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SEROTONIN INHIBITION OF ADENYLATE CYCLASE IN HUMAN PLATELET MEMBRANES; RELATION TO 5-HT-1A RECEPTOR-MEDIATED ACTIVITY

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Abstract—Serotonin inhibited both basal and forskolin-stimulated adenylate cyclase activity in human platelet membranes by approx. 30%, with an EC_{50} of 54 nM. Addition of NaCl to the assay medium reduced the degree of inhibition. 5-carboxamidotryptamine (5-CT) behaved as a full agonist in this system (EC_{50} of 5.4 nM) and BMY 7378 as a partial agonist (inducing 19% inhibition); the putative 5-HT-1a receptor agonists metergoline, spiroxatrine and MDL 73005 were inactive. The 5-HT-1a receptor antagonists metitepin and NAN-190 behaved as antagonists with K_b or K_i values of 11.2 and 1.17 nM, respectively. Spiperone behaved as a partial antagonist only. Epinephrine and 5-HT produced convergent, non-additive inhibition of both basal and forskolin-stimulated cyclase.

Key words: serotonin; 5-HT-1a receptors; adenylate cyclase; platelet; epinephrine; signal transduction

In recent years multiple 5-HT† receptors have been characterized both by pharmacological binding and functional studies and by molecular cloning procedures. The nomenclature of these receptors has recently been reviewed [1, 2]. 5-HT-1 receptors in particular have been shown to have many sub-types, several of which (5-HT-1a, 5-HT-1b, 5-HT-1d, 5-HT-1e and 5-HT-1f) are negatively coupled to the enzyme adenylate cyclase. Human platelets have been shown to possess 5-HT-2 receptors [3] and a 5-HT uptake site which can be labelled by [^{3}H]-8-OH-DPAT [4], but the existence of other sub-types has until now been uncertain.

In a previous publication [5], the ability of 5-HT and some of its analogs to inhibit forskolin-stimulated adenylate cyclase in human platelet membranes was described. This activity paralleled that shown by 5-HT in hippocampal membranes of calf, rat and guinea-pig [6, 7] and also in intact mouse hippocampal neurons [8], an activity mediated by 5-HT-1a receptors. Although the activity of the human platelet enzyme showed some similarities to the 5-HT-1a receptor-mediated activity, important differences were also observed. Thus, the prototypal 5-HT-1a receptor agonist 8-OH-DPAT showed only minimal inhibition, and spiperone and pindolol were only partially active as antagonists. In the present report the human platelet activity was further characterized by the use of additional agonists and antagonists. In addition the relationship between the 5-HT mediated activity and epinephrine-induced inhibition of adenylate cyclase, an activity mediated by alpha-2 adrenoceptors, was investigated in order to determine the nature of the interaction between the two inhibitory receptors in the human platelet.

MATERIALS AND METHODS

Preparation of platelet membranes. Platelet membranes were prepared from platelet concentrates obtained from the blood bank at Hadassah University Hospital. The concentrates were centrifuged at 400 *g* for 20 min, and the supernatants discarded. If red blood cells were present in the pellet after this centrifugation, the pellet was re-suspended in a small volume of buffer comprised of 0.32 M sucrose, 20 mM Tris–HCl, pH 7.4, 5 mM EDTA, 5 mM dithiothreitol and centrifuged for 2 min only at 100 *g*. The pellet containing the red blood cells was discarded. The supernatant from this centrifugation was then diluted with more buffer and re-centrifuged at 400 *g* for 20 min. The pellet was re-suspended in a volume of 10 mL and homogenized using a Polytron apparatus (5 sec at position 5), diluted with approx. 60 mL buffer, and centrifuged for 20 min at 48,000 *g*. The pellet from this centrifugation was resuspended in buffer with Polytron homogenization and frozen at −85° in 1.5 mL aliquots.

