomycin (iono; $30 nM$) (b), OAG ($10 \mu M$) (c) or both OAG and ionomycin at the same time (d). After 7.5 min they were challenged with 5HT. The tracings shown are representative of three different platelet preparations.

mechanism, we used ionomycin and OAG, respectively. Previously, we demonstrated that $10 \mu M$ OAG induces a robust activation of protein kinase C compared with $1 \mu M$ 5HT, as measured by phosphorylation of the 47 kDa protein [25]. However, the results demonstrated in Figure 5(c) show that $10 \mu M$ OAG was not able to desensitize platelets to $1 \mu M$ 5HT. Addition of the Ca^{2+} ionophore ionomycin ($30 nM$), in the presence of Ca^{2+}_{ex} , induced an immediate, long-lasting, increase in $[Ca^{2+}]_{cyt}$ (Figure 5b). Although $[Ca^{2+}]_{cyt}$ was increased to the same level as the control response of 5HT ($1 \mu M$) (Figure 5a), the subsequent addition of 5HT ($1 \mu M$) induced a marked supplementary increase in $[Ca^{2+}]_{cyt}$ (Figure 5b). Comparison with the second addition of 5HT ($1 \mu M$) (Figure 5a) proves that platelets were obviously not desensitized by previous exposure to ionomycin.

The simultaneous addition of OAG ($10 \mu M$) and ionomycin ($30 nM$) (Figure 5d), to mimic as closely as possible the cytosolic changes during platelet activation by 5HT, inhibited the 5HT ($1 \mu M$) response 7.5 min later only modestly, compared with the desensitization induced by simple addition of $1 \mu M$ 5HT (Figure 5a).

Resensitization phase

Owing to 5HT uptake during the early and late desensitization phases of 5HT-stimulated platelets, the resensitization process could be studied without washing procedures to eliminate the agonist from the extracellular environment, which gives a real indication about how much time desensitized platelets need *in vivo* to become resensitized after stimulation with 5HT. Preliminary experiments, in which platelet shape change was measured, had demonstrated that platelets desensitized to 5HT ($1 \mu M$) were at least partly resensitized 30 min later (results not shown).

At the molecular level, we studied the resensitization process by triggering platelets with 5HT ($1 \mu M$) at different time points after challenge with the same concentration of agonist, and measuring the production of second messengers. Platelets were labelled with $[^{32}P]P_i$ as well as being loaded with Fura 2 to measure resensitization of activation of phospholipase C (PA formation; Figure 6) simultaneously with the subsequent bifurcated pathway (Ca^{2+} mobilization, Figure 6, and protein kinase C activity by 47 kDa phosphorylation, Figure 7) on the same batch of platelets.

The results shown in Figure 6 demonstrate that 5HT-induced PA formation, as well as Ca^{2+} mobilization, became resensitized simultaneously within 30 min to about 80% of the first response. The inset of Figure 6 shows the individual data and demonstrates a strong positive correlation ($r = 0.915$) between these receptor-mediated intracellular signals during the resensitization of platelets to 5HT.

Figure 6 Time curve of the recovery of 5HT-induced activation of phospholipase C

At zero time, ^{32}P -labelled platelets loaded with Fura 2 were triggered with 5HT at $1 \mu M$. Samples of the same batch of platelets were stimulated again with the same concentration of 5HT at time points indicated, and Ca^{2+} mobilization (■) and PA formation (□) as well as protein phosphorylation (Figure 7) were determined. For measurement of PA formation and protein phosphorylation, samples were taken 20 s before and 45 s after the addition of 5HT. The data shown are means $\pm$ S.E.M. ($n = 3$), showing the second stimulation as a percentage of the initial stimulation. The inset shows the correlation between the 5HT-induced increase in $[Ca^{2+}]_{cyt}$ and PA formation during resensitization, based on the individual data of the time curve. The line was drawn by linear-regression analysis ($r = 0.915$).

