THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 18, pp. 12722–12728, May 5, 2006

Nuclear Factor-κB Activated by Capacitative Ca^{2+} Entry Enhances Muscarinic Receptor-mediated Soluble Amyloid Precursor Protein (sAPPα) Release in SH-SY5Y Cells*

Received for publication, February 2, 2006 Published, JBC Papers in Press, February 20, 2006, DOI 10.1074/jbc.M601018200

Shinkyu Choi[1], **Jin Hyoung Kim**[1,2], **Eun-Jihn Roh, Moon-Jeong Ko, Ji-Eun Jung, and Hwa-Jung Kim**[3]

From the Research Institute of Pharmaceutical Sciences, College of Pharmacy, Ewha Womans University, Seoul 120-750, Korea

$G_q/_{11}$ protein-coupled muscarinic receptors are known to regulate the release of soluble amyloid precursor protein (sAPPα) produced by α-secretase processing; however, their signaling mechanisms remain to be elucidated. It has been reported that a muscarinic agonist activates nuclear factor (NF)-κB, a transcription factor that has been shown to play an important role in the Alzheimer disease brain, and that NF-κB activation is regulated by intracellular Ca^{2+} level. In the present study, we investigated whether NF-κB activation plays a role in muscarinic receptor-mediated sAPPα release enhancement and contributes to a changed capacitative Ca^{2+} entry (CCE), which was suggested to be involved in the muscarinic receptor-mediated stimulation of sAPPα release. Muscarinic receptor-mediated NF-κB activation was confirmed by observing the translocation of the active subunit (p65) of NF-κB to the nucleus by the muscarinic agonist, oxotremorine M (oxoM), in SH-SY5Y neuroblastoma cells expressing muscarinic receptors that are predominantly of the M3 subtype. NF-κB activation and sAPPα release enhancement induced by oxoM were inhibited by NF-κB inhibitors, such as an NF-κB peptide inhibitor (SN50), an IκBα kinase inhibitor (BAY11-7085), a proteasome inhibitor (MG132), the inhibitor of proteasome activity and IκB phosphorylation, pyrrolidine dithiocarbamate, the novel NF-κB activation inhibitor (6-amino-4-(4-phenoxyphenylethylamino) quinazoline), and by an intracellular Ca^{2+} chelator (TMB-8). Furthermore, both oxoM-induced NF-κB activation and sAPPα release were antagonized by CCE inhibitors (gadolinium or SKF96365) but not by voltage-gated Ca^{2+}-channel blockers. On the other hand, treatment of cells with NF-κB inhibitors (SN50, BAY11-7085, MG132, or pyrrolidine dithiocarbamate) did not inhibit muscarinic receptor-mediated CCE. These findings provide evidence for the involvement of NF-κB regulated by CCE in muscarinic receptor-mediated sAPPα release enhancement.

The amyloid precursor protein (APP)[4] is a transmembrane protein that produces β amyloid (Aβ) by proteolytic cleavage in brains of individuals with Alzheimer disease. APP normally undergoes proteolytic cleavage within the Aβ sequence liberating the α-secretase-cleaved APP (sAPPα). Although evidence indicates that sAPPα plays important roles in regulating neuronal survival and plasticity, the mechanisms that mediate these biological activities and the regulation of sAPPα secretion have not been established. The transcriptional nuclear factor-κB (NF-κB) responds to a large number of environmental cues, and in the nervous system, it is modulated under physiological and pathological conditions, which include developmental cell death and acute of chronic neurodegenerative disorders (1, 2). It was proposed that NF-κB or other κB-binding proteins may be involved in a neuroprotective change in gene expression evoked by various cytokines and by secreted APP in neuronal cells, and therefore, may have a positive effect on Alzheimer disease (3–6).

The NF-κB/Rel family of dimeric transcriptional factors is involved in the immediate early transcription of a large array of genes induced by mitogenic and antiapoptotic pathogen-associated stimuli (7, 8). The eukaryotic NF-κB/Rel family of eukaryotic transcription factors, which includes p50, p65, c-Rel, RelB, and p52, bind DNA with high specificity and affinity as homo- or heterodimers to mediate a diverse range of biological processes (9–11). The most common form of NF-κB consists of a heterodimer of p50 (NF-κB1) and p65 (Rel A) (12–15). In most resting cells, NF-κB is retained in the cytoplasm by its association with inhibitor molecules of the IκB family (16). The formation of NF-κB-IκB complex masks the nuclear localization signal sequence in NF-κB and thus prevents its nuclear translocation. In response to various stimuli, NF-κB dimers are released from cytoplasmic IκB proteins by a process involving site-specific phosphorylation of IκB by IκB kinase, ubiquitination, and subsequent proteolytic degradation via the 26 S proteasome pathway (6, 17, 18).

The induction of NF-κB activity is known to be regulated by the intracellular Ca^{2+} level following various stimuli, and Ca^{2+} oscillations increase the efficiency and specificity of the expression of various genes (19, 20). Lilienbaum *et al.* (21) demonstrated a rise of intracellular Ca^{2+} through the opening of voltage-sensitive L-type Ca^{2+} channels in the plasma membrane and through the indirect opening of inositol 1,4,5-triphosphate receptors associated with intracellular Ca^{2+} stores and that this is responsible for basal and inducible NF-κB activity via cytokines or glutamate. Capacitative calcium entry (CCE), which is mediated by Ca^{2+}-permeable channels termed store-operated Ca^{2+} channels (SOCs), was reported to require the sustained activation of a nuclear factor of activated T cells, an NF-κB-like molecule on T cells (22, 23). Immunoreceptors and receptor tyrosine kinases activate phos-

* This work was supported by grants from the Korea Health 21 R&D Project, the Korean Ministry of Health & Welfare (Grant 02-PJ1-PG3-21304-0001), and the Korean Research Foundation (Grant KRF-2001-005-E00003). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Both authors contributed equally to this wok.

[2] Present address: Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151-742, Korea.

[3] To whom correspondence should be addressed: College of Pharmacy, Ewha Womans University, 11-1, Daehyun-dong, Seodaemun-gu, Seoul, 120-750, Korea. Tel.: 82-2-3277-3021; Fax: 82-2-3277-3051; E-mail: hjkim@ewha.ac.kr.