Adenylate cyclase assay. The reaction was performed in an assay volume of 0.2 mL containing 80 mM Tris–HCl, pH 7.4 at 18°; 1 mM EDTA; 1 mM dithiothreitol; 2 mM $MgSO_4$; 0.1 mM ATP; 10 μM GTP; 1 μCi [alpha-^{32}P]ATP; 1.5 mg/mL creatine phosphate; 0.2 mg/mL creatine phosphokinase and 1 mM 3-isobutyl-1-methylxanthine. Incubations were started by addition of ≈50 μg protein, and terminated after 10 min at 30° by adding 0.1 mL of a solution containing 0.5 mM cyclic AMP and 4 mM ATP and transferring the tubes to a boiling water bath. [^{32}P] cyclic AMP was separated from ATP by the double column method [9]. All concentration points were

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† Abbreviations: 5-CT, 5-carboxamidotryptamine; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; 5-HT, serotonin

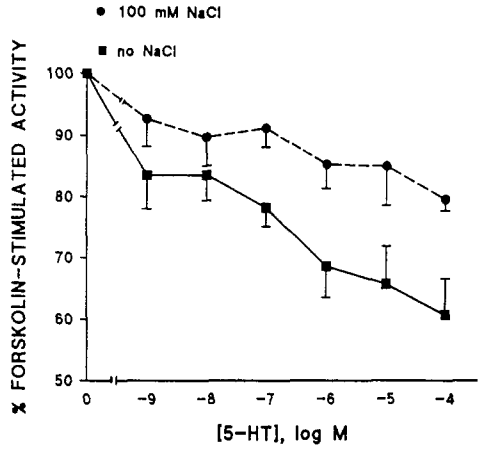

Fig. 1. Effect of 5-HT on forskolin (30 μM)-stimulated adenylate cyclase activity in human platelet membranes in the presence (●) or absence (■) of 100 mM NaCl. Each point represents the mean ± SEM of six determinations.

tested in duplicate, each observation corresponding to the mean of duplicate samples.

Data analysis. EC_{50} values and maximal degrees of inhibition were calculated from dose-response curves by computerized curve-fitting according to the Michaelis–Menten equation using the program ENZFIT (Elsevier Biosoft Ltd). K_b values were calculated from EC_{50} values obtained in the presence and absence of antagonist (Fig. 5) as described by Schoeffter and Hoyer [7]. The Cheng–Prusoff equation was used to calculate K_i values from experiments in which an IC_{50} value for an antagonist was determined (Fig. 6), as described by Craig [10].

Materials. 5-CT, BMY 7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro [4, 5] -decane—7,9-dione dihydrochloride), NAN-190 (1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]-piperazine) and spiroxatrine were purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.). 5-HT, epinephrine and spiperone were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The following chemicals were kind gifts of the companies indicated: MDL 73005 (8-[2-[2,3-dihydro-1,4-benzodioxin-2-yl)methylamino]ethyl]-8-azaspiro[4, 5]decan-7,9-dione methane sulphonate) from the Merell Dow Research Institute, (Strasbourg, France); metergoline from Farmitalia Carlo Erba (Milan, Italy); and metitepin from F. Hoffmann-LaRoche (Basel, Switzerland).

RESULTS

Optimum conditions for determining 5-HT induced inhibition of adenylate cyclase were first selected. In an earlier publication [5] inhibition was determined in the presence of 100 mM NaCl, conditions adapted from those used for measurement of 5-HT-1a receptor-mediated activity in hippocampal membranes [6]. However, Fig. 1 shows that in human platelet membranes the degree of 5-HT-induced inhibition was actually greater in the absence of

Table 1. Effect of addition of 100 mM NaCl on basal and forskolin-stimulated adenylate cyclase activities in human platelet membranes

Addition	None	100 mM NaCl
Basal activity	39.2 ± 1.9 (6)	11.9 ± 0.5 (6)
30 μM Forskolin	318.1 ± 36.2 (5)	110.5 ± 12.6 (5)

Results are expressed in pmol/min/mg protein and are means ± SEM of the number of samples indicated in parentheses, each sample being assayed in duplicate.