The resensitization of protein kinase C activity, as measured by 5HT-induced phosphorylation of the 47 kDa protein, is shown in Figure 7. Protein kinase C was completely desensitized 7.5 min after the first stimulation with 5HT, in agreement with the observation that PA formation, which is a measure of the production of DAG by phospholipase C, was also desensitized at that time. After 23 min, 5HT-induced activation of protein kinase C was restored to a level comparable with the control value. Surprisingly, MLCK was never completely desensitized to 5HT. Even at the shortest time points measured (3 and 7 min), a 5HT-inducible phosphorylation of the 20 kDa protein was apparent (Figure 7), although maximal resensitization was only accomplished after about 23 min, in agreement with PA formation, Ca^{2+} mobilization (Figure 6) and protein kinase C activity (Figure 7). In view of the apparent correlation between the 5HT-induced increase in $[Ca^{2+}]_{cyt}$, PA formation and protein kinase C activation, the resensitization phenomenon was further investigated by measurement of changes in $[Ca^{2+}]_{cyt}$ only.

To check whether resensitization of 5HT-induced mobilization

Figure 7 Recovery of the 5HT-induced phosphorylation of platelet proteins

Samples from the experiment described in the legend to Figure 6 were denatured and proteins were separated by SDS/PAGE and autoradiographed.

Figure 8 Recovery of the 5HT-induced mobilization of $[Ca^{2+}]_{cyt}$ in the presence and absence of Ca^{2+}_{ex}

Fura 2-loaded platelets were divided into batches and stimulated with 5HT ($1 \mu M$) either in the presence (●) or absence (○) of Ca^{2+}_{ex} (addition of 2 mM EGTA 5 min before the first stimulation). At the time points indicated, samples were taken for a second stimulation with the same dose of 5HT, and the increase in $[Ca^{2+}]_{cyt}$ was measured. The results shown are means $\pm$ S.E.M. ($n = 4$), showing the second stimulation as a percentage of the first one.

of Ca^{2+} depends on the availability of Ca^{2+}_{ex} , resensitization of Ca^{2+} mobilization to 5HT was measured on the same batch of platelets in the presence and in the absence of extracellular Ca^{2+} (2 mM EGTA added). The results shown in Figure 8 prove that platelets become resensitized to 5HT equally fast in the presence as in the absence of Ca^{2+}_{ex} .

Whether activation of protein kinase C during the early phase of desensitization might affect resensitization was examined by stimulation of platelets with 5HT, in the presence or in the absence of H7 ($60 \mu M$) and measurement of how long it takes for platelets to restore Ca^{2+} mobilization in response to the same dose of 5HT ($1 \mu M$) in both conditions. Figure 9 clearly demonstrates that, although H7 had no effect on desensitization of 5HT-induced Ca^{2+} -mobilization (Figure 1e), platelets stimulated with 5HT in the presence of H7 became resensitized markedly more slowly than did control platelets.

DISCUSSION

The sequelae of molecular events thought to be involved in desensitization of cellular responses mediated by β -adrenergic

Figure 9 Effect of H7 on resensitization of the 5HT₂ receptor

A suspension of Fura 2-loaded platelets was divided into three batches, A, B and C. They were preincubated for 5 min in the presence of H7 ($60 \mu M$) (B and C) or solvent (A). Subsequently, batches A and C were desensitized to 5HT by stimulation with $1 \mu M$ 5HT. At the time points indicated, samples of A, B and C were stimulated with $1 \mu M$ 5HT again.

receptors have been extensively studied [1]. Although other mechanisms cannot be excluded, covalent modification of the receptor protein by phosphorylation has been shown to uncouple this receptor from its signal-transducing system. Subsequently, internalization of the receptor mediates down-regulation and is linked to desensitization. In contrast with β -adrenergic receptors, there is a lack of information about these processes for phospholipase C-linked G-protein-coupled receptors [3,6,14,26]. In addition, as regards resensitization, neither the molecular mechanisms that control the process nor the functional role have been described.

Exposure of receptors to neurotransmitters or hormones is often short-lived, because of sequestration by avid re-uptake systems, degradation at their site of release, or elimination by transport [27,28]. We suppose that these agonist-elimination mechanisms fit into a cellular strategy to recover signal-transduction systems after previous stimulation and termination of the cellular response. Accordingly, we found that in a suspension of platelets a near-maximally effective 5HT concentration decreased to undetectable levels within 7.5 min (Table 1) and that the desensitization–resensitization cycle of platelets to 5HT takes only 30 min (Figures 6–9). The fact that H7 delayed 5HT uptake as well as resensitization suggests, but does not prove, that the 5HT-uptake system might influence the length of the desensitization–resensitization cycle or is necessary for resensitization to occur. Nevertheless, the rapid resensitization that we observed indicates that desensitization of the 5HT₂ receptor is a transient phenomenon and fits, together with the presence of the 5HT-uptake system, into a signal-transduction recovery strategy that enables platelets to go through repeated stimulus–response cycles upon repeated exposure to 5HT.