[4] The abbreviations used are: APP, amyloid precursor protein; sAPPα, α-secretase-cleaved APP; Aβ, β amyloid; NF-κB, nuclear factor-κB; CCE, capacitative calcium entry; SOC, store-operated Ca^{2+} channels; QNZ, 6-amino-4-(4-phenoxyphenylethylamino) quinazoline; fura-2/AM, 1-(2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy)-2-(2[1]-amino-5[1]-methylphenoxy)-ethane-*N*,*N*,*N*[1],*N*[1]-tetra-acetic acid penta-acetoxymethyl ester; DAPI, 4′, 6-diamino-2-phenylindole dihydrochloride; oxoM, oxotremorine M; FITC, fluorescein-5-isothiocyanate; PDTC, pyrrolidine dithiocarbamate; TMB-8, 8-diethylaminooctyl 3,4,5-trimethoxybenzoate; EMSA, electrophoretic mobility shift assay.

phatidylinositol-specific phospholipase C_γ, whereas G-protein-coupled receptor activates phospholipase C_β followed by CCE (24).

Little information on the mechanism of NF-κB regulation by muscarinic stimulation is known, especially in neuronal cells. Moreover, in neuronal cells, the involvement of CCE in muscarinic receptor-mediated NF-κB activation or *vice versa* is little understood. Our recent data indicated that CCE is involved in the regulation of muscarinic receptor-mediated sAPPα release (25), and we postulated that NF-κB might be an important signaling molecule in the regulation of muscarinic receptor-mediated sAPPα release. Therefore, in the present study, we examined how NF-κB and CCE are involved in muscarinic receptor-mediated sAPPα release in SH-SY5Y human neuroblastoma cells.

EXPERIMENTAL PROCEDURES

Materials—SN50, BAY11-7085, pyrrolidine dithiocarbamate (PDTC), MG132, 8-diethylaminooctyl 3,4,5-trimethoxybenzoate (TMB-8), and 6-amino-4-(4-phenoxyphenylethylamino) quinazoline (QNZ) were purchased from Biomol (Plymouth Meeting, PA). 1-(2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy)-2-(2[1]-amino-5[1]-methylphenoxy)-ethane-*N*,*N*,*N*[1],*N*[1]-tetra-acetic acid penta-acetoxymethyl ester (fura-2/AM) and 4′, 6-diamino-2-phenylindole dihydrochloride (DAPI) were purchased from Molecular Probes (Eugene, OR), and oxotremorine M (oxoM) was from Research Biochemicals International (Natick, MA). Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Invitrogen. Desalting columns (PD-10) and reagents for enhanced chemiluminescence (ECL) were purchased from Amersham Biosciences. Anti-Aβ monoclonal antibodies to sAPPα (clone 6E10), recognizing the amino acid residues 1–17 of APP, were purchased from Chemicon International (Temecula, CA), and anti-pre A4 monoclonal antibodies to sAPP (clone 22C11), which recognize the amino terminus of APP, were purchased from Roche Applied Science. A fluorescein-5-isothiocyanate (FITC)-conjugated anti-human NF-κB monoclonal anti-p65 antibody (sc8008) recognizing an epitope mapping to the amino terminus of human NF-κB P65 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-mouse antibody and goat anti-rabbit antibody used in Western blot analysis were purchased from Sigma. The other reagents used from Sigma or Fisher Scientific.

Mammalian Cell Cultures—SH-SY5Y human neuroblastoma cells (ATCC CRL-2266) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained at 37 °C in humidified conditions under 5% CO_2. Media were changed twice weekly, and cultures were split in the ratio of 1:5 weekly. For experiments, SH-SY5Y cells were plated in 6- or 12-well plates for 24 h. The medium was then removed and replaced with fresh medium without serum, and the cells were maintained for the time periods indicated.

Measurement of sAPPα Release—sAPPα release was measured as described by Nitsch *et al.* (26) with some modification. Briefly, cells were cultured to confluency in multiwell dishes (6-well format). Prior to drug addition, cells were washed with serum-free media and pretreated with various agents in serum-free media for 30 min following which they were treated with oxoM in serum-free media for 1 h. Conditioned media from wells were centrifuged to remove debris, desalted, dried, and reconstituted in SDS loading buffer. Total cell protein per dish was measured using the bicinchoninic acid assay (Pierce). Reconstituted culture media protein extracts containing the same amounts of total protein were separated by SDS-polyacrylamide gel electrophoresis on 10% gels and transferred to polyvinylidene fluoride membranes. The membranes were immunoblotted using antibodies 22C11 (1:125) or 6E10 (1:200), and bands were visualized by chemiluminescence. Data collection and processing were performed using a luminescent image analyzer LAS-1000 and IMAGE GAUSE software.

Measurement of Changes in $[Ca^{2+}]_i$—$[Ca^{2+}]_i$ levels were determined in suspensions of fura-2/AM-loaded cells. Briefly, confluent cells were harvested, washed with a buffer containing 140 mM NaCl, 2.0 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, 40 mM sucrose, and 0.05% bovine serum albumin (pH 7.3), and resuspended in 4.0 ml of the same buffer. Cells were then placed in a quartz microcuvette in a thermostat-controlled cell holder. Fura-2/AM was added to a final concentration of 5 μM, and the cells were then incubated with continuous gentle stirring for 30 min at 37 °C in the dark. Supernatants containing extracellular fura-2/AM were removed following gentle centrifugation of 0.5-ml aliquots, and cells were washed three times. They were then resuspended in 1 ml of buffer and incubated at 37 °C for 10 min prior to further centrifugation and resuspension in 4.5 ml of buffer at 37 °C. Experiments were performed at 37 °C. For Ca^{2+}-free experiments, the same buffer was used, but $CaCl_2$ was omitted. Fluorescence was monitored using fura 2-loaded cells in a Shimadzu RF-5301 spectrofluorometer (Shimadzu Scientific Instruments, Columbia, MD) using the dual wavelength method of Grynkiewicz *et al.* (27) (excitation at 340 and 380 nm and emission at 510 nm every 0.5 s). Data are expressed as ratios of fura-2 fluorescence at 340 *versus* 380 nm (F_{340}/F_{380}).

Preparation of Cytosolic and Nuclear Extracts—Cells were washed twice in ice-cold phosphate-buffered saline and suspended in ice-cold lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of pepstatin A, leupeptin, and aprotinin) and incubated on ice for 30 min. Lysed cells were centrifuged at 8,000 rpm for 5 min at 4 °C, and supernatants were collected as cytosolic protein extracts. To prepare nuclear extracts, the pellets were resuspended in a buffer containing 20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.1 mM β-glycerophosphate, 0.05 mM vanadate, and the protease inhibitor mixture. After extraction on ice for 30 min, samples were centrifuged at 14,000 rpm for 30 min at 4 °C. Supernatant, containing nuclear proteins, was transferred to a microcentrifuge tube, an aliquot was removed for protein determination, and samples were stored at −20 °C.

Electrophoretic Mobility Shift Assay (EMSA)—EMSAs were performed using a double-stranded 15-bp oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) containing the NF-κB binding motif, which was radiolabeled as described previously (28). For the binding reaction, nuclear protein extract (10 μg) was incubated in a total volume of 20 μl in binding buffer containing 10 mM HEPES, pH 7.5, 5% glycerol, 50 mM KCl, 1 mM dithiothreitol, 1 μg of poly(dI-dC), and radiolabeled (about 10,000 cpm) DNA for 30 min at room temperature. DNA-protein complexes were resolved in a pre-electrophoresed 6% nondenaturing polyacrylamide gel at 4 °C. Subsequently, the gel was dried under vacuum and exposed to film.

Immunolocalization of P65 Proteins—SH-SY5Y cells on coverslips were fixed with 3.7% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 20 min at room temperature, permeabilized with 0.3% Triton X-100/phosphate-buffered saline for 5 min, and blocked with 10% bovine serum albumin in phosphate-buffered saline. Translocation of p65 to the nucleus was assessed after fixing stimulated (with 1 mM oxoM for 1 h) and unstimulated cells by overnight incubation with an FITC-conjugated anti-human NF-κB monoclonal anti-p65 antibody (sc-8008, 1:50) at 4 °C followed by a 3-min incubation with DAPI for nuclear staining. Fluorescence images were acquired with a confocal laser scan-

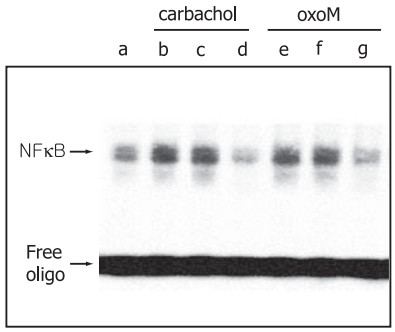

FIGURE 1. **NF-κB activation by muscarinic receptor stimulation.** SH-SY5Y cells were treated with vehicle (*a*), 100 μM carbachol (*b* and *d*), 1 mM carbachol (*c*), 100 μM oxoM (*e* and *g*), or 1 mM oxoM (*f*) for 1 h. EMSA was carried out in nuclear extracts incubated with ^{32}P-end labeled oligonucleotide, as described under "Experimental Procedures." In *d* and *g*, reaction mixtures contained a 100-fold excess of unlabeled NF-κB oligonucleotides (*oligo*).

ning microscope (LSM510; Carl Zeiss Co., Oberkochen, Germany) using a 505 nm long path filter for emission and an argon ion laser for excitation (488 nm).

Statistical Analysis—Data are expressed as means ± S.E. Comparisons between controls and treated groups were performed using the Student's *t* test. $p < 0.05$ was considered statistically significant.

RESULTS

Muscarinic Receptor-mediated NF-κB Activation in SH-SY5Y Cells—NF-κB is activated by physical and chemical stresses, viruses, bacteria, and pro-inflammatory cytokines such as tumor necrosis factor (29), and it is also activated by a novel mechanism through the stimulation of G-protein-coupled receptors as was shown in isolated canine gastric parietal cells (30). Initially, we examined whether muscarinic receptor stimulation can lead to the activation of NF-κB in SH-SY5Y cells by measuring NF-κB binding activities using EMSA. NF-κB was detected as two major bands, which increased after treating the cells with the muscarinic receptor agonists, carbachol or oxoM, as reported previously in SH-SY5Y cells (31). Moreover, muscarinic agonist-induced NF-κB activation was abolished by excess unlabeled NF-κB oligomer (Fig. 1). This result shows that muscarinic receptor stimulation induces the activation of NF-κB in SH-SY5Y cells.

NF-κB is activated by the posttranslational modification of an inactive cytosolic complex that contains p50, p65, and one of several inhibitory subunits that is generally designated as IκB (32). Upon appropriate cell stimulation, the inhibitory subunit of NF-κB, IκBα, is rapidly phosphorylated and undergoes proteolytic breakdown by proteasomes, thereby permitting NF-κB to translocate from the cytoplasm to the nucleus (33). The presence and function of NF-κB in cells of the nervous system are poorly documented, for example, as compared with lymphocytes. To obtain information on the mechanism of NF-κB activation mediated by muscarinic receptors in SH-SY5Y neuroblastoma cells, we carried out immunocytochemistry for the p65 subunit of NF-κB in cells pretreated with muscarinic receptor agonist and/or antagonist. The FITC-labeled p65 subunit, which was located in the cytosol, was translocated to the nucleus after treatment with the muscarinic agonist oxoM. Moreover, pretreatment with atropine, a muscarinic antagonist, reversed the oxoM-induced translocation of p65. To confirm that the muscarinic receptor-mediated translocation of p65 into the nucleus represents NF-κB activation, we used several NF-κB inhibitors, which differently block intracellular NF-κB activation pathways. SN50 is a cell-permeable peptide, which inhibits NF-κB by blocking its translocation to the nucleus (34). BAY11-7085 is an IκBα kinase inhibitor, and PDTC is known as a copper binding compound that inhibits protea-

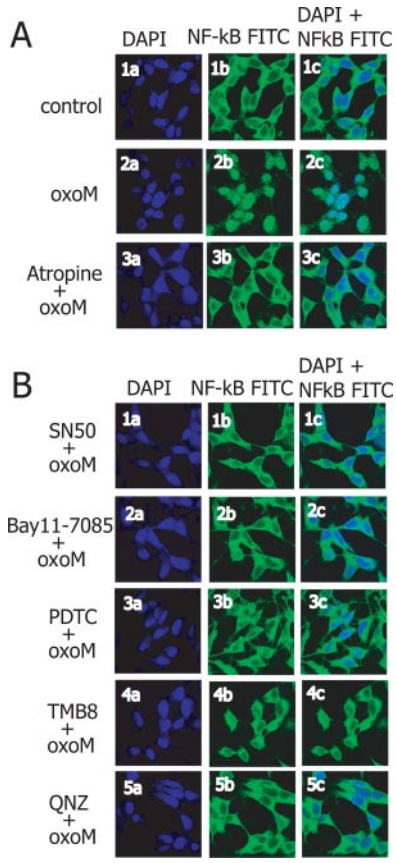

FIGURE 2. **Muscarinic receptor-mediated subcellular localization of NF-κB.** *A*, SH-SY5Y cells were treated with vehicle or 1 mM oxoM in serum-free media for 1 h with or without pretreatment of muscarinic antagonist atropine (10 μM) for 30 min. *B*, SH-SY5Y cells were pretreated with NF-κB inhibitors, SN50 (20 μg/ml), Bay11-7085 (20 μM), PDTC (10 μM), TMB-8 (100 μM), or a novel NF-κB activation inhibitor (QNZ, 20 nM) for 30 min and then treated with 1 mM oxoM in serum-free media for 1 h. DAPI nuclear staining and FITC immunofluorescence are shown in *panels a* and *b*, respectively, and a combined image is presented in *panels c*. Results are representative of 3–5 independent experiments.

some activity and IκB phosphorylation (35, 36). TMB-8 is known to inhibit NF-κB activation by blocking intracellular Ca^{2+} mobilization and chelating intracellular Ca^{2+} (37–39), and the novel NF-κB activation inhibitor (QNZ, $IC_{50} = 15$ nM) was found to inhibit the activation of NF-κB by an unknown mechanism (40). As is shown in Fig. 2*B*, muscarinic receptor-mediated NF-κB activation was inhibited by all NF-κB activation inhibitors tested.

Involvement of NF-κB Activation in the Regulation of Muscarinic Receptor-mediated sAPPα Release—It was reported that the activation of muscarinic receptors (M1 and M3) increases sAPPα release (26, 41). We also confirmed muscarinic receptor-mediated sAPPα release in SH-SY5Y cells. The secretion of sAPPα to culture medium was significantly increased by treating oxoM in a time-dependent manner, and this oxoM-induced sAPPα release was blocked by pretreating with 10 μM atropine, a muscarinic receptor antagonist (Fig. 3*A*).

Several studies have shown that sAPPα can stimulate the activation of the transcription factor NF-κB (3, 4), and sAPPα induced neurotrophic and excitoprotective actions mediated by phosphatidylinositol 3-kinase, Akt kinase, and p42/p44 mitogen-activated protein kinases (42). However, it has not been reported whether NF-κB activity can regulate sAPPα release. Therefore, to examine the involvement of NF-κB in the regulation of muscarinic receptor-mediated sAPPα release in SH-SY5Y cells, we tested whether the suppression of NF-κB activation could antagonize sAPPα release enhancement by muscarinic receptor activation. The oxoM-induced increase of sAPPα release was found to be

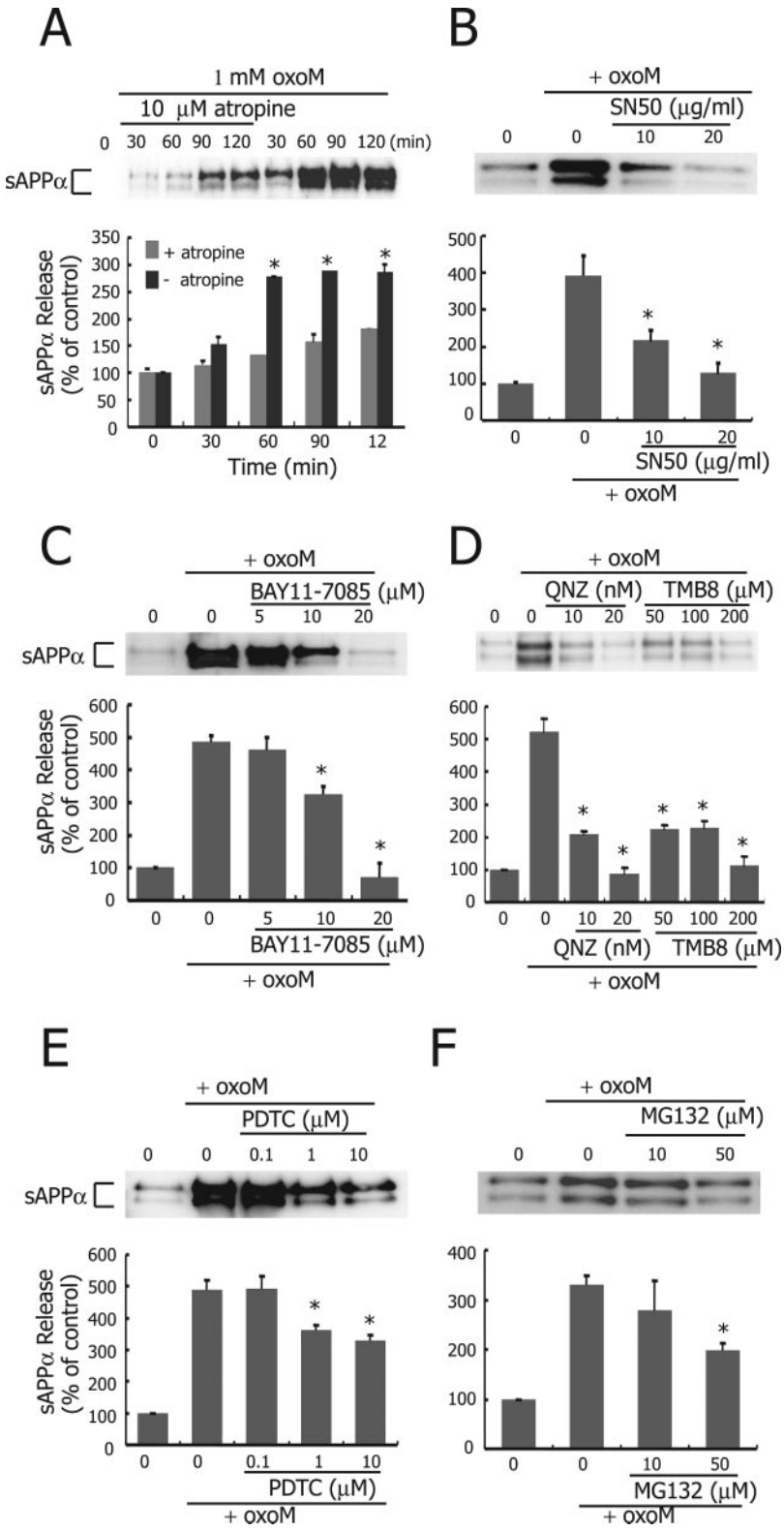

FIGURE 3. **Effects of NF-κB inhibitors on muscarinic receptor-stimulated sAPPα release.** SH-SY5Y cells were pretreated with vehicle or 10 μM atropine and then stimulated with 1 mM oxoM (*A*). SH-SY5Y cells were pretreated with indicated concentrations of SN50 (*B*), BAY11-7085 (*C*), QNZ, TMB-8 (*D*), PDTC (*E*), or MG132 (*F*) for 30 min and then stimulated with 1 mM oxoM for 1 h, and the secreted sAPPα was measured as described under "Experimental Procedures." Results are representative of 3 independent experiments. The data shown are means ± S.E. of 3 independent experiments. Significance indicates statistically different from oxoM-treated cells in the absence of inhibitors. *, $p < 0.05$.

concentration-dependently inhibited by all of the NF-κB inhibitors tested: SN50, BAY11-7085, QNZ, TMB-8, PDTC, or MG132 (Fig. 3). MG132 is an inhibitor of proteasome activity (43).

Regulation of Muscarinic Receptor-mediated NF-κB Activation by Extracellular Ca^{2+} Influx through CCE—Cells respond to various types of stimulation with a remarkable diversity of $[Ca^{2+}]_i$ signals. Ca^{2+} is a highly pleiotropic second messenger that plays an important role in a wide variety of cellular events. One of the important functions of Ca^{2+} is to activate signaling pathways that lead to the expressions of genes (44, 45). Although it is known that Ca^{2+} is involved in the induction of NF-κB activity following various stimuli in diverse types of cells, it is not known whether muscarinic receptor-mediated NF-κB activation can be regulated by CCE through SOCs in SH-SY5Y cells. Therefore, we examined whether CCE is involved in muscarinic receptor-mediated NF-κB

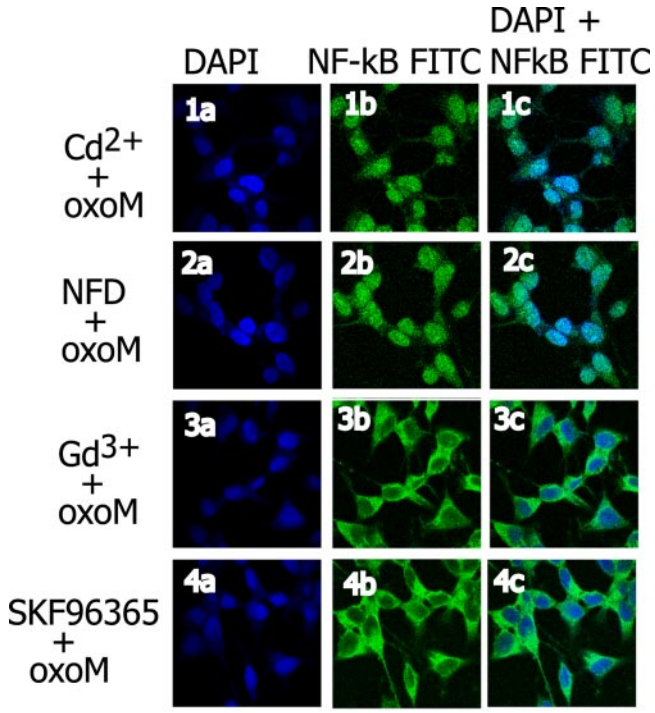

FIGURE 4. **Effects of CCE inhibitors on the muscarinic receptor-mediated subcellular localization of NF-κB.** SH-SY5Y cells were pretreated with voltage-sensitive Ca^{2+} channels inhibitors, Cd^{2+} (10 μM), or nifedipine (*NFD*, 10 μM) and CCE inhibitors, Gd^{3+} (50 μM), or SKF96365 (50 μM) for 30 min and then stimulated with 1 mM oxoM in serum-free medium for 1 h. DAPI nuclear staining and FITC immunofluorescence are shown in *panels a* and *b*, respectively, and a combined image is presented in *panels c*. Results are representative of 3 independent experiments.

translocation to the nucleus. NF-κB translocation to the nucleus by oxoM was blocked by Gd^{3+} or SKF96365, both of which are known potent inhibitors of SOC (46–48), but was not blocked by Cd^{2+}, or nifedipine, both voltage-sensitive Ca^{2+} channel inhibitors (Fig. 4). These results indicate that CCE plays a key role in NF-κB activation induced by muscarinic receptor stimulation and that NF-κB is engaged in muscarinic receptor-mediated sAPPα release.

Relationship between NF-κB Activation and CCE Stimulation Mediated by Muscarinic Receptor Stimulation—Because it was found that NF-κB activity regulates muscarinic receptor-mediated sAPPα release and that CCE regulates muscarinic receptor-mediated NF-κB activation in SH-SY5Y cells, we investigated whether NF-κB activation affects muscarinic receptor activation-induced Ca^{2+} entry by examining the effects of the NF-κB inhibitors, QNZ, TMB-8, SN50, BAY11-7085, PDTC, and MG132, on the intracellular Ca^{2+} responses evoked by oxoM. After preincubation in Ca^{2+}-free buffer containing 1 mM oxoM (to deplete Ca^{2+} stores), the re-addition of Ca^{2+} induced CCE, and this oxoM-induced CCE stimulation was inhibited by QNZ (a newly synthesized NF-κB inhibitor with an unknown mechanism) (Fig. 5*A*). In the presence of extracellular Ca^{2+}, pretreatment with QNZ also led to a significant reduction in the second phase of plateau Ca^{2+} entry (considered as CCE) of the oxoM-induced $[Ca^{2+}]_i$ response, without reducing transient initial Ca^{2+} release (Fig. 5*B*). Similar experiments were carried out using an intracellular Ca^{2+} chelator, TMB-8. In Ca^{2+}-free buffer, TMB-8 abolished all oxoM-induced $[Ca^{2+}]_i$ responses including Ca^{2+} release from intracellular stores and the Ca^{2+} entry induced when Ca^{2+} was restored to the medium (Fig. 5*C*). Also, in the presence of extracellular Ca^{2+}, both the initial transient Ca^{2+} release phase and the plateau Ca^{2+} entry phase of $[Ca^{2+}]_i$ response during oxoM application were completely abolished by TMB-8 pretreatment (Fig. 5*D*). On the other hand, other NF-κB inhibitors including the proteasome inhibitors, MG-132 and PDTC, a translocation inhibitor, SN50, and an IκB kinase inhibitor, BAY11-7085, which inhibited sAPPα release evoked by muscarinic receptor activation in SH-SY5Y cells, did not inhibit muscarinic receptor-mediated Ca^{2+} influx via CCE both in Ca^{2+}-free-buffers (Fig. 5*E*) and in Ca^{2+}-containing buffers (Fig. 5*F*). Results indicate that CCE induced by muscarinic receptor activation is not mediated by NF-κB. Therefore, the NF-κB activation pathway is downstream of CCE induced by muscarinic receptor activation, leading to enhancement of sAPPα release.

DISCUSSION

Although large secreted β-APP (sAPPα) is well known to play important roles in the regulation of neuronal survival and plasticity, the signal transduction mechanisms that mediate its release or biological activities are still not established. Numerous studies have demonstrated that stimulation of phospholipase C-linked G-protein-coupled receptors, including muscarinic M1 and M3 receptors, increases the release of the sAPPα by α-secretase cleavage. The purpose of this research was to determine whether muscarinic receptor stimulation activates NF-κB in neuroblastoma SH-SY5Y cells expressing abundant M3 muscarinic receptors and whether such muscarinic receptor-mediated NF-κB activation is involved in sAPPα release and contributes to change the CCE, which our recent data (25) indicate plays an important role in the muscarinic receptor-mediated stimulation of sAPPα release.

Although the muscarinic agonist carbachol has previously been shown to activate NF-κB in isolated canine gastric parietal cells (30) and the activation of NF-κB appears to be an important event in numerous intracellular inflammatory processes, there is insufficient evidence about correlating NF-κB and muscarinic receptor in neuronal cells. Therefore, we first confirmed that muscarinic receptor stimulation evoked the activation of NF-κB in human neuroblastoma SH-SY5Y cells by observing that muscarinic receptor agonists increased NF-κB binding activity (Fig. 1) and induced the translocation of p65 a subunit of NF-κB (Fig. 2), which is a direct evidence of NF-κB activation.

Given that muscarinic receptor stimulation increases sAPPα release and NF-κB activation, it is unclear whether this stimulation induces sAPPα release and subsequent NF-κB activation or *vice versa*. We observed that increased sAPPα release by oxoM was suppressed dose-dependently by pretreating with various NF-κB inhibitors with different mechanisms of NF-κB activation (Fig. 3). This result provides evidence that the NF-κB pathway is involved in the regulation of muscarinic receptor-mediated sAPPα release and that cross-talk exists between sAPPα release and NF-κB activation after muscarinic receptor activation. It was reported that several neuroprotective genes and proteins are markedly up-regulated in mice overexpressing APP (49). Our results suggest that NF-κB, which is known to have a neuroprotective role, is a signaling molecule responsible for sAPPα release by facilitating the secretase process. It has been reported that exposure to sAPPα protects hippocampal neurons against Ca^{2+}-mediated damage (3) and against Aβ-induced apoptosis in PC12 cells expressing mutant presenilin-1 (4) by inducing activation of the transcription factor NF-κB. It can be assumed that secreted sAPPα increased by muscarinic agonist-induced NF-κB activation may then further activate NF-κB in disease state such as Alzheimer disease.

NF-κB activation is modulated under both physiological and pathological conditions. Moreover, the activation of NF-κB before an experimental insult, such as exposure to glutamate, glucose deprivation, β-amyloid peptide, or oxidative molecules, has been shown to protect neurons against apoptosis (1–4, 50). The results of the present study

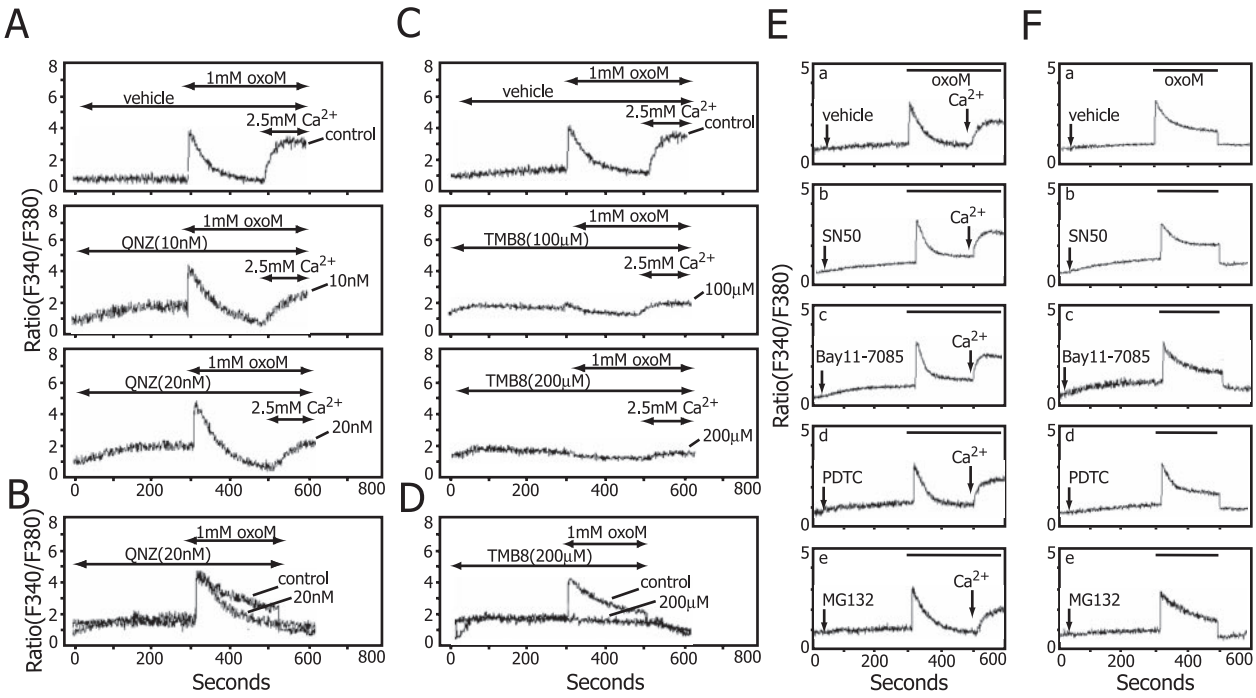

FIGURE 5. **Effects of NF-κB inhibitors on muscarinic receptor-induced Ca^{2+} entry.** Fura-2/AM-loaded SH-SY5Y cells were incubated for 300 s in the presence or absence of indicated concentrations of QNZ (10, 20 nM) or TMB-8 (100, 200 μM), SN50 (20 μg/ml), BAY11-7085 (20 μM), PDTC (10 μM), or MG132 (50 μM) and then stimulated with 1 mM oxoM in Ca^{2+}-free buffer (*A*, *C*, and *E*) and Ca^{2+}-containing buffer (*B*, *D*, and *F*). After 200 s, $CaCl_2$ (final concentration 2.5 mM) was added to the buffer to initiate Ca^{2+} entry in Ca^{2+}-free buffer. Elevations in $[Ca^{2+}]_i$ were monitored using 340/380 nm absorbance ratios. Traces are representative of 3 independent experiments.

show that muscarinic receptor stimulation in the absence of such apoptotic or critical conditions in neuronal SH-SY5Y cells induced NF-κB translocation to the nucleus. Several studies have demonstrated that various transcription factors are regulated by intracellular Ca^{2+} movement (19, 21, 51). We observed that extracellular Ca^{2+} influx (CCE) via SOC, which is functionally coupled with endoplasmic reticulum Ca^{2+} depletion, plays a key role in muscarinic receptor-mediated sAPPα release (25). Thus, we attempted to characterize the nature of the Ca^{2+} influx that participates in muscarinic receptor-mediated NF-κB activation by administering Ca^{2+} channel blockers. Nonselective and selective voltage-gated Ca^{2+} channel blockers had no effect, but SOC blockers inhibited muscarinic receptor-mediated NF-κB activation (Fig. 4). This finding suggests that the CCE induced by muscarinic receptor stimulation regulates NF-κB activation.

The activation of cell surface receptors coupled to phospholipase C, including muscarinic (M1 and M3) receptors, leads to the generation of the second messenger inositol 1,4,5-triphosphate and allows Ca^{2+} to be released into the cytosol (52). Numerous studies, including our study, have shown that the activation of muscarinic receptors with a maximal concentration of agonist (100 μM–1 mM) induced a biphasic $[Ca^{2+}]_i$ rise, composed of the release of Ca^{2+} from intracellular stores followed by Ca^{2+} influx from the extracellular space (*i.e.* CCE). NF-κB responds to intracellular Ca^{2+} elevation by stimulating several kinds of ligands (21, 53). Thus, we were interested to determine whether muscarinic receptor-mediated CCE is affected by NF-κB inhibitors. Our results show that CCE response to muscarinic receptor activation is not blocked by most NF-κB activation inhibitors except ones acting via intracellular Ca^{2+}-regulating pathway, such as TMB-8, an intracellular Ca^{2+} chelator. The newly synthesized NF-κB inhibitor with quinazoline moiety, QNZ, may inhibit CCE by altering $[Ca^{2+}]_i$ and then lead to the inhibition of NF-κB activation.

The results from the present study suggest that CCE induced by muscarinic receptor activation is upstream of the NF-κB activation pathway that participates in muscarinic receptor-mediated sAPPα release. Camandola *et al.* (54) recently reported that activation of NF-κB in fibroblasts and neurons leads to decreased levels of the type I inositol 1,4,5-triphosphate receptor and decreased Ca^{2+} release from the endoplasmic reticulum. They also observed no change in the magnitude of CCE analyzed in fibroblast cells lacking NF-κB p65/RelA subunit after exposure to thapsigargin in Ca^{2+}-free medium, which correlates well with our result showing no blockade of muscarinic receptor-mediated CCE by NF-κB inhibitors in SH-SY5Y cells. In conclusion, NF-κB activation plays a role in muscarinic receptor-mediated sAPPα release, muscarinic receptor-mediated NF-κB activation is regulated by CCE, and the CCE produced by muscarinic receptor stimulation is not influenced by NF-κB in SH-SY5Y cells. It is assumed that the sAPPα release enhanced by NF-κB activation via CCE produced by muscarinic receptor stimulation may protect neurons against Aβ-induced cell death by further activation of NF-κB stabilizing Ca^{2+} homeostasis through down-regulation of endoplasmic reticulum Ca^{2+} release responses. NF-κB-mediated sAPPα release following muscarinic stimulation can provide a potential therapeutic mechanism for muscarinic agonists in Alzheimer disease.

Acknowledgment—We thank Dr. Soo Young Lee in the Division of Molecular Life Sciences and Center for Cell Signaling Research, Ewha Womans University, Seoul, Korea for help on EMSA.

REFERENCES

1. Barkett, M., and Gilmore, T. D. (1999) *Oncogene* **18,** 6910–6924
2. Mattson, M. P., Culmsee, C., Yu, Z., and Camandola, S. (2000) *J. Neurochem.* **74,** 443–456
3. Barger, S. W., and Mattson, M. P. (1996) *Mol. Brain Res.* **40,** 116–126
4. Guo, Q., Robinson, N., and Mattson, M. P. (1998) *J. Biol. Chem.* **273,** 12341–12351
5. Zhao, Q., and Lee, F. S. (2003) *Biochemistry* **42,** 3627–3634
6. Stein, T. D., Anders, N. J., DeCarli, C., Chan, S. L., Mattson, M. P., and Johnson, J. A. (2004) *J. Neurosci.* **24,** 7707–7717

7. Liou, H. C., and Baltimore, D. (1993) *Curr. Opin. Cell Biol.* **5,** 477–487
8. Siebenlist, U., Franzoso, G., and Brown, K. (1994) *Annu. Rev. Cell Biol.* **10,** 405–455
9. Thanos, D., and Maniatis, T. (1995) *Cell* **80,** 529–532
10. Lee, J. I., and Burckart, G. J. (1998) *J. Clin. Pharmacol.* **38,** 981–993
11. Beg, A. A., and Baltimore, D. (1996) *Science* **274,** 782–784
12. Sen, R., and Baltimore, D. (1986) *Cell* **47,** 921–928
13. Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., Le Bail, O., Urban, M. B., Kourilsky, P., Baeuerle, P. A., and Israel, A. (1990) *Cell* **62,** 1007–1018
14. Nolan, G. P., Ghosh, S., Liou, H. C., Tempst, P., and Baltimore, D. (1991) *Cell* **64,** 961–969
15. Koulich, E., Nguyen, T., Johnson, K., Giardina, C., and D'mello, S. (2001) *J. Neurochem.* **76,** 1188–1198
16. Whiteside, S. T., and Israel, A. (1997) *Semin. Cancer Biol.* **8,** 16–18
17. Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. (1994) *Cell* **78,** 773–785
18. Traenckner, E. B., Wilk, S., and Baeuerle, P. A. (1994) *EMBO J.* **13,** 5433–5441
19. Dolmetsch, R. E., Keli Xu, and Lewis, R. S. (1998) *Nature* **392,** 933–936
20. Komarova, S. V., Pilkington, M. F., Weidema, A. F., Dixon, S. J., and Sims, S. M. (2003) *J. Biol. Chem.* **278,** 8286–8693
21. Lilienbaum, A., and Israel, A. (2003) *Mol. Cell. Biol.* **23,** 2680–2698
22. Stankunas, K., Graef, I. A., Neilson, J. R., Park, S. H., and Crabtree, G. R. (1999) *Cold Spring Harbor Symp. Quant. Biol.* **64,** 505–516
23. Okamura, H., and Rao, A. (2001) *Curr. Opin. Cell Biol.* **13,** 239–243
24. Dolmetsch, R. E., Pajvani, U., Fife, K., Spotts, J. M., and Greenberg, M. E. (2001) *Science* **294,** 333–339
25. Kim, J. H., Choi, S., Jung, J.-E., Roh, E.-J., and Kim, H.-J. (2006) *J. Neurochem.* **97,** 245–254
26. Nitsch, R. M., Slack, B. E., Wurtman, R. J., and Growdon, J. H. (1992) *Science* **258,** 304–307
27. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450
28. Unlap, T., and Jope, R. S. (1995) *Neurosci. Lett.* **198,** 41–44
29. Ghosh, S. (1999) *Immunol. Res.* **19,** 183–189
30. Todisco, A., Ramamoorthy, S., Pausawasdi, N., and Tadey, K. (1999) *Biochem. Biophys. Res. Commun.* **261,** 877–884
31. Li, X., Song, L., and Jope, R. S. (1996) *J. Neurosci.* **16,** 5914–5922
32. Gillmore, T. D., and Morin, P. J. (1993) *Trends Genet.* **9,** 427–433
33. Matthews, J. R., and Hay, R. T. S. (1995) *Int. J. Biochem. Cell Biol.* **27,** 865–879
34. Lin, Y. Z., Yao, S. Y., Veach, R. A., Torgerson, T. R., and Hawiger, J. (1995) *J. Biol. Chem.* **270,** 14255–14258
35. Daniel, K. G., Chen, D., Orlu, S., Cui, Q. C., Miller, F. R., and Dou, Q. P. (2005) *Breast Cancer Res.* **7,** R897–R908
36. Schreck, R., Meier, B., Mannel, D. N., Droge, W., and Baeuerle, P. A. (1992) *J. Exp. Med.* **175,** 1181–1194
37. Wang, R., Zhang, L., Zhang, X., Moreno, J., Celluzzi, C., Tondravi, M., and Shi, Y. (2002) *Eur. J. Immunol.* **32,** 1090–1098
38. Kim, Y. H., Moon, J. S., Lee, K. S., Park, S. Y., Cheong, J. H., Kang, H. S., Lee, H. Y., and Kim, H. D. (2003) *Biochem. Biophys. Res. Commun.* **314,** 695–703
39. Petegnief, V., Ursu, G., Bernal, F., and Mahy, N. (2004) *Neurochem. Int.* **44,** 287–291
40. Tobe, M., Isobe, Y., Tomizawa, H., Nagasaki, T., Takahashi, H., and Hayashi, H. (2003) *Bioorg. Med. Chem.* **11,** 3869–3878
41. Slack, B. E., Nitsch, R. M., Livneh, E., Kunz, G. M., Breu, J., Eldar, H., and Wurtman, R. J. (1993) *J. Biol. Chem.* **268,** 21097–21101
42. Cheng, G., Yu, Z., Zhou, D., and Mattson, M. P. (2002) *Exp. Neurol.* **175,** 407–414
43. Grisham, M. B., Palombella, V. J., