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Desipramine selectively potentiates norepinephrine-elicited ERK1/2 activation through the α_{2A} adrenergic receptor

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ABSTRACT

The precise physiological effects of antidepressant drugs, and in particular their actions at non-monoamine transporter targets, are largely unknown. We have recently identified the tricyclic antidepressant drug desipramine (DMI) as a direct ligand at the α_{2A} adrenergic receptor (AR) without itself driving heterotrimeric G protein/downstream effector activation [5]. In this study, we report our novel finding that DMI modulates α_{2A}AR signaling in response to the endogenous agonist norepinephrine (NE). DMI acted as a signaling potentiator, selectively enhancing NE-induced α_{2A}AR-mediated ERK1/2 MAPK signaling. This potentiation of ERK1/2 activation was observed as an increase in NE response sensitivity and a prolongation of the activation kinetics. DMI in a physiologically relevant ratio with NE effectively turned on ERK1/2 signaling that is lacking in response to physiological NE alone. Further, the DMI-induced ERK1/2 potentiation relied on heterotrimeric $G_{i/o}$ proteins and was arrestin-independent. This modulatory effect of DMI on NE signaling provides novel insight into the effects of this antidepressant drug on the noradrenergic system which it regulates, insight which enhances our understanding of the therapeutic mechanism for DMI.

1. Introduction

A complete picture regarding the mechanism of action of antidepressant medications remains elusive. Indeed, although more than half a century has passed since the introduction of the tricyclic antidepressant drug class [1], a mechanistic physiological model for how these drugs function is nonexistent. These drugs are, speaking generally, understood to be antidepressants by virtue of their ability to inhibit the reuptake of the monoamine neurotransmitters norepinephrine (NE) and serotonin via blockade of the NE and serotonin transporters [1]. However, reuptake inhibition alone is not sufficient to explain the antidepressant effects of these medications.

Our previous work has characterized the interaction of the tricyclic antidepressant drug desipramine (DMI), a class member with the strongest specificity for the noradrenergic over the serotonergic system [1], with the α_{2A} adrenergic receptor (AR). The α_{2A}AR, a prototypical GPCR, is a well-established key player in noradrenergic transmission as a regulator of NE synthesis and release from noradrenergic neuronal terminals [2] and of neuronal activity within the locus coeruleus [3,4], the primary brain region from which central noradrenergic neurons originate. Our work has established that DMI, as a direct α_{2A}AR ligand, selectively drives recruitment of the important interacting regulator protein arrestin to the receptor leading to receptor trafficking responses, while not driving coupling/activation of heterotrimeric G proteins to the receptor and classical signaling responses [5].

The present work is aimed at investigating the potential impact of DMI, acting as a direct α_{2A}AR ligand, on receptor-mediated signaling by the endogenous agonist NE. Given that DMI was shown to bind as an orthosteric ligand at the α_{2A}AR, we hypothesized that DMI would act as a competitive antagonist and block signaling induced by the endogenous α_{2A}AR agonist NE. Further, any antagonism will be dependent on the relative concentrations of DMI and NE when given together (e.g. the [DMI]/[NE] ratio), given that the α_{2A}AR has essentially identical intrinsic affinities for the two ligands (K_i values of 4.62 and 3.63 μM for DMI and NE, respectively) [5], contrasting with typical antagonist affinities that are 100- to 1000-fold higher. The α_{2A}AR classically couples to heterotrimeric G proteins of the $G_{i/o}$ family, leading to inhibition of adenylyl cyclase and voltage-gated Ca^{2+} channels, and activation of downstream effectors including inwardly-rectifying K^+ channels, MAPKs, and Akt [5–10]. Here, we have utilized activation of the ERK1/2 MAPK and Akt pathways as straightforward and reliable readouts for α_{2A}AR signaling. As well, these signaling pathways have both been more generally implicated in physiological antidepressant

Abbreviations: AR, adrenergic receptor; DMI, desipramine; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; NE, norepinephrine; PTx, pertussis toxin.

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responses [11,12]. By utilizing an *in vitro* heterologous cell model, we have been able to isolate and specifically study α_{2A}AR-mediated signal transduction in the absence complex confounding effects from the full complement of endogenous DMI molecular targets.