Since desensitization to 5HT is strictly dependent on the dose of the initial stimulation (Figure 4), occupation of the 5HT₂ receptor by 5HT appears to play an essential role in the desensitization process. This is confirmed by the fact that all efforts that we made to induce desensitization to 5HT, by activating the phospholipase C-linked signal-transduction pathway without activating 5HT₂ receptors, failed. Desensitization to 5HT could not be induced with ionomycin to increase $[Ca^{2+}]_{cyt}$ (Figure 5), with OAG to activate protein kinase C (Figure 5), or with a much stronger platelet stimulus, such as VP (Figure 3), which uses exactly the same downstream phospholipase C signal-transduction mechanism linked to the V1 receptor [15]. Compartmentalization of the signal-transduction mechanism that is linked to the receptor might be a possible explanation for the crucial role of receptor occupation in desensitization to 5HT. Evidence that activation of protein kinase C by thrombin might be spatially isolated in human platelets has been presented by others previously [29], and extensive compartmentalization of receptor-coupled intracellular Ca^{2+} stores has also been proposed for parotid acinar cells [30]. If it is accepted that each receptor is linked to one set of signal-transduction proteins that is not interchangeable between different receptors, signal transduction should be considered as a strictly localized phenomenon in the intracellular space. As a consequence, a general increase in $[Ca^{2+}]_{cyt}$ or of protein kinase C activity, by ionomycin or OAG respectively, might be insufficient indeed to trigger the strictly localized mechanism that is responsible for desensitization of the signal-transduction pathway linked to the 5HT₂ receptor. Likewise, this mechanism would not necessarily be affected by strong activation of the set of signal-transduction proteins, including phospholipase C and protein kinase C, linked to the V1 receptor.

The desensitization–resensitization process of the signal-transduction mechanism for 5HT could be divided into three phases, based on the presence or absence of receptor–effector coupling.

During the early desensitization phase, the effects of ketanserin (Figure 1b) and of adrenaline (Figure 1c) indicate that 5HT₂ receptors are still coupled to phospholipase C. Therefore, the decrease in the intracellular concentration of second messengers (Figure 1a; Figure 2, left panel) during this phase cannot be explained by decreased receptor–effector coupling alone. Other mechanisms, depending on which intracellular messenger is used to monitor the early phase of desensitization, are also involved. During the late phase of desensitization, receptor–effector coupling was completely disconnected (Figure 2, right panel). Whether or not this disconnection was mediated by down-regulation via internalization of receptors during desensitization–resensitization, by analogy with β -adrenergic receptors, could not be determined, due to the presence of the platelet 5HT-uptake system.

The resensitization process of the 5HT-induced responses enabled us to follow the recovery of the receptor–effector coupling and to compare the reconstitution of the different post-phospholipase-C events as a function of time. The signal-transducing system coupled to the 5HT₂ receptor could be re-activated as quickly as 12.5 min after the first exposure to 5HT (Figure 6). We found a near-perfect correlation between the extent of activation of phospholipase C, measured as an increase in PA, and the increase in $[Ca^{2+}]_{cyt}$ (Figure 6). Since DAG is the metabolic precursor of PA, as well as the endogenous activator of protein kinase C, 5HT-induced 47 kDa-protein phosphorylation became resensitized, as could be expected, within the same time course (Figure 7).

Our finding that activation of phospholipase C, activation of protein kinase C and mobilization of Ca^{2+} are all resensitized within the same time course strongly confirms that these steps are controlled by the same coupling mechanism, and indicates that recovery of the coupling between the 5HT₂ receptor and phospholipase C is most probably the rate-determining step in resensitization. For 5HT-induced activity of MLCK, the situation appeared to be different. MLCK only remained partly desensitized. Even 3 and 7 min after the first addition of 5HT, a 5HT-inducible phosphorylation of myosin light chain was obvious. How MLCK, which is a Ca^{2+} -dependent enzyme [12], was activated is not clear. As reported previously in smooth muscle [31], Ca^{2+} fluxes that are not detectable by Fura 2 might be responsible for the increase in MLCK. Unless an alternative signal-transducing system for the 5HT₂-receptor-mediated activation of MLCK was operating, possibly even coupled to another 5HT-receptor subtype, this observation might indicate that activation of MLCK is a far more sensitive measure for activation of the platelet 5HT₂ receptor than is measurement of PA formation or $[Ca^{2+}]_{cyt}$ with Fura 2.

It has been repeatedly suggested that protein kinase C is involved in termination of primary responses that are induced by occupation of G-protein-coupled receptors [11,13,14,21]. Receptor-induced activation of protein kinase C either directly or indirectly should lead to phosphorylation of the inducing receptor peptide, the G-protein or the effector protein, resulting in functional desensitization of this receptor [13]. In platelets stimulated by thrombin, activation of protein kinase C has been shown to inhibit phospholipase C activity by a negative-feedback loop, possibly involving G-proteins [13,14]. In contrast, stimulation of phospholipase C by the endoperoxide analogue U44069, a weaker platelet agonist, was unaffected by the protein kinase C inhibitor staurosporine [14]. Since 5HT is also a weak agonist, the latter is in accordance with our observation (Figure 1e) that H7 had no effect on early desensitization of the 5HT-induced mobilization of Ca^{2+} . Apparently, in desensitization to weak platelet agonists, the contribution of protein kinase C activity

generated by occupation of the receptor is minimal. In addition, this might be an alternative explanation, apart from compartmentalization of signal transduction, for the fact that platelets were not desensitized to 5HT after stimulation of protein kinase C by OAG (Figure 5). A role for protein kinase C in the desensitization–resensitization cycle, although not apparent when desensitization is considered alone, is suggested by the fact that resensitization was markedly attenuated in the presence of the protein kinase C inhibitor H7 (Figure 9). A phosphorylation step that is catalysed by protein kinase C during the acute phase of desensitization might be involved later in the recoupling process of 5HT₂ receptors in platelets desensitized to 5HT. However, a contribution of the H7-induced delay of the 5HT uptake in the delayed resensitization to 5HT cannot be excluded. Therefore a clear-cut conclusion concerning the role of protein kinase C in resensitization to 5HT awaits the development of potent and selective protein kinase C inhibitors.

The contribution of Ca²⁺ influx from the external medium in the 5HT-induced increase in [Ca²⁺]_{cyt} as measured in Fura 2-loaded human platelets is very small [11] (Figure 4). Therefore, the Ca²⁺ ions for the 5HT-induced Ca²⁺ response must almost entirely be supplied by intracellular Ca²⁺ pools. Depletion of intracellular Ca²⁺ pools cannot explain late desensitization to 5HT, since VP could still mobilize Ca²⁺ from intracellular stores (Figure 4), unless intracellular Ca²⁺ pools are extensively compartmentalized. In view of this possibility, we tested whether the presence of Ca²⁺_{ex} could play a role in resensitization to 5HT by replenishing these hypothesized intracellular 5HT-specific Ca²⁺ pools. Obviously (Figure 9), resensitization to 5HT was completely independent of the presence of Ca²⁺_{ex}. Thus, refilling of postulated 5HT-specific Ca²⁺ pools with Ca²⁺ from the extracellular medium is not rate-determining in the resensitization of platelets to 5HT.

In conclusion, the results of this study prove that desensitization of human platelets to 5HT is homologous, dependent on occupation of the 5HT₂ receptor and reversible. Based on whether or not 5HT₂ receptors are coupled to phospholipase C, the desensitization–resensitization cycle can be divided into an early and a late desensitization phase followed by a resensitization phase. The late desensitization phase and the resensitization phase are characterized by the complete absence or the recovery, respectively, of coupling at a level up to or preceding phospholipase C. Early desensitization cannot be explained either by disconnection of this coupling or by the decrease of the 5HT concentration during 5HT uptake. Most probably, 5HT-induced activation of protein kinase C is not involved in early or late desensitization to 5HT, but possibly enhances resensitization. Ca²⁺ influx does not seem to play an important role in this desensitization–resensitization cycle, but the 5HT-induced

increase in platelet [Ca²⁺]_{cyt} proved to be an excellent monitor for study of the phenomenon.