NaCl (maximal inhibition with 100 mM NaCl, 14.5 ± 2.1%; in the absence of NaCl, 34.7 ± 2.5%, mean ± SEM of six observations in each case). Separate experiments (data given in Table 1) showed that NaCl alone inhibited both basal and forskolin-stimulated activity. This effect of NaCl probably accounted for its action in relatively reducing the amount of inhibition induced by 5-HT. A similar effect for epinephrine inhibition of platelet adenylate cyclase activity has been documented by Steer and Wood [11] and Bockaert *et al.* [12]. Figure 2 shows that there was no difference in the degree of inhibition produced by 5-HT between basal and forskolin-stimulated adenylate cyclase when NaCl was omitted from the assay medium. Under basal assay conditions, maximal inhibition was 28.9 ± 4.7% (mean ± SEM of seven determinations) while for forskolin-stimulated activity the corresponding value was 28.7 ± 2.3% (mean ± SEM of three determinations). The EC_{50} for 5-HT was 53.9 ± 26.3 nM (mean ± SEM of nine determinations). Thus, in experiments involving the use of other putative agonists, their effects were determined on basal

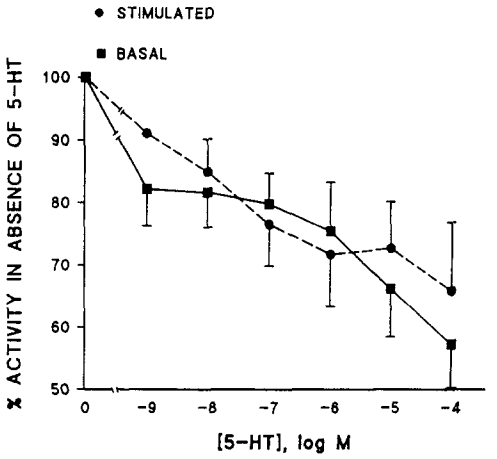

Fig. 2. Effect of 5-HT on basal (■) and forskolin (30 μM)-stimulated (●) adenylate cyclase activity in human platelet membranes. Assays were conducted in the absence of NaCl. Each point represents the mean ± SEM of seven determinations in the case of basal activity and three determinations for forskolin-stimulated activity.

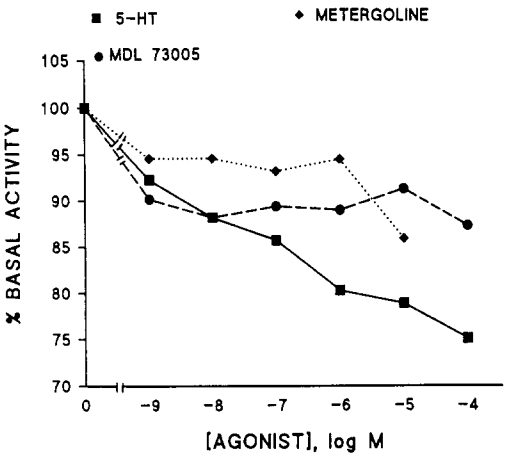

Fig. 3. Effects of various concentrations of 5-HT (■), metergoline (◆) and MDL 73005 (●) on basal adenylate cyclase activity in human platelet membranes. Each point represents the mean of five observations in each case. Standard errors are omitted for clarity.

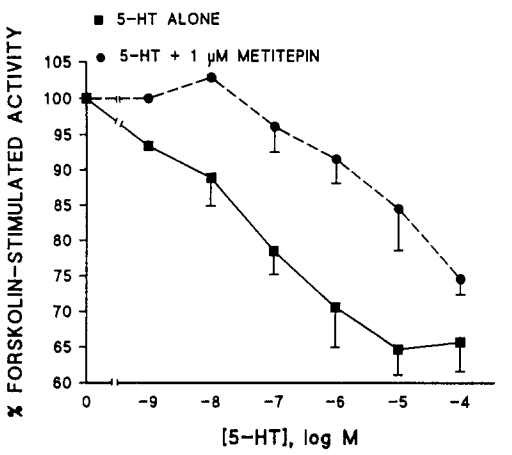

Fig. 5. Effect of 5-HT on forskolin (30 μM)-stimulated adenylate cyclase activity in human platelet membranes in the presence (●) or absence (■) of 1 μM metitepin. Each point represents the mean ± SEM of three determinations.

