Tyrosine kinase inhibition reduces the plateau phase of the calcium increase in response to progesterone in human sperm

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Abstract Progesterone (P) has previously been shown to induce a rapid increase in $[Ca^{2+}]_i$ as well as tyrosine phosphorylation of proteins in human spermatozoa. Both these effects are essential for induction of the acrosome reaction by P. We investigated a possible relationship between the P-induced calcium increase and tyrosine kinase activation, by evaluating the effect of the tyrosine kinase inhibitor genistein on these two effects. We found that preincubation with genistein abolished P-induced tyrosine phosphorylation of two sperm proteins of 97 and 75 kDa molecular weight and significantly inhibited the plateau phase of P-induced $[Ca^{2+}]_i$ increase without affecting the peak phase. Conversely, the plateau phase was enhanced by the tyrosine phosphatase inhibitor Na_3VO_4. The effect of genistein was specific for P, since no inhibition was observed on the $[Ca^{2+}]_i$ increase induced by thapsigargin, an inhibitor of endoplasmic Ca^{2+}-ATPase previously shown to mobilize Ca^{2+} in spermatozoa. These results indicate that tyrosine kinase activation is involved in the generation of the plateau phase of Ca^{2+} influx induced by P, and suggest the possibility that two different pathways are involved in the induction of Ca^{2+} entry by P in human sperm.

Key words: Spermatozoa; Progesterone; Intracellular calcium; Tyrosine phosphorylation; Nongenomic steroid effect

1. Introduction

The acrosome reaction of spermatozoa is a specialized exocytotic event promoted by an increase in intracellular Ca^{2+} essential for the process of fertilization [1]. This irreversible reaction occurs after interaction of the spermatozoon with the oocyte and consists of the fusion and fenestration of the outer acrosomal membrane with the plasma membrane, resulting in the release of acrosomal enzymes which aid the spermatozoon in penetrating the various investments of the oocyte [1]. Among the different stimuli of acrosome reaction, progesterone (P) is considered, together with zona pellucida proteins, to be one of the physiological inducers of this event, being present in high levels in the cumulus matrix that surrounds the oocyte [2]. P triggers calcium influx [3–5] in human spermatozoa by stimulating a poorly characterized cell surface receptor [6], which appears to be different from nuclear P receptors [5,7]. P-induced calcium waves are characterized by an initial transient peak followed by a sustained plateau phase lasting for several minutes [4,5]. Both these effects are dependent on extracellular calcium since they do not occur when calcium is removed from the external medium [4]. In addition to an increase in calcium, P has been shown to promote tyrosine phosphorylation of sperm proteins [8,9]. Such an effect is, at least partially, involved in induction of the acrosome reaction, since inhibitors of tyrosine kinases blunted the exocytotic effect of P [8,9]. However, P-induced tyrosine phosphorylation of proteins is independent of extracellular calcium [8], raising the question of whether the increase in $[Ca^{2+}]_i$ stimulated by P, which is also essential for induction of the acrosome reaction of spermatozoa [10,11], could be downstream of tyrosine kinase activation, as recently demonstrated in other cell systems [12–15]. We have investigated the involvement of tyrosine kinase activity in the regulation of Ca^{2+} entry induced by P, by studying the effect of the tyrosine kinase inhibitor genistein on tyrosine phosphorylation of sperm proteins and on the increase in $[Ca^{2+}]_i$ stimulated by P. We found that tyrosine kinase inhibition significantly inhibits the plateau phase of Ca^{2+} entry without affecting the rapid peak phase of the P-induced calcium response.