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REFERENCES

- 1 Lefkowitz, R. J., Hausdorff, W. P. and Caron, M. G. (1990) *Trends Pharmacol. Sci.* **11**, 190–194
- 2 Briscoe, C. P., Plevin, R. and Wakelam, M. J. O. (1994) *Biochem. J.* **298**, 61–67
- 3 Benya, R. V., Kusui, T., Shikado, F., Batteys, J. F. and Jensen, R. T. (1994) *J. Biol. Chem.* **269**, 11721–11728
- 4 Neer, E. J. and Clapham, D. E. (1988) *Nature (London)* **333**, 129–134
- 5 Hug, H. and Sarre, T. F. (1993) *Biochem. J.* **291**, 329–343
- 6 Wojcikiewicz, R. J. H., Tobin, A. B. and Nahorski, S. R. (1993) *Trends Pharmacol. Sci.* **14**, 279–285
- 7 De Clerck, F., David, J.-L. and Janssen, P. A. J. (1982) *Agents Actions* **12**, 388–397
- 8 de Chaffoy de Courcelles, D., Leysen, J. E., De Clerck, F., Van Belle, H. and Janssen, P. A. J. (1985) *J. Biol. Chem.* **260**, 7603–7608
- 9 de Chaffoy de Courcelles, D., Roevens, P., Wynants, J. and Van Belle, H. (1987) *Biochim. Biophys. Acta* **927**, 291–302
- 10 de Chaffoy de Courcelles, D., Roevens, P. and van Belle, H. (1984) *FEBS Lett.* **171**, 289–292
- 11 Kagaya, A., Mikuni, M., Kusimi, I., Yamamoto, H. and Takahashi, K. (1990) *J. Pharmacol. Exp. Ther.* **255**, 305–311
- 12 Siess, W. (1989) *Physiol. Rev.* **69**, 58–178
- 13 Crouch, M. F. and Lapetina, E. G. (1988) *J. Biol. Chem.* **263**, 3363–3371
- 14 Crouch, M. F. and Lapetina, E. G. (1989) *J. Biol. Chem.* **264**, 584–588
- 15 Pollock, W. K. and MacIntyre, D. E. (1986) *Biochem. J.* **234**, 67–73
- 16 Yakel, J. L., Lagrutta, A., Adelman, J. P. and North, R. A. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5030–5033
- 17 Stahl, S. M. (1985) in *The Platelets, Physiology and Pharmacology: Platelets as Pharmacologic Models for the Receptors and Biochemistry of Monoaminergic Neurons* (Longenecker, G. L., ed.), pp. 307–340, Academic Press, Mobile, AL
- 18 Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- 19 Rink, T. J. and Pozzan, T. (1985) *Cell Calcium* **6**, 133–144
- 20 De Clerck, F., Loots, W., Somers, Y., Beetens, J., Wouters, L., Wynants, J. and Janssen, P. A. J. (1990) *Br. J. Pharmacol.* **99**, 631–646
- 21 de Chaffoy de Courcelles, D., Roevens, P., Van Belle, H., Kennis, L., Somers, Y. and De Clerck, F. (1989) *J. Biol. Chem.* **264**, 3274–3285
- 22 Leysen, J. E. and Gommeren, W. (1986) *Drug Dev. Res.* **8**, 119–131
- 23 Roevens, P., De Clerck, F. and de Chaffoy de Courcelles, D. (1993) *Eur. J. Pharmacol.* **245**, 273–280
- 24 Watson, S. P., McNally, J., Shipman, L. J. and Godfrey, P. P. (1988) *Biochem. J.* **249**, 345–350
- 25 de Chaffoy de Courcelles, D., Roevens, P. and Van Belle, H. (1984) *Biochem. Biophys. Res. Commun.* **123**, 589–595
- 26 Brass, L. F. (1992) *J. Biol. Chem.* **267**, 6044–6050
- 27 Burnstock, G. (1981) *J. Physiol. (London)* **313**, 1–35
- 28 Kanner, B. and Shuldiner, S. (1987) *Crit. Rev. Biochem.* **22**, 1–38
- 29 Crouch, M. F. and Lapetina, E. G. (1988) *Biochem. Biophys. Res. Commun.* **156**, 149–156
- 30 Muallem, S., Pandol, S. J. and Beeker, T. G. (1989) *J. Biol. Chem.* **264**, 205–212
- 31 Rasmussen, H. (1989) *Sci. Am.* October 66–73