Through a combination of kinetic and response sensitivity analyses, we report here that DMI is in fact a potentiator that specifically facilitates NE-induced α_{2A}AR-mediated ERK1/2 signaling. We further show that this DMI-potentiated signaling remains dependent on heterotrimeric $G_{i/o}$ proteins and does not simply represent a switch to arrestin-mediated signal transduction. By comparison, we observed a general inhibitory effect of DMI on cellular Akt signaling. These data provide a novel example of a complex ligand/receptor relationship between DMI and the α_{2A}AR which cannot be explained by classical agonist/antagonist regulation of receptor function. As well, in the context of antidepressant pharmacotherapy, our findings add valuable and novel information to the understanding of the physiological mechanism of the antidepressant drug DMI.

2. Materials and methods

2.1. Cell culture

Mouse embryonic fibroblast (MEF) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals) and 1% penicillin/streptomycin (Invitrogen), and maintained in a humidified 5% CO_2 incubator. MEFs isolated from wild-type and arrestin2,3-null ($Arr2,3^{-/-}$) [13] animals and stably expressing N-terminal hemagglutinin (HA) epitope-tagged murine α_{2A}ARs were used for this study. Generation of HA-α_{2A}AR MEF lines expressing the receptor at an average density of 400 fmol/mg has been described [5].

2.2. Drugs and treatments

All drugs were obtained from Sigma unless otherwise noted. NE and DMI stocks of 10 mM were prepared freshly in distilled water prior to each experiment. For antagonist experiments, cells were pretreated with the selective α_{2A}AR antagonist BRL44408 (1 μM) for 5 min prior to stimulation with an NE/BRL combination. For pertussis toxin (PTx) experiments, cells were pretreated with PTx (List Biological Laboratories, Inc.) at a final concentration of 200 ng/ml or vehicle (in serum-free DMEM) for 24 h, and PTx was maintained during drug stimulation. All signaling experiments were done in the presence of 1 μM propranolol (βAR antagonist) and prazosin (α_1 and $\alpha_{2B/C}$AR antagonist) to pharmacologically isolate the α_{2A}AR.

2.3. SDS–PAGE and Western blot

SDS–PAGE (10% acrylamide gel) and Western blot were performed as previously described [5]. MEF cells were serum-starved overnight prior to all signaling assays. At least three independent samples were analyzed for each experimental group.

The following primary antibodies and dilutions were used: phospho-ERK1/2, p44/42 MAPK (T202/Y204) mouse monoclonal antibody (Cell Signaling), 1:16000; phospho-Akt (T308) rabbit polyclonal antibody (Cell Signaling), 1:4000; β-tubulin mouse antibody (University of Iowa Hybridoma Bank), 1:50000; total ERK, p44/42 MAPK rabbit polyclonal antibody (Cell Signaling). HRP-conjugated secondary antibodies and Immobilon Western detection system were obtained from Millipore. β-tubulin was selected as total protein/loading control for most experiments as both phospho-ERK1/2 and phospho-Akt were being analyzed simultaneously. Total ERK blots were performed after membrane stripping as previously described [5].

2.4. Data analysis

Densitometric analysis of dose response Western blot results was done using Scion Image software. Kinase activation for both ERK1/2 and Akt was determined as the ratio of optical density values obtained for phosphorylated kinase to those for total protein. All statistical analyses were carried out using GraphPad Prism software (GraphPad, San Diego, California), using Student's *t*-tests with $p < 0.05$ considered statistically significant.

3. Results and discussion

3.1. DMI alone does not initiate signaling but potentiates NE-induced α_{2A}AR-mediated ERK1/2 activation

We have previously established that NE, in the absence of input from β, α_1, and $\alpha_{2B/C}$ ARs, drives activation of ERK1/2 MAPK in an α_{2A}AR-dependent and G_i-dependent fashion [9,10]. α_{2A}AR-mediated ERK1/2 activation proceeds through the $G_{\beta\gamma}$ subunits and Ras [14,15]. For this study, we began confirming the absence of signal activation by DMI alone, as we have previously reported [5]. As shown in Fig. 1A, DMI alone does not initiate ERK1/2 signaling. We next assayed the kinetics of NE-induced signaling, alone or in combination with DMI, through time course analysis. 10 μM NE alone induces a robust activation of ERK1/2, peaking at 5 min and fully desensitized to baseline by 15 min (Fig. 1B). The kinetics of ERK1/2 activation observed here are consistent with our previous findings on NE-induced α_{2A}AR signaling [9,10]. When DMI was added (NE and DMI together at 10 μM), signaling was not blocked, but rather the time course was prolonged, with ERK1/2 activation

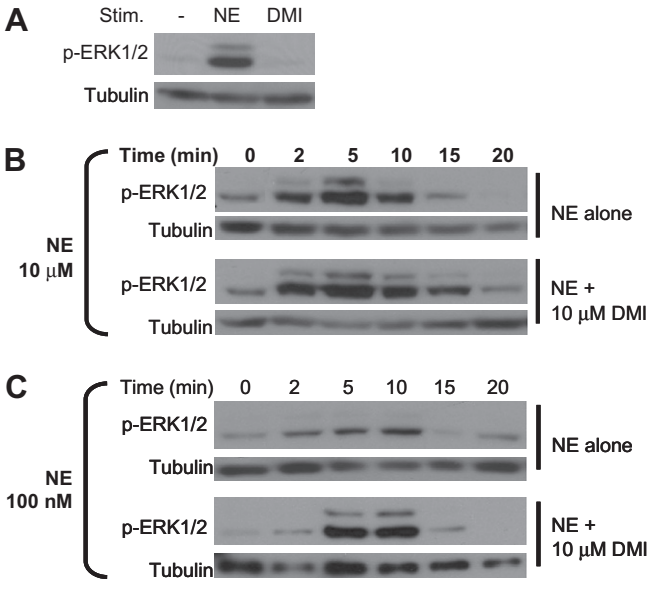

Fig. 1. DMI impacts the kinetics of NE-induced α_{2A}AR-mediated ERK1/2 signaling. All NE stimulations were done in the presence of propranolol (βAR antagonist) and prazosin (α_1 and $\alpha_{2B/C}$AR antagonist). (A) DMI does not itself drive ERK1/2 signaling. MEF cells were stimulated for 5 min with NE alone (as a positive control) or DMI alone. Whole cell homogenates were then analyzed by SDS–PAGE/Western blot, probing for phospho-ERK1/2 and tubulin (loading control). (B) and (C) MEF cells were stimulated for the indicated times with either 100 nM or 10 μM NE, alone or in combination with 10 μM DMI, and analyzed for ERK1/2 activation as in panel A. Blots shown are representative of three independent experiments.

elevated above baseline through 15 min (Fig. 1B). These data seem to contradict our initial hypothesis that DMI acts as a competitive antagonist.

The ability of competitive antagonists to block agonist actions is typically observed either when receptor/antagonist affinity greatly exceeds receptor/agonist affinity or at elevated [antagonist]/[agonist] ratios. Given the essentially identical affinity values for NE and DMI at the α_{2A}AR [5], we therefore chose to assay a much lower level of NE (100 nM), which alone induced a weak and transient activation of ERK1/2 having similar kinetics to those observed at 10 μM (Fig. 1C). Surprisingly, the addition of DMI (now at a 100-fold higher level than NE, 10 μM against 100 nM) did not block NE-induced signaling but rather resulted in a markedly enhanced level of ERK1/2 activation (Fig. 1C). Clearly, these data indicate that the interaction of DMI with the α_{2A}AR is not that of a classical antagonist. Although DMI itself binds to the orthosteric site without driving heterotrimeric G protein/downstream effector activation, it lacks the ability to block agonist-induced signaling at the receptor. In fact, it appears to function as a signaling potentiator, and so our hypothesis has already been refuted.

We further investigated this modulation of NE-induced signaling by dose response analysis, performed at the 5 min (peak activation) time point. This analysis was intended to determine if DMI alters the response sensitivity of NE-induced ERK1/2 activation by the α_{2A}AR. NE dose-dependently induced ERK1/2 activation, with modest activation beginning at 100 nM and more robust activation observed at 1, 10, and 100 μM, while the addition of DMI caused a clear leftward shift in the dose response (Fig. 2A).

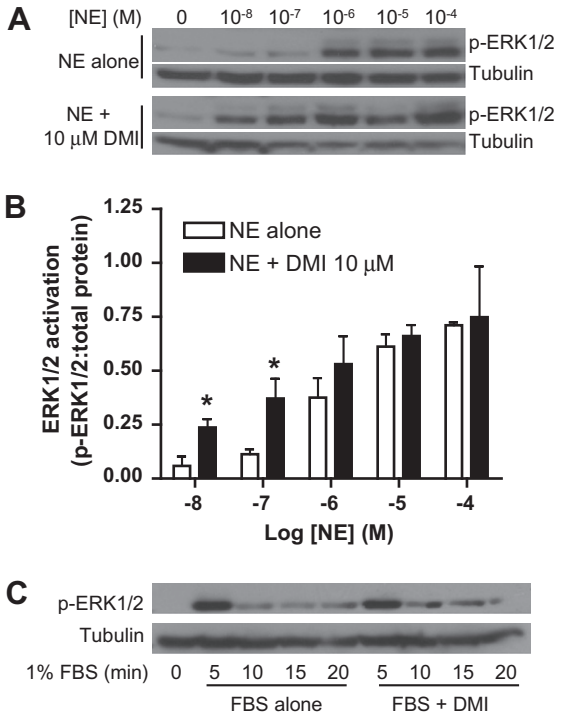

Fig. 2. DMI enhances α_{2A}AR-mediated ERK1/2 response sensitivity to NE. (A) MEF cells were stimulated for 5 min with NE at varying concentrations, ranging from 10 nM (10^{-8} M) to 100 μM (10^{-4} M), alone or in combination with 10 μM DMI, as indicated. Whole cell homogenates were then analyzed by SDS–PAGE/Western blot, probing for phospho-ERK1/2 and tubulin (loading control). Blots shown are representative of three independent experiments. (B) Densitometric quantitation for panel A, with signaling activation calculated as a ratio of phospho-kinase:total protein. Data are mean ± SEM obtained over three independent experiments. $^{*}p < 0.05$ vs. NE alone. (C) MEF cells were subjected to stimulation with 1% FBS for the indicated times, alone or in combination with 10 μM DMI, and analyzed as in panel A. Blots are representative of three independent experiments.

Densitometric analysis confirmed significantly greater ERK1/2 activation at by NE at 10 and 100 nM in the presence of DMI (Fig. 2B). The maximum efficacy of NE-induced ERK1/2 activation was unaffected by the addition of DMI (Fig. 2A and B). These findings correspond nicely with the data presented in Fig. 1, wherein the 5-min ERK1/2 response was clearly potentiated by DMI at 100 nM NE but not at 10 μM NE. Overall, our dose response analysis indicates that DMI enhances the ERK1/2 activation response sensitivity of the α_{2A}AR to NE stimulation and provides further evidence that the interaction of DMI with the α_{2A}AR has a potentiating rather than antagonizing effect on agonist-induced signaling.

Our observations at 10 nM NE have particular physiological relevance, given that this is the same concentration reached in the brain following reuptake inhibition by antidepressants like DMI [3,16]. The addition of DMI to 10 nM NE initiates ERK1/2 activation which is lacking with 10 nM NE alone. As well, the NE/DMI ratios at the 10 and 100 nM data points (100- to 1000-fold higher DMI) are representative of the physiological situation, given that therapeutic levels of DMI reach the micromolar range [5]. These data, then, are strongly supportive of a physiological and pharmacological role for the DMI-mediated potentiation of ERK1/2 signaling.

The possibility exists that DMI is modulating ERK1/2 signal transduction via a non-selective mechanism at some level other than the receptor. As well, ERK1/2 activation is not coupled exclusively to the α_{2A}AR, and can be activated and modulated by a number of upstream mediators. To address this issue of selectivity, we tested the ability of DMI to modulate the growth factor receptor-mediated signaling activity associated with acute FBS exposure. FBS stimulation alone induced a robust and transient ERK1/2 activation, signaling which was unaffected by the addition of DMI (Fig. 2C). Although these data do not definitively rule out an α_{2A}AR-independent mechanism for the potentiation of NE-induced ERK1/2 activation by DMI, they do lend support to the contention that DMI is selectively modulating α_{2A}AR-mediated ERK1/2 signaling.

3.2. Modulation of Akt signaling by DMI lacks selectivity

We next expanded our investigation to include a separate downstream effector activated by α_{2A}AR signal transduction, the Akt kinase. We confirmed that the observed Akt response to NE is both α_{2A}AR-mediated (blocked by the α_{2A}AR antagonist BRL44408) and G_i-dependent (PTx-sensitive) (Fig. 3A). Although G protein-mediated Akt signaling by the α_{2A}AR has not been specifically reported, based on previous studies at other $G_{i/o}$-coupled receptors [17], it seems likely that the receptor couples to Akt via $G_{\beta\gamma}$ subunit-mediated activation of PI3K. Additionally, we again confirmed our previous finding [5] that DMI alone does not drive activation of this downstream effector (Fig. 3B).

Analyzing the samples from our dose response analyses above for Akt activation revealed yet another surprise. While NE alone dose-dependently induced α_{2A}AR-mediated Akt activation, the addition of DMI attenuated this signaling (Fig. 3C), contrasting with its effects on ERK1/2 (Fig. 2). Densitometric analysis confirmed significantly reduced Akt activation at the highest NE levels of 10 and 100 μM (Fig. 3D). These data initially suggested that DMI selectively modulates NE-induced signaling through the α_{2A}AR, altering the signaling profile of the receptor to favor activation of ERK1/2 over Akt. However, in our FBS assay for selectivity, we found that the Akt activation driven by FBS was blocked by the addition of DMI (Fig. 3E). Therefore, DMI appears to inhibit cellular Akt activation through a general mechanism that lacks selectivity for the agonist-induced α_{2A}AR-mediated signaling.

Nevertheless, a more general physiological role for the observed Akt inhibitory effect should not be ruled out. Although a mechanistic explanation of course remains to be seen, that DMI exerts a

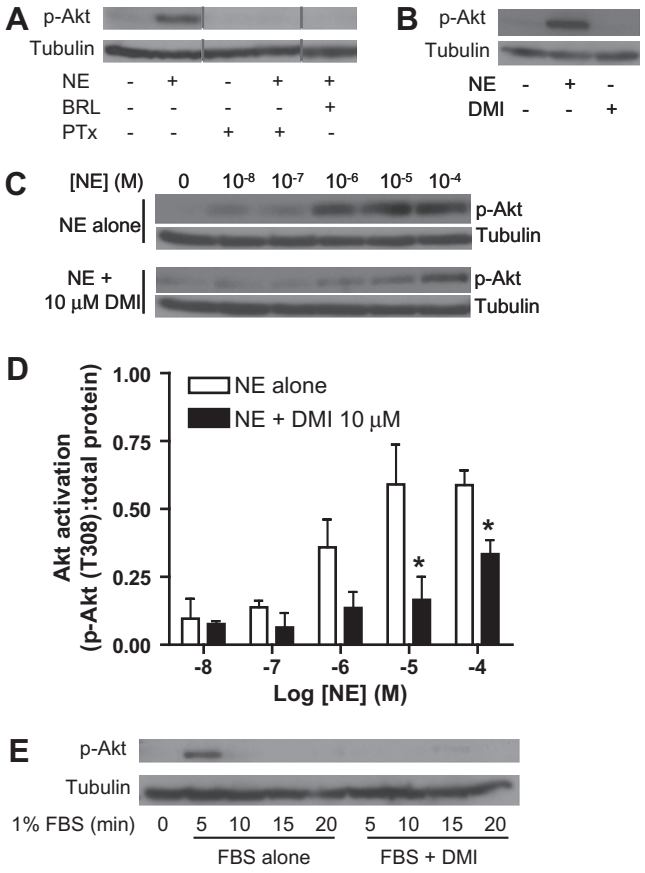

Fig. 3. Modulation of Akt signaling by DMI lacks selectivity. (A) MEF cells were subjected to 5 min stimulation with 10 μM NE alone, NE in combination with the α_{2A}AR antagonist BRL44408, or NE following treatment with pertussis toxin (PTx) as described in the Section 2. Whole cell lysates were analyzed by SDS–PAGE/Western blot for phospho-Akt and tubulin. Note that the lanes shown are from a single experiment but not all adjacent lanes. (B) MEF cells were stimulated for 5 min with 10 μM NE alone (positive control) or 10 μM DMI alone and analyzed as in panel A. (C) MEF cells were stimulated for 5 min with NE at varying concentrations, ranging from 10 nM (10^{-8} M) to 100 μM (10^{-4} M), alone or in combination with 10 μM DMI, as indicated, and analyzed as in panel A. (D) Densitometric quantitation for panel A, with signaling activation calculated as a ratio of phospho-kinase:total protein. Data are mean ± SEM obtained over three independent experiments. $^{*}p < 0.05$ vs. NE alone. (E). MEF cells were subjected to stimulation with 1% FBS for the indicated times, alone or in combination with 10 μM DMI, and analyzed as in panel A. All blots are representative of three independent experiments.

general inhibitory effect on cellular Akt activation is itself a novel finding with potential importance to antidepressant pharmacology. Activated Akt targets and phosphorylates (inactivates) a downstream kinase, GSK3, and this Akt/GSK3 pathway has been extensively implicated in antidepressant mechanisms of action [12]. Therefore, further study of the DMI/Akt phenomenon reported here seems to be warranted.

3.3. Desipramine-modulated α_{2A}AR-mediated ERK1/2 signaling is G_i-dependent and arrestin-independent

We have previously identified DMI as an arrestin-biased ligand at the α_{2A}AR, selectively driving arrestin recruitment to the receptor without inducing G protein coupling [5], and demonstrated critical involvement of the α_{2A}AR/arrestin complex for the *in vivo* antidepressant behavioral effects of DMI [18]. Therefore, a straightforward mechanistic explanation for the data presented thus far could be a switch from classical G protein-mediated to arrestin-mediated signal transduction by the α_{2A}AR in the presence of DMI. Although the α_{2A}AR-mediated ERK1/2 signaling reported thus far in the present and previous studies has been exclusively G protein-dependent, it is now a well-established general fact that GPCRs can signal via G protein-independent mechanisms utilizing arrestin for signal transduction [19,20].

Therefore, we postulated that a DMI-dependent switch from classical G protein-mediated signaling to novel arrestin-mediated signaling might provide a mechanistic explanation for the observed potentiation of NE-induced ERK1/2 signaling by DMI. Such a mechanism could also provide an attractive potential biochemical basis for our recent *in vivo* findings mentioned above [18]. However, our data unfortunately do not support this postulated mechanism, as the DMI-potentiated ERK1/2 signaling remains PTx-sensitive and therefore dependent on $G_{i/o}$ proteins (Fig. 4A). This finding also rules out the possibility of a change in the identity of the heterotrimeric G proteins coupling to the receptor, as PTx is inhibitory toward $G_{i/o}$ subfamily G proteins only. Additionally, repeating the dose response experiment in MEF cells lacking both ubiquitously-expressed arrestins (arrestin2 and 3, also known as β-arrestin 1 and 2) revealed that both NE-stimulated ERK1/2 activation and the potentiation of ERK1/2 signaling by DMI are preserved in the absence of arrestins. The mechanistic details of this G protein-dependent and arrestin-independent potentiation of NE-induced ERK1/2 by DMI remain to be determined.

3.4. Conclusions and implications

The data presented here provide the first evidence that the antidepressant drug DMI can selectively potentiate ERK1/2 signaling through the α_{2A}AR induced by its endogenous agonist NE. The potentiation effect is observed as enhanced activation by an NE/DMI combination when [DMI] exceeds [NE] (Figs. 1C, 2A and B), and a prolongation of the activation time course when [DMI] equals [NE] (Fig. 1B). The potentiation presents mainly as an enhancement of response sensitivity, as DMI does not have any significant effect the maximal NE-induced ERK1/2 activation (Fig. 2A and B), and is G protein-dependent (Fig. 4A) and arrestin-independent (Fig. 4B).

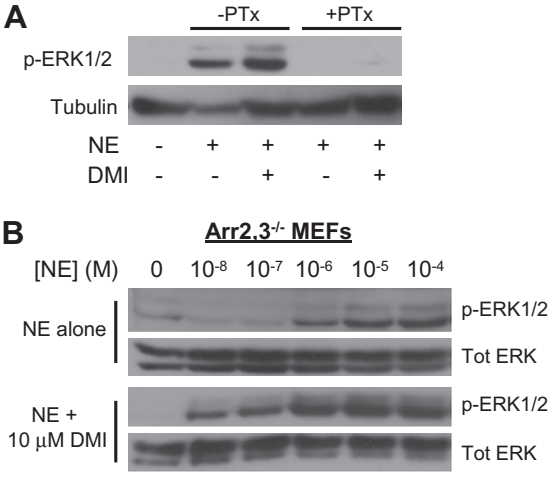

Fig. 4. DMI-modulated ERK1/2 signaling through the α_{2A}AR remains G_i-dependent and arrestin-independent. (A) MEF cells were subjected to pertussis toxin (+PTx) or vehicle (−PTx) treatment as described in methods, then stimulated with 10 μM NE alone or in combination with 10 μM DMI for 5 min. Whole cell homogenates were analyzed by SDS–PAGE/Western blot, probing for phospho-ERK1/2 and tubulin. (B) Arrestin2,3 double knockout (Arr2,3$^{-/-}$) MEFs were stimulated for 5 min with NE at varying concentrations, ranging from 10 nM (10^{-8} M) to 100 μM (10^{-4} M), alone or in combination with 10 μM DMI, as indicated. Homogenates were analyzed as in panel A, with the addition of stripping/probing for total ERK. Blots are representative of three independent experiments.

Selectivity is supported by the findings that while α_{2A}AR-mediated ERK1/2 activation is enhanced, an opposite and more general effect of DMI on Akt is observed (Fig. 3).

Our original hypothesis that DMI would serve as a functional antagonist of endogenous agonist-mediated signaling at the α_{2A}AR is soundly refuted by our data. In fact, it seems that the interaction of DMI with the α_{2A}AR is much more complex than any associated with classical ligands. Given that a switch in the signaling from G protein-mediated to arrestin-mediated transduction does not occur (Fig. 4), a mechanistic model of this complex interaction remains to be elucidated. The functional signaling data in this study could be interpreted as representing a positive allosteric modulation of the receptor by DMI. Although our past analysis indicates that DMI binds to the α_{2A}AR orthosterically [5], it is possible that DMI may interact with both the orthosteric site and an allosteric site, a phenomenon which has been reported for ligands at the M_2 muscarinic receptor [21]. DMI may bind to an allosteric site which is available only within the active conformation of the receptor, given that our previous analysis was carried out with an antagonist as the competing radioligand. These possibilities raised by our present data suggest a more rigorous analysis of the DMI/α_{2A}AR interaction is warranted, utilizing such approaches as the kinetic receptor binding techniques described by Limbird and more specifically designed to detect allosterism [22].

Alternatively, DMI may in fact be binding to the orthosteric site on the α_{2A}AR and modulating signaling in some other way, for example through a dimerization of DMI-bound receptor with NE-bound receptor which alters the receptor signaling profile. α_{2A}ARs are among the many GPCRs which have been shown to homodimerize [23], and dimerization has the potential to alter the signaling profiles of GPCRs as a kind of allosterism [24].

Regardless of the underlying mechanism, our findings have potential implications for antidepressant pharmacology. Our previous study [5] postulated a model whereby DMI exerts its therapeutic antidepressant effects through a downregulation of central nervous system α_{2A}AR expression, correcting the pathobiological increases in α_{2A}AR density and activity associated with clinical depression (reviewed in [25]). The present data demonstrate that DMI in a physiologically relevant ratio with NE can effectively turn on MAPK signaling at that is lacking in response to the physiological level (e.g. 10 nM, see Fig. 2A and B). This α_{2A}AR-activating effect could serve to counterbalance the beneficial α_{2A}AR-decreasing effect until a certain level of α_{2A}AR downregulation is attained. Such a model may represent an important component of the mechanistic basis underlying the significant delay of 3–6 weeks between the start of clinical antidepressant therapy and onset of symptom relief.

Role of the funding sources

The funding sources had no involvement in the study design, collection, analysis, and interpretation of data, writing of the report, or decision to submit the article for publication.

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