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Lithium Suppresses Signaling and Induces Rapid Sequestration of β2-Adrenergic Receptors

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Lithium is a monovalent cation used therapeutically to treat a range of affective disorders (1), although the cellular mechanisms of lithium regulation that might contribute to its therapeutic effects at the level of neurotransmitter receptors are not known. Herein we report the ability of lithium to stimulate the internalization of β2-adrenergic receptors. Lithium treatment of A431 human epidermoid carcinoma cells resulted in a rapid, prominent desensitization and internalization of β2-adrenergic receptors. The ability of these receptors to generate a cyclic AMP response was strongly inhibited by lithium, at concentrations therapeutic in humans. Receptors for the serotonin (5HT1c) and for opiates (μ-opioid), in sharp contrast, resisted the effects of lithium on internalization. These data provide the first receptor-based mechanism to be described for lithium that could explain, in part, the therapeutic effects of lithium on affective disorders.

Key Words: **lithium; β2-adrenergic receptors; signaling; sequestration; Na-channels.**

Lithium has a long history as a useful drug in the treatment of various affective disorders, especially bipolar disease. In the last decade, insights in possible mechanisms of lithium action have been revealed, including its effects on nerve conduction, neurotransmitter uptake, and neuronal excitability (2). Neurotransmitters, such as the catecholamine norepinephrine, have been implicated in affective disorders (3) and act via a well-known signaling paradigm, in which norepinephrine binds to receptors that couple via heterotrimeric G-proteins to effectors such as adenylylcyclase (4, 5). In spite of the level of knowledge that has accumulated, little is known to what extent, if any, are the therapeutic effects of lithium exerted directly at the level of these G-protein coupled receptors (GPCRs).

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MATERIALS AND METHODS

Cell culture. Human epidermoid carcinoma cells (A431) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), penicillin (60 μg/ml), and streptomycin (100 μg/ml) and grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C (6).

Transfection of GFP-tagged β2-adrenergic, 5HT1c, and μ-opioid receptor. A431 cells were transfected with expression vectors harboring the GFP-tagged β_2AR using Lipofectin (Life Technologies, Inc.), according to the manufacturer's protocol and viable clones selected in 400 μg/ml of the neomycin analogue G418. Resistant colonies were subcloned and screened for GFP-fusion protein expression by epifluorescence microscopy. Transfections with the 5HT1c and opioid receptor expression vectors were transient.

Epifluorescence imaging. Microscopy of live cells was performed on the Eclipse TE300 (Nikon) inverted microscope equipped with 40× oil objective. Images were acquired using MicroMAX Imaging System (Princeton Instruments Inc.) and WinView32 software (7).

Inhibitor studies. A431 cells and stably transfected clones were routinely challenged without or with either lithium (5 min) and then with or without isoproterenol (10 μM) for 30 min and the trafficking of the GFP-tagged β_2AR monitored by epifluorescence. Cells were serum-deprived for 8 h prior to remove growth factors and catechols from the cell media. For studies of the effects of inhibitors on the trafficking of the GFP-tagged receptor in response to either isoproterenol or lithium (or both), the inhibitors were added 30–40 min in advance of the challenge with drugs. The inhibitors and the concentrations at which they were employed are as follows: amiloride (100 μM); genistein (5 μM); and LY294002 (10 μM) to inhibit 1-phosphatidylinositol 3-kinase (8).

RESULTS AND DISCUSSION

To address the effects of lithium on GPCRs, A431 human epidermoid carcinoma cells propagated in culture were treated with a β-adrenergic agonist and their cyclic AMP responses measured (Fig. 1A). Treatment with lithium at a final concentration of 1 mM resulted in a >50% decline in the ability of the cells to respond

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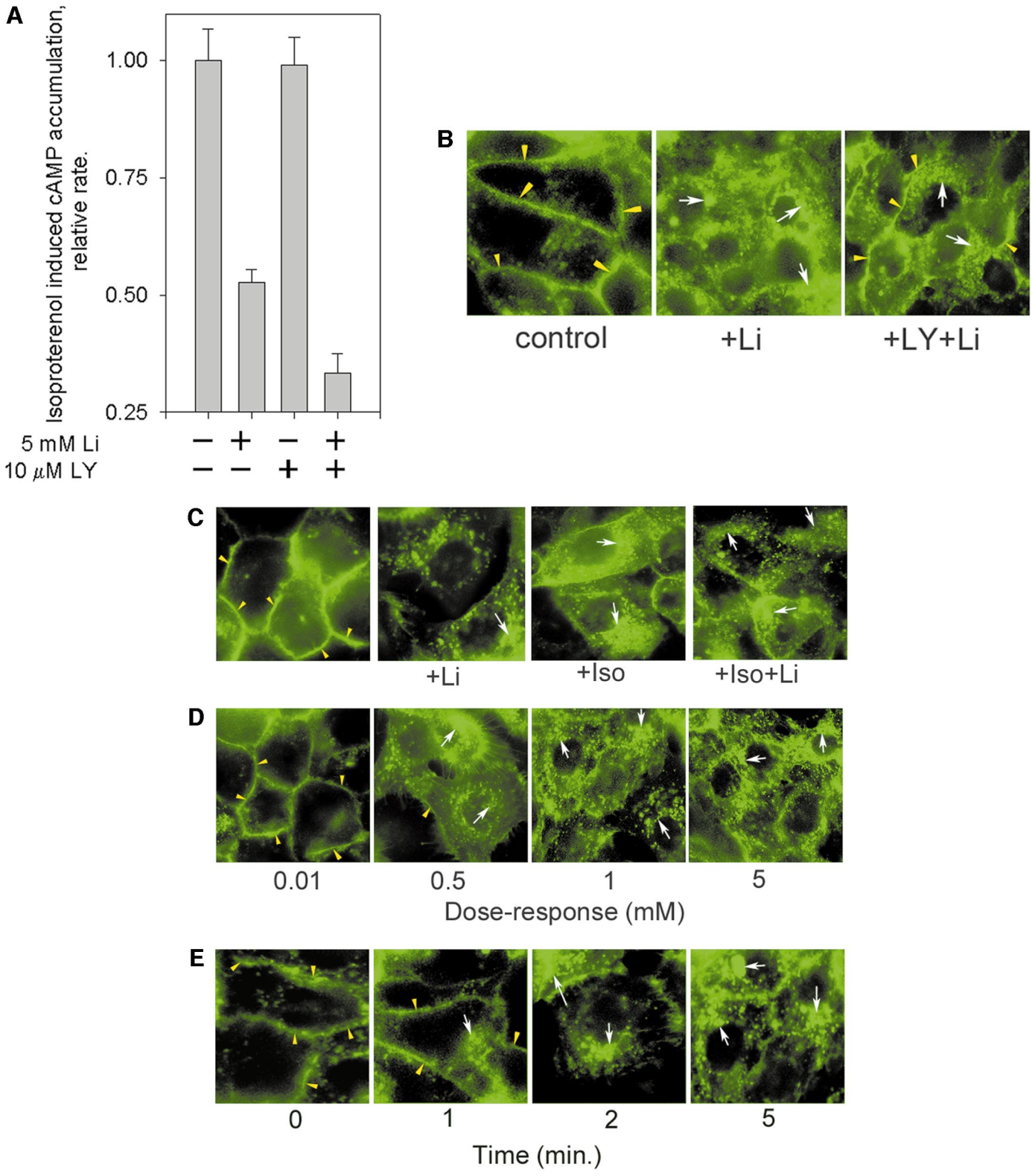

FIG. 1. Lithium treatment inhibits agonist-stimulated cAMP synthesis and internalization of β_2-adrenergic receptor. Serum-starved A431 cells were treated with lithium chloride and the cyclic AMP response to isoproterenol and the internalization of β_2AR assayed. (A) Inhibition of isoproterenol-stimulated cyclic AMP synthesis (10 μM Iso) in cells treated with and without lithium (15 min, 1 mM Li.) (B, C) Localization of GFP-tagged β_2AR receptors after treatment with lithium (15 min, 1 mM Li) compared to treatment with isoproterenol (15 min, 10 μM Iso). (D) Dose-response for lithium-stimulated internalization of β_2ARs. (E) Time course (0–5 min) for lithium-induced internalization of β_2AR. In A and B, the effects of the addition of the PI3-kinase inhibitor LY294002 (10 μM, 40 min) was examined on lithium effects on the cyclic AMP response and receptor localization. The images presented are representative of the results from at least four separate experiments.

to the β-adrenergic agonist isoproterenol (10 μM). Basal cyclic AMP levels were unaffected by lithium treatment (not shown). The dose–response curve for the effects of lithium on the agonist-stimulated cyclic AMP response revealed the half-maximal inhibition at ~0.5 mM lithium (not shown), well within the range of concentrations employed therapeutically (1). Receptor internalization was investigated as one possible element in the ability of lithium to reduce the cyclic AMP response. β_2-adrenergic receptors (β_2AR) were tagged with the green fluorescent protein (GFP) and used in tandem with epifluorescence microscopy to make visible the cellular localization of the β_2AR (Fig. 1B). β_2AR are localized predominantly to the cell membrane (yellow arrowheads, Fig. 1B). Lithium treatment (1 mM) provoked a prominent internalization of the β_2AR (white arrows, Fig. 1B). The extent of the internalization induced by treatment with lithium was greater than that in response to the β-adrenergic agonist isoproterenol (10 μM), which stimulates agonist-induced internalization (Fig. 1C). The addition of both lithium and isoproterenol yielded the same level of β_2AR internalization as with lithium alone.

Based upon the internalization of the GFP-tagged β_2AR made visible by epifluorescence, the dose-response for lithium-induced sequestration was investigated (Fig. 1D). No lithium-induced internalization of β_2AR was detected at 0.01 mM, whereas 1 mM lithium ion was sufficient to induce maximal internalization. The time-course for lithium-induced internalization revealed a very rapid response, peaking within a few minutes of challenge with the monovalent cation (Fig. 1E). Thus, lithium ions induce rapid, marked internalization of this GPCR, β_2AR, more profound than that induced by agonist alone. In addition, the internalization is observed with concentrations of lithium well within the range of therapeutic concentrations of this ion used to treat various affective disorders.

Lithium effects are often ascribed to the ability of this ion to alter aspects of inositol phosphate metabolism, via inhibition of inositol-1-phosphatase (9, 10) and activation of phosphatidyinositol-3 (PI3)-kinase (11, 12). We tested the effects of an inhibitor of PI3-kinase to influence lithium effects. Addition of LY294002 compound to the media blocks PI3-kinase activity, but did not influence the ability of lithium ions to suppress either the cyclic AMP response or the internalization of the β_2AR (Figs. 1A and B).

Entrance and extrusion of lithium ions from cells occurs primarily through Na-transport pathways (13). Amiloride and its analogs are known inhibitors of a number of transmembrane Na+ transport systems, including the epithelium Na+ channel, the Na+/H+ exchange system and the Na+/Ca2+ exchange system. To test for the role of the sodium channels in lithium-induced internalization of the β_2AR, cells were pretreated with amiloride and then challenged with lithium (1 mM). Epifluorescence microscopy of GFP-tagged β_2AR revealed that amiloride itself had little effect on β_2AR internalization, but can block the internalization of the receptors stimulated in response to lithium (Fig. 2A). Earlier studies implicated the interaction of GPCR with their cognate G-proteins (14), or adenylylcyclase (15) as targets for lithium action. Lithium reduced the isoproterenol-stimulated cyclic AMP response by ~50%, but this effect was not altered by amiloride (Fig. 2B). These observations suggest that lithium, like β_2AR agonist, provokes an uncoupling and internalization of β_2AR. Amiloride blocks the latter, but not the former. Amiloride has been shown to inhibit Src, much like PP2 (16). Src is an obligate element of β_2AR internalization in response to agonist stimulation (8), so inhibition of Src itself would be expected to alter receptor internalization, but not desensitization as observed herein.

We examined the effects of the tyrosine kinase inhibitor genistein on lithium-induced sequestration of the β_2AR. Lithium effects, such as activation of the Na+/H+-antiporter (NHE1) or the mitogenic pathways, have been linked to the action of tyrosine kinases that are sensitive to genistein (17). Genistein is known to inhibit adenylylcyclase activity (18) and a 50% decline in the isoproterenol-induced cyclic AMP response precluded our assessment of its ability to alter lithium effects on cyclic AMP responses (not shown). Analysis of β_2AR localization revealed, however, that genistein does inhibit lithium-induced internalization, although not completely (Fig. 3A). The Tyr350 residue of the β_2AR is essential for tyrosine kinase-induced internalization of this GPCR, providing an SH2-binding site involved in receptor sequestration (19). We tested if the Tyr350Phe (Y350F) mutant form of β_2AR that is not sequestered in response to tyrosine kinase activation was sensitive to lithium ion treatment (Fig. 3B). Unlike the wild-type (wt) receptor, the Tyr350Phe mutant β_2AR displayed no significant internalization in response to lithium. Taken together these data suggest a central role of tyrosine phosphorylation and the integrity of the Tyr350 residue of the β_2AR for the receptor to undergo lithium-induced internalization.

Many G-protein coupled receptors are thought to control aspects of mood and have been implicated as possible sites of affective disorders, such as bipolar disease. We compared the effects of lithium ions on the β_2AR and on a member of the serotonin (5HT1c) receptor family (Fig. 4). The GFP-tagged 5HT1c receptor was distributed to the cell membrane and to the perinuclear region of the cell. Lithium ion stimulated a rapid, marked internalization of the β_2AR, but had little effect on the cellular localization of GFP-tagged 5HT1c receptors. The complement of the 5HT1c receptors localized to the cell membrane was not changed in response to lithium. We explored next the effects of lithium treatment on cellular localization of a member

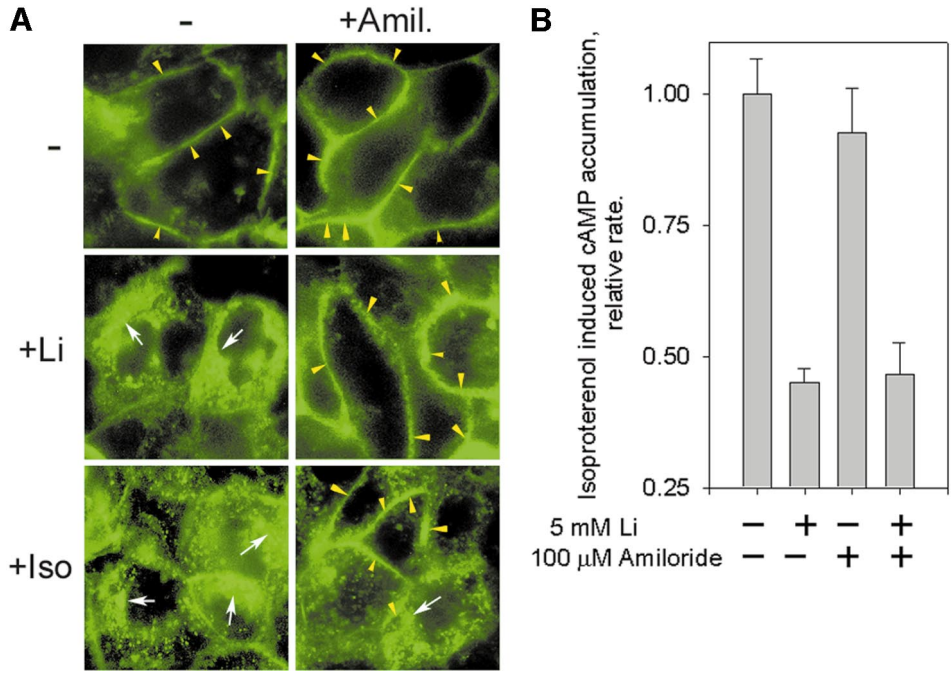

FIG. 2. Amiloride abolishes lithium-stimulated internalization of β_2AR, but not the lithium-dependent inhibition of isoproterenol-induced cAMP synthesis. (A) Effects of preincubation with amiloride (100 μM, for 40 min) on lithium (1 mM, 15 min)-stimulated internalization of β_2AR. (B) Effects of amiloride (100 μM) on lithium (1 mM, 15 min)-stimulated inhibition of isoproterenol-stimulated cAMP synthesis. The images presented are representative of the results from at least four separate experiments. The cyclic AMP data are representative of results from three separate experiments.

of the opioid receptor family, the μ-opioid receptor (Fig. 4). A GFP-tagged version of the μ-opioid receptor was expressed in the A431 cells and then the cells were challenged with lithium ions (1 mM). The cellular distribution of μ-opioid receptor, like that of the 5HT1c receptor, was unaffected by treatment with lithium ion. Based upon this limited sampling of G-protein-coupled receptors, the effects of lithium on receptor internalization seems to be selective for the β_2AR.

Lithium salts were one of the earliest pharmacological agents identified as therapeutic for various affective disorders. Treatment with lithium remains an im-

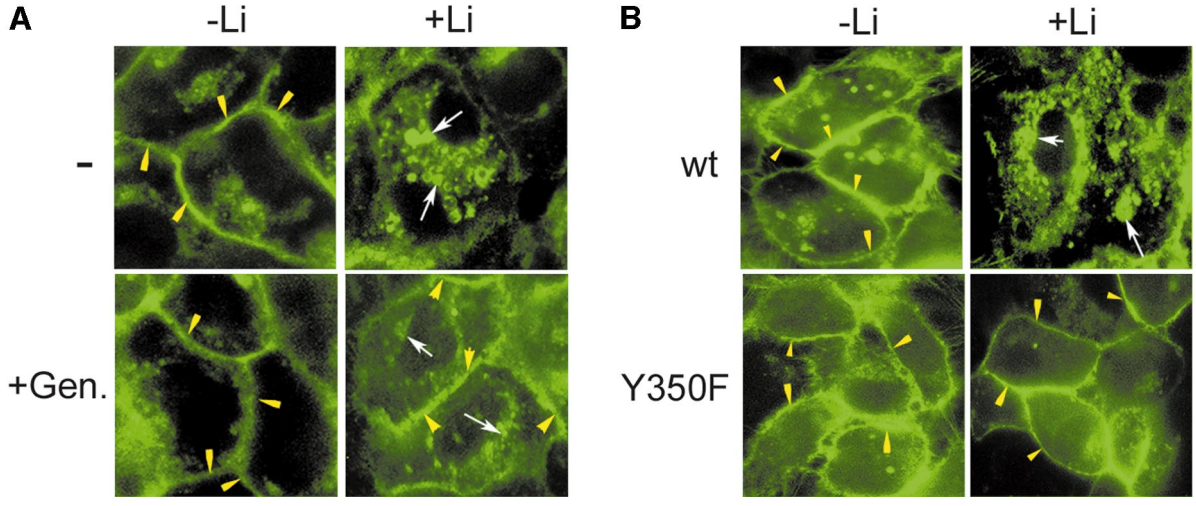

FIG. 3. Lithium-induced internalization of β_2AR is blocked by genistein as well as the Y350F mutation of the receptor. (A) Effects of preincubation of cells with genestein (5 μM, 40 min) on lithium (1 mM, 15 min)-stimulated internalization of β_2AR. (B) Effects of lithium (1 mM, 15 min) on the internalization of wild-type (wt) and the Y350F mutant form of the β_2AR. The images presented are representative of the results from at least four separate experiments.

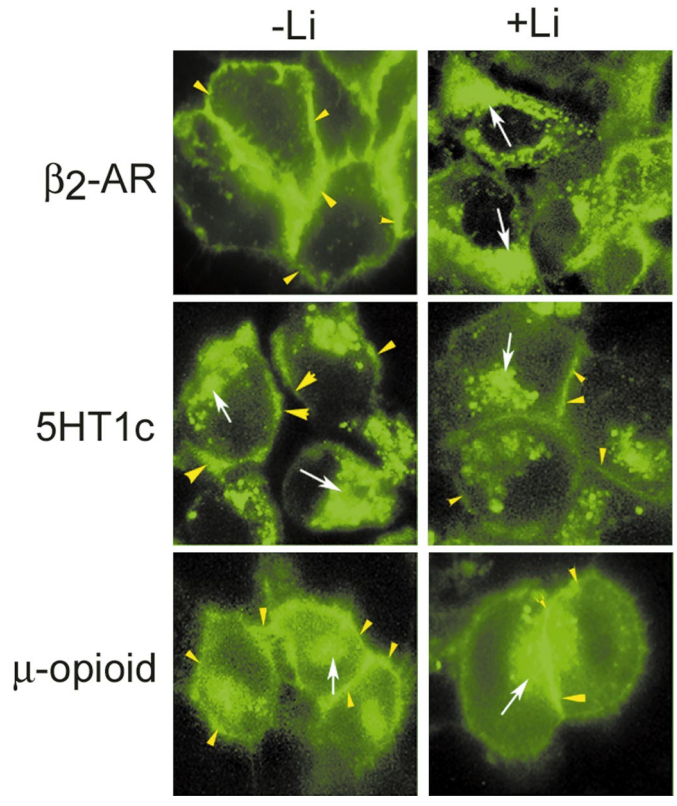

FIG. 4. Lithium treatment provokes rapid internalization of β_2AR, but not internalization of the 5HT1c serotonin receptor or the μ-opioid receptor. Localization of GFP-tagged receptors after treatment with lithium (15 min, 1 mM Li). Epifluorescence images of the cellular localization of GFP-tagged β_2-AR, 5HT1c serotonin, and μ-opioid receptors are displayed. The images presented are representative of the results from at least four separate experiments.

portant element of therapy for mood disorders, especially bipolar disease. There are many studies that address the macroscopic effects of lithium salts on nerve conduction and Na-channels (20, 21). Herein we report the first analysis of the effects of lithium on the trafficking of an important members of the neurotransmitter family, the β_2AR. As previously noted in brains from animals treated with lithium (2), there is a reduction in the cyclic AMP response for A431 cells treated with therapeutic concentrations of lithium ions. Remarkably, the treatment with lithium ion resulted in a profound internalization of the β_2AR. The extent of the internalization induced by lithium is greater than that obtained during β-adrenergic agonist-induced desensitization. In addition, the effects of lithium are observed within minutes, whereas the well-known agonist-induced internalization occurs over 30 min or more. Lithium-induced internalization was not observed for two other G-protein-coupled receptors studied herein, the 5HT1c serotonin receptor and the μ-opioid receptor. Defining the precise mechanism(s) by which lithium provokes a rapid and marked internalization of β_2ARs may provide valuable new insights into the trafficking of G-protein-coupled receptors.

ACKNOWLEDGMENT

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