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Tyrosine kinase inhibitors and Ca^{2+} signaling: direct interactions with fura-2

Alf Berts, Kenneth P. Minneman *

Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322, USA

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Abstract

Selective inhibitors were used to study the role of tyrosine kinases in α_{1A}-adrenoceptor-mediated responses in transfected PC12 cells. Ca^{2+} responses to noradrenaline were measured using fura-2, and the effects of genistein, tyrphostin A25, and herbimycin A were examined. Neither genistein nor herbimycin A pretreatment altered noradrenaline-induced Ca^{2+} responses, although tyrphostin A25 pretreatment caused some reduction. However, acute addition of genistein quickly reversed the apparent noradrenaline response, apparently, through a direct interaction with cytoplasmic fura-2. Both genistein and tyrphostin A25, at concentrations similar to those used to inhibit tyrosine kinases, markedly reduced fluorescence of fura-2 excited by both 340 and 380 nm, and genistein also reduced the 340/380 ratio. Tyrosine kinase inhibitors did not alter noradrenaline stimulated inositol phosphate formation in α_{1A}-PC12 cells. These results suggest that tyrosine kinases are not involved in second messenger responses to α_{1A}-adrenoceptors, but that tyrosine kinase inhibitors can interact directly with fura-2.

Keywords: Ca^{2+}; Tyrosine kinase; Adrenoceptor; Inositol phosphate; Fura-2

1. Introduction

Tyrosine kinases are involved in mitogenic responses to many G-protein-coupled receptors and attention has recently been focused on their possible role in stimulation of second messenger formation. Many different heterotrimeric G-proteins induce tyrosine phosphorylation of various cellular proteins (Zachary and Rozengurt, 1992; Wan et al., 1996). In some cases, tyrosine kinases have been suggested to feedback onto G-protein activation and provide a novel mechanism for regulating their signaling functions.

Recently, Umemori et al. (1997) studied the role of tyrosine kinases in $G_{q/11}$-coupled receptor signaling. They found that activation of $G_{q/11}$-coupled receptors induced tyrosine phosphorylation of $G_{q\alpha}$, that tyrosine kinase inhibitors blocked inositol 1,4,5-trisphosphate and Ca^{2+} responses, and that the activity of $G_{q/11}$ was regulated by tyrosine phosphorylation. A variety of other studies have also implicated tyrosine kinases in $G_{q/11}$ receptor-mediated second messenger formation and Ca^{2+} signaling (Tsunoda, 1998). Tyrosine kinase inhibitors have been found to inhibit (Hashii et al., 1996; Goutsouliak and Rabkin, 1997; Nelson et al., 1997; Taketo et al., 1997; Yousufzai and Abdel-Latif, 1998; Palmier et al., 1999) or potentiate (Dickenson and Hill, 1998) inositol phosphate and/or Ca^{2+} responses to a variety of $G_{q/11}$-coupled receptors. This effect may involve direct tyrosine phosphorylation of the inositol 1,4,5-trisphosphate receptor (Jayaraman et al., 1996).

We have recently been studying the mitogenic actions of α_1-adrenoceptors stably expressed in PC12 cells. α_1-Adrenoceptors (Zhong and Minneman, 1999a), like other G-protein-coupled receptors, activate mitogenic responses in many cells, and play important roles in regulating growth and proliferation (LaMorte et al., 1994; Milano et al., 1994). In rat PC12 cells transfected with α_{1A}-adrenoceptors, noradrenaline activates all three arms of the mitogen-activated protein kinase pathway. Noradrenaline treatment of α_{1A}-PC12 cells activates extracellular regulated kinases 1 and 2, c-Jun-NH_2-terminal kinases, and p38 mitogen-activated protein kinases (Williams et al., 1998), and causes the cells to differentiate into a neuronal like phenotype. These mitogenic responses appear to be independent of the inositol phosphate and Ca^{2+} signaling

* Corresponding author. Tel.: +1-404-727-5985; fax: +1-404-727-0365.

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pathways activated by these receptors (Berts et al., 1999), but are associated with activation of a variety of tyrosine kinases. Activation of Src, in particular, appeared to be necessary for mitogenic responses (Zhong and Minneman, submitted for publication). Because of the possible feedback on second messenger formation, we have used selective inhibitors to determine whether tyrosine kinases alter Ca^{2+} or inositol phosphate responses to noradrenaline in these cells.