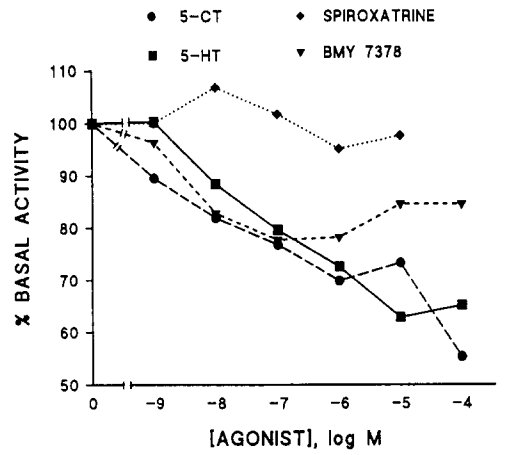

Fig. 4. Effects of various concentrations of 5-HT (■), 5-CT (●), BMY 7378 (▼) and spiroxatrine (◆) on basal adenylate cyclase activity in human platelet membranes. Each point represents the mean of four observations in each case. Standard errors are omitted for clarity.

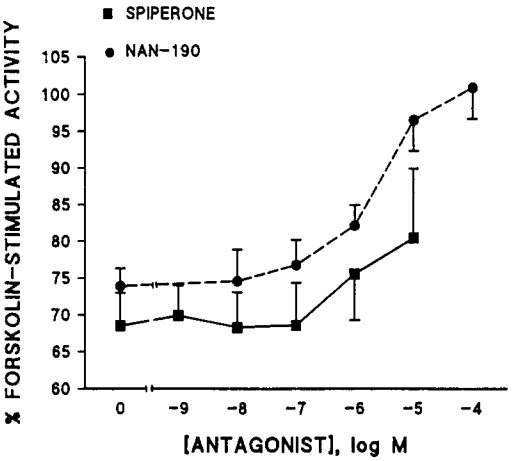

Fig. 6. Effects of various concentrations of spiperone (■) and NAN-190 (●) on 100 μM 5-HT-mediated inhibition of forskolin-stimulated adenylate cyclase activity in human platelet membranes. Each point represents the mean ± SEM of 12 observations for spiperone and five observations for NAN-190.

adenylate cyclase in the absence of NaCl. Stimulation of adenylate cyclase by 5-HT was not observed under the present assay conditions.

The effects of the addition of various concentrations of compounds which have been characterized as 5-HT-1a receptor agonists in other systems are shown in Figs 3 and 4. 5-CT behaved as a full agonist, causing maximal inhibition of 31.5 ± 4.3% with an EC_{50} of 5.4 nM, compared to 5-HT which in the same experiment caused maximal inhibition of 33.5 ± 2.6% with an EC_{50} of 45.0 ± 26.4 nM. BMY 7378, on the other hand, induced maximal inhibition of only 19.1 ± 1.8%, indicating that this compound was behaving as a partial agonist. None of the compounds—spiroxatrine, metergoline and MDL 73005—showed any significant agonist activity.

Figure 5 shows the effect of the addition of 1 μM metitepin on the 5-HT inhibition of forskolin-stimulated activity. Metitepin shifted the dose–response curve to the right but only reduced the maximal inhibition produced by 5-HT by a small amount (35.3 ± 2.4% in the absence of metitepin, 23.8 ± 3.7% in its presence, mean ± SEM of three observations in each case), consistent with its action as a competitive antagonist. The calculated K_b for metitepin was 11.2 nM. The effects of the addition