2. Materials and methods

2.1. Chemicals

Fura-2/AM, thapsigargin, free acid ionomycin, genistein and herbimycin were purchased from Calbiochem (La Jolla, CA). Sodium orthovanadate (Na_3VO_4), progesterone, fatty acid-free bovine serum albumin (BSA), gelatin, glycine, trizmabase, Temed, APS, *o*-phospho-DL-tyrosine, Ponceau S and Cooamassie R250, ethylene glycol-bis (β-aminoethyl ether) tetraacetic acid (EGTA) were from Sigma (St. Louis, MO). Sangivamycin was obtained from National Institutes of Health Drug Synthesis and Chemistry Branch (Bethesda, MD). Peroxidase-conjugated monoclonal (PY 20) anti-phosphotyrosine antibody was from ICN (Costa Mesa, CA). Reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were from Bio-Rad Labs (Hercules, CA). Protein molecular weight standards and Tween 20 were from Fluka Chemie AG (Bucks, Switzerland). Percoll was obtained from Pharmacia LKB (Uppsala, Sweden). Enhanced chemiluminescence (ECL) Western blotting detection reagents were from Amhersham (Buckinghamshire, UK). Sperm preparation medium (229.5 mg/l $CaCl_2 \cdot 2H_2O$, 346.55 mg/l KCl, 173.27mg/l $MgSO_4 \cdot 7H_2O$, 5891.28 mg/l NaCl, 2200 mg/l $NaHCO_3$, 137.15 mg/l Na $H_2PO_4 \cdot 2H_2O$, 866.36 mg/l D-glucose, 8.66 mg/l Phenol red, 97.0 mg/l Na-pyruvate, 1000 mg/l HEPES sodium salt, 3010 mg/l HEPES acid, 1.0 ml/l SSR2 (Comp. A), 1.0 ml/l SSr2 (Comp. B), 50 mg/ml streptomycin, 50,000 IU/l penicillin and 10 mg/ml human serum albumin) was obtained from MEDI-CULT (Denmark). Genistein and herbimycin were dissolved in DMSO at the initial concentration of 10 mg/ml and further diluted in buffer.

2.2. Preparation of spermatozoa

Human semen was obtained from normospermic men undergoing semen analysis for couple infertility. Spermatozoa were washed from seminal plasma and prepared by the swim-up procedure as described previously [16]. In some experiments, spermatozoa were separated on a Percoll gradient as described [5,17]. Sperm samples were capacitated for 2 h in sperm preparation medium at 37°C in an atmosphere of CO_2. Motility was evaluated using a fully automated computer-assisted semen analyzer (Hamilton-Thorn) [16].

2.3. Measurement of intracellular calcium concentration

Spermatozoa, prepared as described above, were loaded with 2 μM Fura 2/AM for 45 min at 37°C, washed, resuspended in FM medium (125 mmol/l NaCl, 10 mmol/l KCl, 2.5 $CaCl_2$, 0.25 mmol/l $MgCl_2$,

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19 mM Na-lactate, 2.5 mM Na-pyruvate, 2 mM HEPES, 3 mg/ml BSA) and $[Ca^{2+}]_i$ measured as described previously using a spectrofluorimetric method [5,16]. Fura-2 loaded spermatozoa were preincubated for 3 min with genistein, sangivamycin, Na_3VO_4 or control solvents, centrifuged, resuspended in FM buffer and transferred to a quartz cuvette for calcium measurements at a concetration of 5×10^6 cells/ml. Spermatozoa were stimulated with 1 μg/ml P. Fluorescence measurements were converted to $[Ca^{2+}]_i$ by determining maximal fluorescence (F_{max}) with ionomycin (8 mM final concentration) followed by minimal fluorescence (F_{min}) with 10 mM EGTA, pH 10. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. [18] assuming a dissociation constant of Fura 2 for calcium of 224 nM.

2.4. SDS-PAGE and Western blot analysis

After incubation with P (1 μg/ml) under the indicated conditions, the samples were processed for SDS-PAGE as described previously [9]. Briefly, samples were centrifuged at $400 \times g$ at 4°C, washed in BSA-free medium, resuspended in 10 μl lysis buffer Sb 2 × (62.5 mM Tris, pH 6.8, containing 10% glycerol, 20% SDS, 2.5% pyronin and 200 mM dithiothreitol), and incubated at 95°C for 5 min. After measurement of proteins, the suspension was loaded onto 7.5% polyacrilamide-bisacrylamide gels, subjected to electrophoresis, transferred to nitrocellulose (Sigma, St. Louis, MO), and immunostained with peroxidase-conjugated monoclonal anti-phosphotyrosine antibodies (PY20). The immunoreactions were revealed by the enhanced chemiluminescence system (ECL, Amersham). Quantification of the bands was performed by image analysis as described previously [9].

2.5. Statistical analysis

Data are expressed as mean ± S.E.M. Differences between means were assessed by Student's *t*-test for paired data and $P < 0.05$ was considered as statistically significant.

3. Results

In a previous study, we reported that P induces an increase in tyrosine phosphorylation in two sperm protein bands, respectively of 75 and 97 kDa, and that this effect was inhibited by the tyrosine kinase inhibitor erbstatin [9]. Short term (5 min) preincubation of spermatozoa with 10 μM genistein also prevented the increase in tyrosine phosphorylation induced by P in these two bands (Fig. 1), confirming activation of tyrosine kinase(s) by the steroid. To investigate whether tyrosine kinase activation is involved in the $[Ca^{2+}]_i$ increase induced by P, capacitated spermatozoa were preincubated for 5 min with genistein (1–30 μM) or control solvent. As shown in Fig. 2A and B,

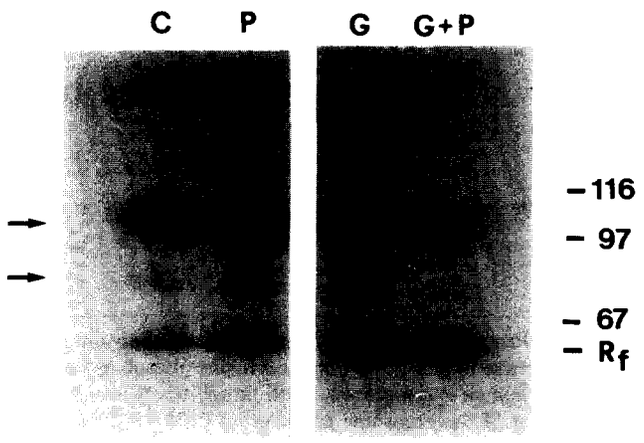

Fig. 1. Effect of genistein (10 μM final concentration, 5 min preincubation) on basal and progesterone-stimulated tyrosine phosphorylation of proteins in capacitated human spermatozoa. Tyrosine phosphorylated proteins were revealed using the monoclonal anti-phosphotyrosine antibody PY20-HRP followed by the ECL detection system. Molecular weight markers ($\times 10^3$) are indicated to the right of the blot.

the addition of P to Fura 2-loaded spermatozoa from two different subjects induced an increase in $[Ca^{2+}]_i$ characterized by an initial transient peak and a long-lasting plateau phase. Pretreatment with increasing concentrations of genistein did not affect the Ca^{2+} rise to a peak, but markedly decreased the plateau phase (Fig. 2A and B) at concentrations as low as 1 μM. The average P-stimulated peak $[Ca^{2+}]_i$ and plateau in 9 different subjects in the presence or absence of increasing concentrations of genistein are reported in Fig. 3A for the peak and Fig. 3B for the plateau. No significant effect of genistein was observed on the peak $[Ca^{2+}]_i$ at any concentration tested (Fig. 3A), whereas the inhibitory effect on the plateau phase was significant starting from 1 μM and was not further enhanced at higher genistein concentrations (Fig. 3B). The effect of genistein was specific for the P-induced sperm $[Ca^{2+}]_i$ increase, since it did not affect the peak or the plateau phase of $[Ca^{2+}]_i$ obtained following stimulation with the endoplasmic Ca^{2+}-ATPase inhibitor thapsigargin (10 μM), previously shown to increase $[Ca^{2+}]_i$ [19] and the acrosome reaction [20] in human spermatozoa. Fig. 4

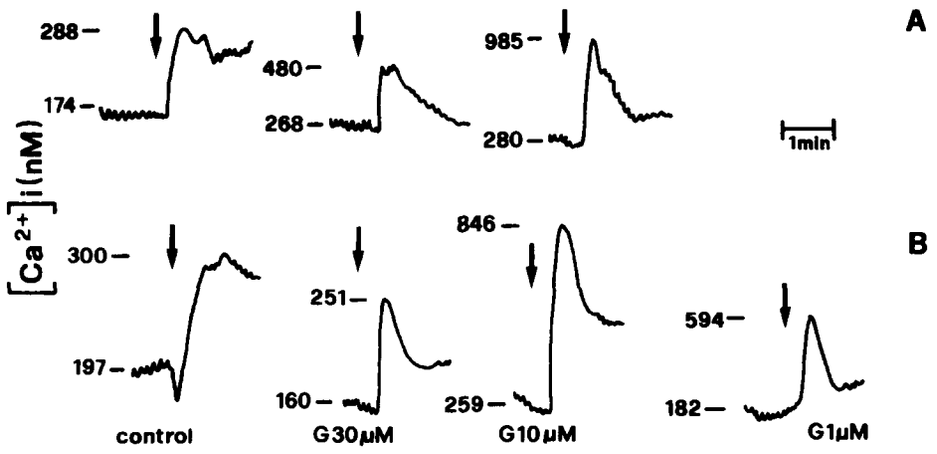

Fig. 2. Effect of progesterone (0.1 μg/ml final concentration) on $[Ca^{2+}]_i$ in Fura 2-loaded, capacitated human spermatozoa in basal conditions or following preincubation with increasing concentrations of genistein (G) in two different subjects (A and B). $[Ca^{2+}]_i$ was evaluated by a fluorimetric method as described in the text. The arrows indicate addition of progesterone.

shows the effect of 1 and 10 μM genistein on Ca^{2+} waves induced by 10 μM thapsigargin (panel A) or (for comparison) by 1 μg/ml P (panel B) in spermatozoa obtained from the same subject. The effect of genistein does not appear to be due to a toxic effect on spermatozoa, since motility parameters, evaluated by a fully automated motion analyzer (Hamilton-Thorn), were not affected by treatment with genistein (results not shown). Pretreatment with genistein did not affect basal $[Ca^{2+}]_i$, or response to ionomycin (data not shown). We also tested the effect of herbimycin A, an inhibitor of tyrosine kinase structurally unrelated to genistein, on the sperm $[Ca^{2+}]_i$ increase induced by P. A 3 min preincubation with 30 μM herbimycin A did not affect the peak phase of the P-induced $[Ca^{2+}]_i$ increase, but inhibited, although to a much lesser extent than genistein, the plateau phase (1.84 ± 0.13 fold increase in control vs. 1.52 ± 0.08 fold increase in treated samples; % inhibition 27.5 ± 3.4; $n = 4$, $P < 0.05$). Herbimycin A did not significantly affect basal $[Ca^{2+}]_i$ nor response to ionomycin (data not shown). To confirm that the effects of genistein and herbimycin are not due to aspecific inhibition of protein kinase C, we evaluated the effect of a short term (3 min) preincubation with the protein kinase C inhibitor sangivamycin. At a concentration of 0.1 μM, sangivamycin did not affect the plateau or the peak phase of the $[Ca^{2+}]_i$ increase induced by P (results not shown), indicating that the effect of genistein is not due to aspecific inhibition of protein kinase C. Conversely, the tyrosine phosphatase inhibitor Na_3VO_4 (0.1 mM, 3 min preincubation), induced a significant increase in the plateau phase of $[Ca^{2+}]_i$ induced by P without affecting the peak phase (Table 1) or basal $[Ca^{2+}]_i$ (not shown). The average increase in the plateau phase induced by Na_3VO_4 was 1.32 ± 0.05 fold over control ($n = 5$).

4. Discussion

The rapid, non-genomic action of progesterone (P) on human spermatozoa has recently been identified [6]. Previous studies demonstrated that P induces an increase in $[Ca^{2+}]_i$ [3–5] as well as tyrosine phosphorylation of a protein in the 95–97 kDa molecular weight range [8,9]. In the present study we show evidence that activation of tyrosine kinases by P is upstream of the generation of the sustained phase of the $[Ca^{2+}]_i$ increase. Indeed, we have demonstrated that incubation with molar concentrations of the tyrosine kinase inhibitor genistein strongly inhibited the plateau phase of the $[Ca^{2+}]_i$ increase induced by P. In contrast, the peak phase of $[Ca^{2+}]_i$ in response to P was unaffected by this treatment. Moreover, preincubation with the tyrosine phosphatase inhibitor Na_3VO_4 increases the plateau phase of the P response, further confirming the involvement of tyrosine kinase in this effect. Our results indicate that the

Table 1
Effect of protein tyrosine phosphatase inhibitor Na_3VO_4 on the peak and plateau phases of the $[Ca^{2+}]_i$ increase induced by progesterone in human spermatozoa

	Control	Na_3VO_4
Peak $[Ca^{2+}]_i$	3.47 ± 0.59	3.84 ± 0.62
Plateau $[Ca^{2+}]_i$	1.50 ± 0.15	1.97 ± 0.24*

Fura 2-loaded spermatozoa were preincubated for 3 min with Na_3VO_4, centrifuged, and $[Ca^{2+}]_i$ evaluated by a spectrofluorimetric method as described in the text. Data are expressed as fold increase over basal $[Ca^{2+}]_i$. $n = 5$ experiments; *$P < 0.05$.

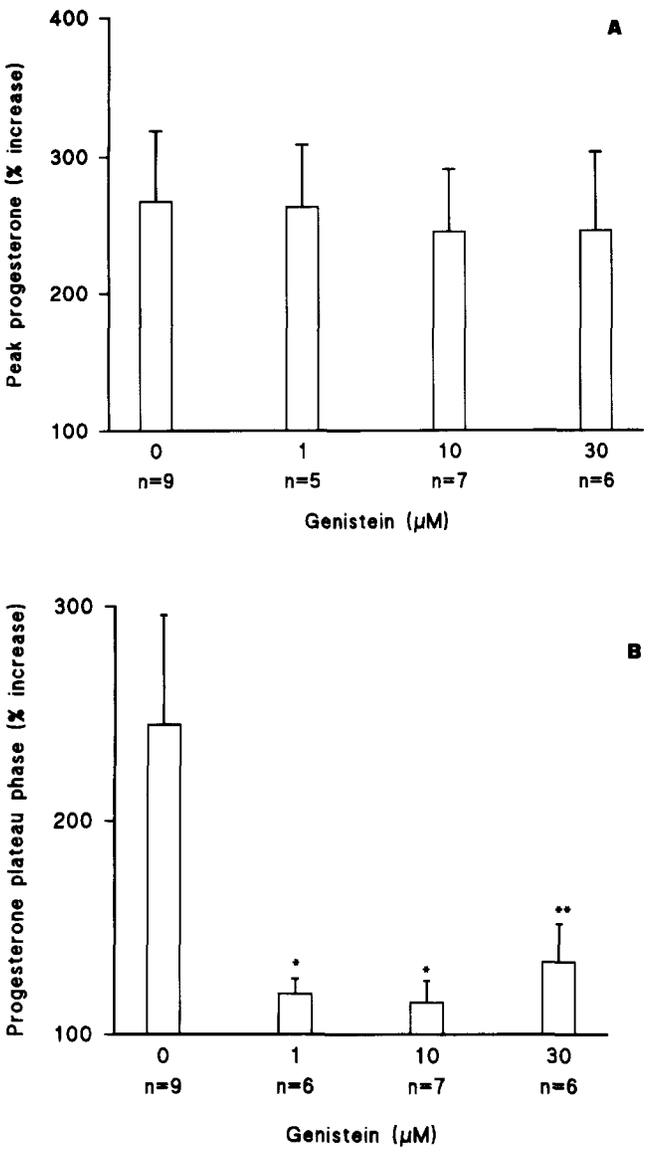

Fig. 3. Average peak $[Ca^{2+}]_i$ (panel A) and plateau phase (panel B) in response to progesterone in Fura 2-loaded, capacitated human spermatozoa under basal conditions or following preincubation with increasing concentrations of genistein. $[Ca^{2+}]_i$ was evaluated by a fluorimetric method as described in the text.

P-mediated increase in tyrosine phosphorylation may be involved in opening a membrane calcium channel responsible for the plateau phase of $[Ca^{2+}]_i$ waves in response to P. Since the increase in tyrosine phosphorylation mediated by P is independent of extracellular calcium, and since both $[Ca^{2+}]_i$ increase and tyrosine kinase activation are essential for induction of acrosome reaction [8–11], it is tempting to speculate that the plateau phase of the calcium increase could be more important than the peak phase in determining the increase in the acrosome reaction in response to P. We have recently reported that an increase in calcium and the acrosome reaction in response to P are highly correlated, with the exception of few cases, with in vitro fertilization of human oocytes [16], indicating a functional relationship between sperm fertilizing ability and responsiveness to P. In the light of present results, it would be interest-

ing to investigate whether a stricter correlation exists between the plateau phase of the calcium increase in response to P and in vitro fertilization outcome.

The inhibitory effect of genistein on the P-induced $[Ca^{2+}]_i$ response in human sperm appears to be due to inhibition of P-stimulated tyrosine kinase activity. Indeed, we have shown that at similar concentrations genistein also inhibits the P-induced increase in tyrosine phosphorylation of sperm proteins, confirming previous results obtained with a different tyrosine kinase inhibitor [9]. To control for non-specific effects of genistein, we employed the chemically and mechanistically dissimilar tyrosine kinase inhibitor herbimycin A. While herbimycin A is thought to inhibit PTK activity through benzoquinone interactions with protein sulfhydral groups, genistein is a competitive inhibitor with respect to ATP. A slight but significant inhibitory effect on the plateau phase of the P response was also observed with herbimycin, further indicating that tyrosine kinase is involved in such an effect. Furthermore, genistein did not inhibit the sustained phase of Ca^{2+} entry stimulated by the endoplasmic Ca-ATPase inhibitor thapsigargin, indicating that the effect of this agent is not due to aspecific inhibition of Ca^{2+} entry. An inhibitory effect of genistein and other tyrosine kinase inhibitors of agonist-mediated calcium signaling has been shown in several other reports [12–15]. In these studies, the inhibitory effect of genistein has been observed either on the agonist- induced peak $[Ca^{2+}]_i$ increase, without affecting the plateau phase [13,15], as well as on the plateau phase without affecting the peak [12,14], with similar results to those obtained in our study.

The mechanism of P-mediated stimulation of human spermatozoa is still unclear. Apparently, such action is not mediated by a genomic pathway, since the antagonist of nuclear P receptors RU486 does not inhibit P responses except at very high doses [21,22]. The presence of P binding sites in human sperm has been recently reported [23], however, a precise characterization of these receptors is still lacking. Our results suggest the possibility that the P-mediated sperm $[Ca^{2+}]_i$ increase is mediated by two different pathways, a first one responsible for the peak transient that apparently does not involve tyrosine kinase activation, and a second one, responsible for the long-lasting plateau phase, which appears to be partially mediated by P-induced tyrosine kinase activation. The fact that the thapsigargin-induced $[Ca^{2+}]_i$ increase in human sperm is not inhibited by genistein suggests that P and thapsigargin induce calcium influx through different pathways. Indeed, we have evidence that thapsigargin does not activate tyrosine kinase(s) in human sperm (unpublished observation).

In conclusion, we report evidence that tyrosine kinase activity is, at least partially, involved in the P-induced sustained phase of the $[Ca^{2+}]_i$ increase, providing new insights into the mechanisms of P-induced responses in human sperm. As recently stated, real progress in understanding male infertility, will only come 'if we attempt to understand more about how a spermatozoon is made and how it works' [24].

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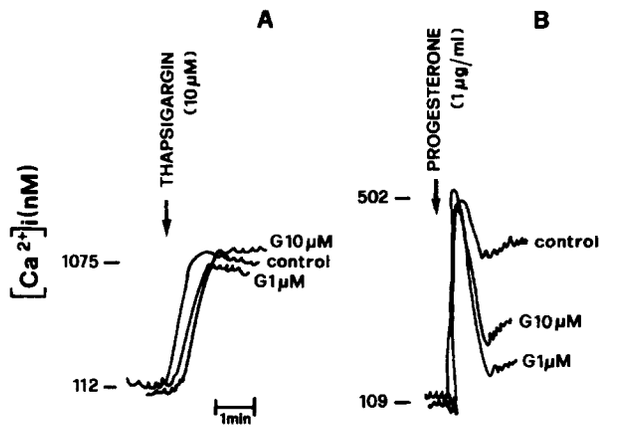

Fig. 4. Sperm $[Ca^{2+}]_i$ increase in response to thapsigargin (10 μM final concentration, panel A) and progesterone (1 μg/ml, panel B) under control conditions or following preincubation with 1 and 10 μM genistein (G). Representative of two similar experiments.

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