2. Materials and methods

2.1. Materials

PC12 cells were obtained from Cindy Miranti and Michael Greenberg (Harvard Medical School). The human α_{1A}-adrenoceptor cDNA (Hirasawa et al., 1993) was obtained from Gozoh Tsujimoto (National Children's Medical Research Center, Tokyo, Japan). Other materials were: Lac-Switch vector system (Stratagene, La Jolla, Ca); fura-2/AM and fura-2 free acid, tyrphostin A25 (Calbiochem, La Jolla, CA); (−)-noradrenaline bitartrate, genistein, Dulbecco's modified Eagles medium, penicillin and streptomycin (Sigma, St. Louis, MO), phentolamine mesylate, and herbimycin A (Research Biochemicals International, Natick, MA).

2.2. Transfection

PC12 cells were cotransfected with the Lac-Switch repressor (p3′SS) and the pRSVICAT operator vectors containing the human α_{1A}-adrenoceptor cDNA by lipofectamine (GIBCO/BRL, Gaithersburg, MD). Cells were propagated for several weeks in the presence of 250 μg/ml hygromycin and 500 μg/ml geneticin, and subclones expressing low constitutive and high inducible receptor levels were screened by radioligand binding (Williams et al., 1998; Zhong and Minneman, 1999b).

2.3. Cell culture

Stably transfected PC12 cells were propagated in 75-cm^2 flasks at 37°C in a humidified 5% CO_2 incubator in Dulbecco's modified Eagles medium, containing 4.5 g/l glucose, 1.4% glutamine, 20 mM HEPES, 100 mg/l streptomycin, 105 U/l penicillin, 10% donor horse serum, and 5% fetal bovine serum. Cells were detached by mild trypsinization (0.25%) in the presence of 2.6 mM EDTA, and subcultured at a ratio of 1:3 upon reaching confluency. Receptor expression was induced by treatment with 1 mM isopropyl-beta-D-thiogalactoside at least 24 h before each experiment (Zhong and Minneman, 1999b). For studies involving Ca^{2+} measurements, 100-mm dishes were seeded at a density of 6×10^6 cells/10 ml and grown to confluency before use.

2.4. Fura-2 measurements

Intracellular Ca^{2+} transients were measured with fura-2 as described previously (Esbenshade et al.,1993). Confluent 100-mm plates were washed with balanced salt solution (BSS; 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 20 mM HEPES, 10 mM glucose, 0.1% bovine serum albumin) and cells detached by mild trypsinization (0.25%). Cells were rinsed three to four times with BSS and stored on ice. One milliliter of cell suspension (1×10^6 cells/ml) was incubated with 1 μM fura-2/acetoxymethylester for 10 min at 37°C, rinsed 10 min with BSS, and diluted to 3 ml before the experiment. The cell suspension was transferred to a cuvette and placed in a Perkin-Elmer (Beaconsfield, Buckingshamshire, England) LS 50B luminescence spectrofluorometer with a thermostatted (37°C) stirred cell holder. The excitation wavelengths were 340 and 380 nm and the emission wavelength was 510 nm. Calibration of the fluorescence signals for calculation of $[Ca^{2+}]_i$ was performed by equilibrating intracellular and extracellular Ca^{2+} with 30 μM digitonin (R_{max}), followed by addition of 30 μl of 300 mM EGTA, 1 M Tris, pH 9.0 (R_{min}), using a K_D of 225 nM for fura-2.

For measuring direct interactions with tyrosine kinase inhibitors, fura-2 free acid (0.33 μM) was diluted into 3 ml of BSS in a cuvette. The free acid was excited with 340 and 380 nm. The 340/380 ratio or fluorescence at 340 or 380 nm was measured using the same equipment.

2.5. Inositol phosphate formation

Accumulation of [^{3}H]inositol phosphates was determined in confluent 35-mm dishes. Cells were prelabeled with *myo*-[^{3}H]inositol (2 mCi/plate) for 1 day. Production of total [^{3}H]inositol phosphates in the presence of 10 mM LiCl was determined as described previously (Esbenshade et al., 1993) following pretreatment with the inhibitors indicated. Results are expressed as percentage of total [^{3}H]inositol incorporated into lipid converted to the phosphate form.

3. Results

3.1. Effect of pretreatment with tyrosine kinase inhibitors on Ca^{2+} responses in α_{1A}-PC12 cells

We first examined whether pretreating cells with a variety of broad spectrum tyrosine kinase inhibitors would alter the shape or magnitude of Ca^{2+} responses to noradrenaline. α_{1A}-PC12 cells were pretreated overnight with 1 μM herbimycin A, or pretreated for 10 min with 100 μM genistein, 50 μM tyrphostin A25, or 50 μM tyrphostin A47. Fura-2 loaded cells were then loaded in the cuvette and noradrenaline (100 μM) induced increases in

intracellular Ca^{2+} monitored. It is clear from Fig. 1 that none of the tyrosine kinase inhibitors examined caused large changes in the time course or magnitude of noradrenaline-induced increases in intracellular Ca^{2+}. The data with tyrphostin A47 is not shown, since pretreatment of cells with this inhibitor resulted in very noisy fura-2 signals. Data from multiple experiments were normalized and combined to determine the significance of the results. The Ca^{2+} response to noradrenaline was not reduced by either genistein ($128 \pm 26\%$ of Control) or herbimycin A ($99 \pm 5\%$ of Control), but was significantly reduced by tyrphostin A25 to $68 \pm 12\%$ of Control.

3.2. Effect of acute administration of tyrosine kinase inhibitors on Ca^{2+} responses in α_{1A}-PC12 cells

Next, we examined whether acute administration of tyrosine kinase inhibitors would alter Ca^{2+} responses to noradrenaline. Fura-2 loaded α_{1A}-PC12 cells were treated with noradrenaline (100 μM) to increase intracellular Ca^{2+}, and then genistein (100 μM) or tyrphostin A25 (50 μM) was added. Fig. 2 shows that genistein caused a large and rapid reduction in the 340/380 fluorescence ratio. Tyrphostin A25 caused little significant effect, except for an increase in noise (Fig. 2), while herbimycin A had no observable effect (data not shown). Because of the large effect of genistein on the 340/380 ratio, these data were not converted to Ca^{2+} levels.

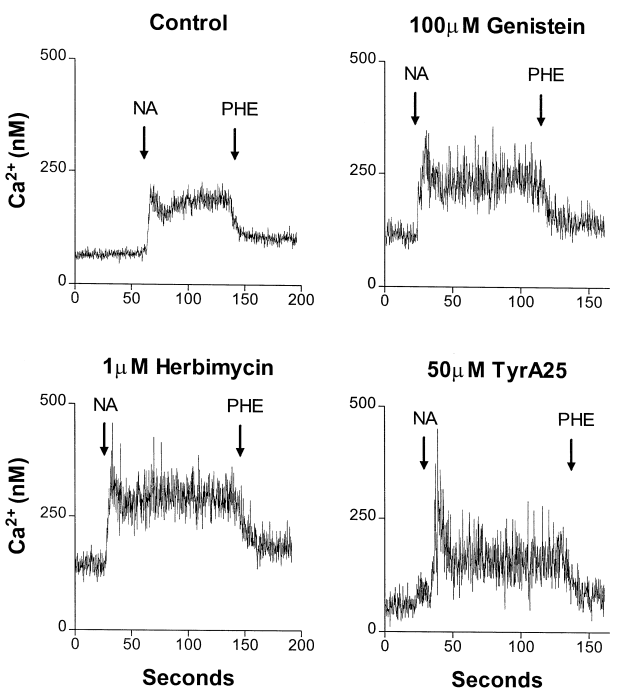

Fig. 1. Effect of noradrenaline on cytoplasmic Ca^{2+} following pretreatment with tyrosine kinase inhibitors. α_{1A}-Adrenoceptor transfected PC12 cells were pretreated for 10 min with 100 μM genistein or 50 μM Tyrphostin A25, or 24 h with 1 μM herbimycin. Fura-2 loaded cells were exposed to 100 μM noradrenaline (NA) and 10 μM phentolamine (PHE) in the presence of inhibitors at the times indicated. Each graph is representative of three to five separate experiments.

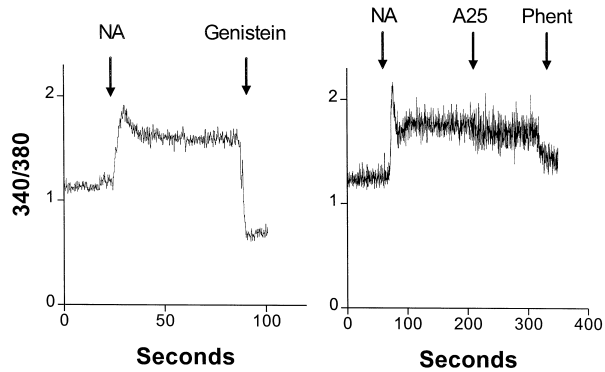

Fig. 2. Effect of acute addition of genistein (left) and or tyrphostin A25 (right) on noradrenaline-induced increases in the 340/380 fura-2 ratio in α_{1A}-PC12 cells. Genistein (100 μM), tyrphostin A25 (50 μM), noradrenaline (NA; 100 μM), and phentolamine (Phent; 10 μM) were added at the times indicated. Each graph is representative of three to five separate experiments.

3.3. Effect of tyrosine kinase inhibitors on fluorescence of fura-2-free acid

To determine whether the effect of genistein might be due to a direct interaction with fura-2, we examined the effect of tyrosine kinase inhibitors on fluorescence of fura-2-free acid. Fig. 3 shows that 100 μM genistein caused a dramatic decrease in the 340/380 ratio of fura-

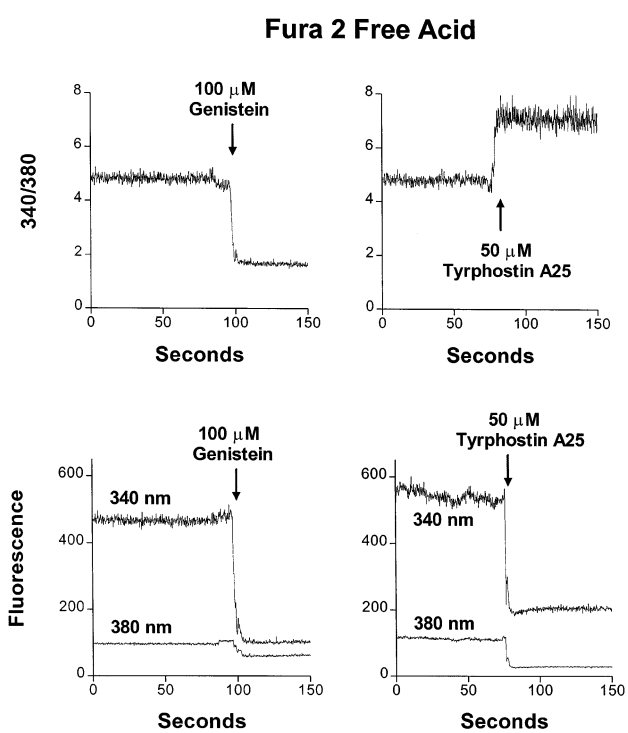

Fig. 3. Effect of genistein and tyrphostin A25 on the 340/380 ratio and fluorescence of fura-2-free acid. Genistein (100 μM) or tyrphostin A25 (50 μM) were added to a solution of 0.33 μM fura-2-free acid at the times indicated. Each graph is representative of three to five separate experiments.

2-free acid. This was due to a reduction in both the 340 and 380 signals, but a greater reduction of the 340 signal (Fig. 3). Wavelength scans of fura-2 fluorescence in the presence of different calcium concentrations showed that genestein altered only the magnitude, but not the shape, of the excitation spectrum (data not shown). Interestingly, tyrphostin A25 increased the 340/380 ratio of fura-2 free acid substantially (Fig. 3). This was also due to a reduction in both the 340 and 380 signals, but in this case, the 380 signal was reduced to a greater degree than the 340 signal (Fig. 3). Tyrphostin A47 (50 μM) had similar effects to tyrphostin A25, reducing both 340 and 380 signals (data not shown), while herbimycin A (1 μM) had no effect on fluorescence of fura-2 free acid (data not shown).

3.4. Concentration-dependence

The concentration-dependence of the effects of both genistein and tyrphostin A25 on fura-2-free acid fluorescence are shown in Fig. 4. Genistein reduced the 340/380 ratio with an IC_{50} around 30 μM, while tyrphostin A25 steadily increased the ratio in concentrations from 25–100 μM. No IC_{50} could be calculated for tyrphostin A25 because no maximum response was achieved.

We also examined whether the source of genistein might influence the interaction. We compared synthetic genistein from Sigma (G-6649) with genistein purified from soybean (Sigma, G-6776) with genistein from Research Biochemicals (G-103). All three preparations showed similar effects on fluorescence of fura-2-free acid, with similar potencies (data not shown).

3.5. Effect of tyrosine kinase inhibitors on inositol phosphate formation

Finally, we studied the effect of pretreatment with the various tyrosine kinase inhibitors on basal and noradrenaline-stimulated inositol phosphate formation in α_{1A}-PC12 cells. Fig. 5 shows that pretreatment with either 100 μM genistein, 50 μM tyrphostin A25, or 1 μM herbimycin did not alter either basal or 100 μM noradrenaline stimulated formation of inositol phosphates in these cells.

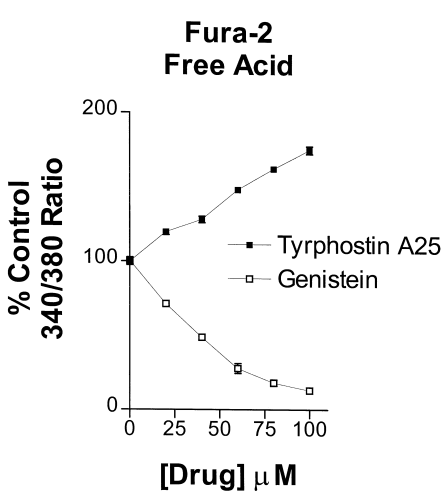

Fig. 4. Concentration–response relationships of genistein and tyrphostin A25 on the 340/380 ratio of fura-2-free acid. Fura-2-free acid (0.33 μM) was exposed to the indicated concentrations of genistein (open symbols) or tyrphostin A25 (solid symbols). Each point is the mean ± S.E.M. of data from three to five separate experiments.

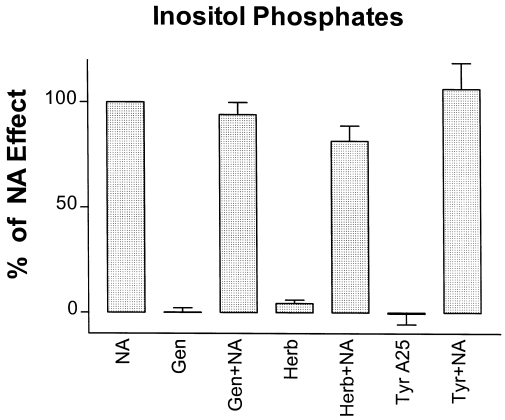

Fig. 5. Effects of tyrosine kinase inhibitors on noradrenaline-stimulated inositol phosphate formation in α_{1A}-PC12 cells. Cells were pretreated for 10 min with 100 μM genistein or 50 μM tyrphostin A25, or 24 h with 1 μM herbimycin. ^{3}H-inositol preloaded cells were exposed to 0 or 100 μM noradrenaline (NA), and inositol phosphate formation determined as described in the text. Each value is the mean ± S.E.M. from four separate observations.

4. Discussion

The results presented here suggest that tyrosine kinases do not play a major role in regulation of α_{1A}-adrenoceptor-mediated Ca^{2+} signaling in transfected PC12 cells. However, they also suggest that tyrosine kinase inhibitors must be used with great care in studying Ca^{2+} responses, since several widely used broad spectrum tyrosine kinase inhibitors directly quench fura-2 fluorescence.

A variety of reports have implicated tyrosine kinases in $G_{q/11}$-mediated second messenger responses. The most direct demonstration was by Umemori et al. (1997), who showed that genistein and tyrphostins AG213 and AG60 blocked Ca^{2+} signals and inositol phosphate responses caused by activation of metabotropic glutamate receptor 1 and other $G_{q/11}$-linked receptors expressed in Chinese hamster ovary (CHO) cells. These authors also showed that $G\alpha_{q/11}$ itself was directly tyrosine phosphorylated in response to receptor activation, which altered its biological activity. Many other investigators have also found tyrosine kinase inhibitors to interfere with Ca^{2+} and inositol phosphate signals generated by $G_{q/11}$-coupled receptors (Hashii et al., 1996; Goutsouliak and Rabkin, 1997; Nelson et al., 1997; Taketo et al., 1997; Yousufzai and Abdel-Latif, 1998; Palmier et al., 1999).

These studies are clearly complicated by the direct interactions of genistein and the structurally related tyr-

phostins with fura-2 reported here. These interactions occur at concentrations of these drugs necessary for inhibition of tyrosine kinase activity (10–100 μM), and are characterized by a marked reduction in fluorescence from excitation at both 340 and 380 nm. Despite the similar fluorescence quenching of the two drugs, the net effect of genistein and tyrphostin A25 on the fura-2 340/380 ratio is different. Genistein causes a marked reduction in this ratio, while tyrphostin A25 causes a substantial increase. Since intracellular Ca^{2+} concentration is usually calculated directly from the fura-2 340/380 ratio, these effects could dramatically influence the apparent experimental results. Genistein-induced quenching of fura-2 fluorescence and subsequent reduction in the 340/380 ratio may be responsible for some of the widely reported inhibitory effects of genistein on fura-2 Ca^{2+} responses (Hashii et al., 1996; Nelson et al., 1997; Taketo et al., 1997; Umemori et al., 1997; Di Salvo and Nelson, 1998; Takemura et al., 1998; Tornquist et al., 1998; Palmier et al., 1999).

These effects may also help explain why different results are sometimes obtained with different inhibitors and/or treatment protocols. For example, pretreatment of cells with genistein caused no significant reduction in the subsequent noradrenaline-induced Ca^{2+} response (Fig. 2). Conversely, addition of genistein after the fura-2 Ca^{2+} signal had already been increased by noradrenaline addition, caused an immediate and dramatic reduction in the 340/380 ratio (Fig. 3). Similarly, pretreatment with tyrphostin A25 was found to reduce the fura-2 Ca^{2+} response to noradrenaline by about 32% (Fig. 2), but addition of tyrphostin A25 after increasing the fura-2 Ca^{2+} signal with noradrenaline caused no apparent effect on the fura-2 340/380 ratio (Fig. 3). The net effect of each compound would be a combination of actions on intracellular Ca^{2+} and direct actions on fura-2 fluorescence.

Interestingly, the structurally unrelated tyrosine kinase inhibitor herbimycin A had no effect on fura-2 fluorescence, or on noradrenaline induced Ca^{2+} responses in α_{1A}-PC12 cells. This supports the hypothesis that tyrosine kinases do not play a major role in regulating Ca^{2+} responses in these cells. In addition, neither herbimycin A, genistein, nor tyrphostin A25 inhibited noradrenaline-stimulated inositol phosphate formation in these cells. This confirms that there is no obvious modulatory role for tyrosine kinases in $G_{q/11}$-mediated second messenger production, at least in these PC12 cells.

Although their direct quenching of fura-2 fluorescence will complicate the use of genistein and tyrphostins on Ca^{2+} measurements with fura-2, this does not necessarily mean that tyrosine kinases do not influence cellular Ca^{2+} homeostasis. Effects of genistein have also been reported on L-type Ca^{2+} currents in cardiac myocytes measured electrophysiologically (Wang and Lipsius, 1998; Ogura et al., 1999). Whether such effects are due to inhibition of tyrosine kinases is not always clear, however, since genistein and tyrphostins also have other effects in the concentration ranges usually employed (Nichols and Morimoto, 1999).

In summary, our results do not support the hypothesis that tyrosine kinases play an important modulatory role in α_{1A}-adrenoceptor second messenger production in PC12 cells. They also suggest that genistein and tyrphostins should be used very cautiously, if at all, in studying Ca^{2+} responses with the commonly used fluorescent indicator fura-2. Finally, these studies reinforce the general principle that several structurally dissimilar drugs with similar cellular actions should be examined before mechanistic conclusions are drawn from pharmacological studies.

Acknowledgements

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