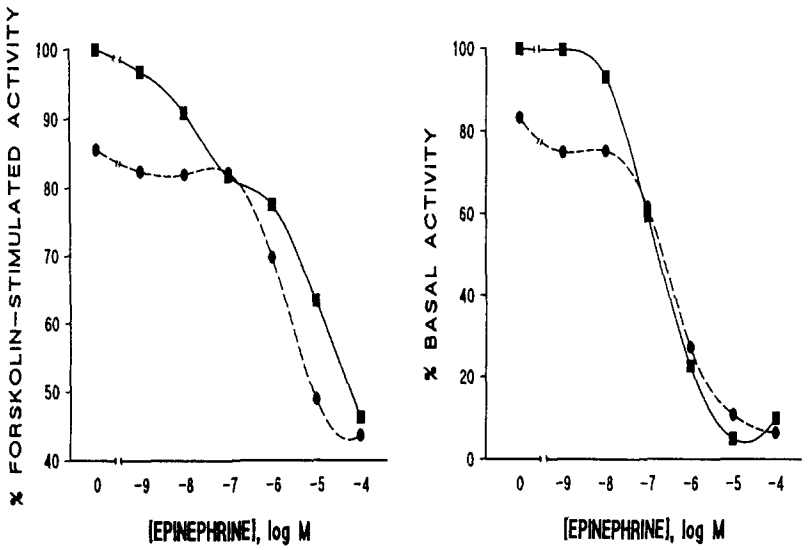

Fig. 7. Effects of various concentrations of epinephrine on adenylate cyclase activity in human platelet membranes measured alone (■) or in the additional presence of 100 μM 5-HT (●). The left-hand panel shows results from experiments performed with 30 μM forskolin and 100 mM NaCl. Each point represents the mean of at least four determinations. Standard errors are omitted for clarity.

of various concentrations of 5-HT receptor antagonists on the degree of inhibition induced by 100 μM 5-HT are shown in Fig. 6. The selective 5-HT-1a receptor antagonist NAN-190 produced a complete reversal of 5-HT-induced inhibition, with an IC_{50} of 2.17 ± 0.63 μM, corresponding to a K_i for this compound of 1.17 nM. The non-selective 5-HT-1 receptor antagonist spiperone only partially reversed 5-HT-induced inhibition. 5-HT (100 μM) caused 31.5 ± 4.5% inhibition of forskolin-stimulated activity in the absence of spiperone and 19.5 ± 9.5% inhibition in the presence of 10 μM spiperone (mean ± SEM of 12 observations in each case). The IC_{50} for spiperone was 0.98 ± 0.50 μM, corresponding to a K_i of 0.53 nM. Partial antagonism by spiperone was also observed when the effect of various concentrations of 5-HT on the inhibition of forskolin-stimulated cyclase was measured in the presence of 10 μM spiperone [5].

Figure 7 shows the effect of adding 100 μM 5-HT on the action of epinephrine in inhibiting cyclase activity under both basal and forskolin-stimulated conditions. In both cases no additional effect of 5-HT on the degree of inhibition was seen when epinephrine concentration exceeded 0.1 μM, a concentration at which the effect of epinephrine was greater than that given by 100 μM 5-HT alone. An inhibitory effect of 5-HT was thus only observed at low epinephrine concentrations. As in the case of 5-HT inhibition, a greater effect was obtained when NaCl was omitted from the assay (basal conditions, right-hand panel) than when NaCl was included (forskolin stimulation, left-hand panel). Under basal conditions in the absence of NaCl, the EC_{50} for epinephrine when assayed alone was 134 ± 86 nM and maximal inhibition 91.6 ± 2.0%, while in the additional presence of 100 μM 5-HT the EC_{50} was 106 ± 69 nM and maximal inhibition 88.3 ± 9.9%. Corresponding values for forskolin stimulation in the presence of 100 mM NaCl were: EC_{50} 291 ± 26 nM and maximal inhibition 42.9 ± 6.9% for epinephrine alone; EC_{50} 249 ± 25 nM and maximal inhibition 51.0 ± 9.3% in the additional presence of 100 μM 5-HT.

DISCUSSION

The present results demonstrate that human platelet membranes possess a "5-HT-1a-like" receptor coupled to inhibition of adenylate cyclase via the same pool of Gi proteins which are responsible for transduction of the signal carried by alpha-2 adrenergic receptors. Although preliminary experiments [13] had suggested that 5-HT may stimulate adenylate cyclase when assayed under basal conditions in a manner analogous to the stimulatory activity reported in rat and guinea-pig hippocampal membranes [14, 15], this phenomenon was not observed in the present series of experiments. This finding is in accordance with the failure of several groups to observe 5-HT-induced stimulation of the enzyme after transfection of the human 5-HT-1a receptor gene into clonal cell lines [16–20]. As in work previously published [5], the dose–response curves obtained for 5-HT-induced inhibition in the present experiments encompassed a wide concentration range, from 1 nM to 100 μM, and did not always reach a clear maximal effect. This suggests that two sites, one possibly corresponding to a 5-HT-1a receptor, may be involved in the effect.

The similarity of the 5-HT mediated response in the human platelet membrane with activity mediated in other systems by 5-HT-1a-like receptors is further strengthened by the results described here. 5-CT, a

full agonist with high affinity at the 5-HT-1a receptor in guinea-pig [6], calf [7] and mouse [8] hippocampus, was equally effective with 5-HT and indeed showed a higher affinity. BMY 7378, which in behavioural studies in pigeons exhibited partial agonist activity at the 5-HT-1a receptor [21] behaved as a partial agonist with respect to human platelet adenylate cyclase as well. This activity, however, contrasts with that at adenylate cyclase in hippocampal membranes, where BMY 7378 has been shown to act as a competitive antagonist [22]. Other differences from the activity of the adenylate cyclase coupled to 5-HT-1a receptors in animal hippocampal membranes are indicated by the action of metergoline, spiroxatrine and MDL 73005. Metergoline was a full agonist in both guinea-pig and calf hippocampal membranes [6, 7], while spiroxatrine was a full agonist in calf membranes [7] and MDL 73005 a partial agonist in rat and calf hippocampal membranes [23, 24]. None of these compounds showed any agonist activity in human platelet membranes in the present experiments, although it should be noted that in recent studies MDL 73005 was also found to be devoid of agonist activity in rat hippocampal membranes [25]. Differences in receptor reserve and coupling efficiency may explain the different results obtained in platelet and hippocampal membranes.

The effects of antagonists provide more supporting evidence for the belief that the platelet enzyme activity is in fact mediated by a "5-HT-1a-like" receptor. Both NAN-190 and metitepin produced complete antagonism of 5-HT-mediated inhibition, with spiperone having a partial effect. The K_i obtained for NAN-190 antagonism of 5-HT inhibition in the present work, 1.17 nM, was somewhat lower than that obtained in transfected cells bearing the human 5-HT-1a receptor gene (17.8 nM, [17]), but very similar to the K_b values obtained in either guinea-pig (1.9 nM, [26]) or calf (2.09 nM, [24]) hippocampal membranes. Although NAN-190 has also been shown to have antagonistic effects at the alpha-1 adrenergic receptor [27], this receptor is not coupled to adenylate cyclase. NAN-190 was inactive as an antagonist at the 5-HT-1b or 5-HT-1c receptors, although it did behave as a limited competitive antagonist at the 5-HT-1d receptor [24]. The K_b value obtained for metitepin, 11.2 nM, corresponds closely to that obtained for the inhibition of cyclase at the 5-HT-1a receptor in guinea-pig (13 nM, [6]) or calf (23 nM, [7]) hippocampal membranes. It should be noted that another "5-HT-1a-like" receptor has been described in mouse cortical neurons grown in culture by Dumuis *et al.* [28]. The classical 5-HT-1a receptor agonist 8-OH-DPAT was a poor partial agonist both at this receptor and in platelet membranes [5], while metitepin and pindolol behaved as antagonists in both systems. Similarly, metergoline, an agonist at the "classical" 5-HT-1a receptor, was inactive in platelet membranes and even behaved as an antagonist in the mouse cortical system.

Incubation of platelet membranes in the present experiments with both epinephrine and 5-HT resulted in convergent, non-additive inhibitory responses. A similar effect has been observed in rat hippocampus for somatostatin and muscarinic receptors by Eva and Costa [29], and for adenosine A1 and 5-HT-1a receptors by Zgombick *et al.* [30]. This phenomenon can be explained by the fact that in each case the receptors are linked to the same pool of the inhibitory GTP-binding protein Gi. Non-additivity of the inhibitory cyclase responses produced by activation of adenosine A1 and GABA-B receptors was also observed by Wojcik *et al.* [31] in rat cerebellum, again indicating that Gi activation constitutes the rate-limiting step in the sequence of events leading to cyclase inhibition. The present results contrast with the synergistic responses obtained with 5-HT and epinephrine in the human platelet when the activity of another enzyme involved in signal transduction, phospholipase C, was measured [3]. This discrepancy may be related to differential coupling of the two enzymes to different G proteins.

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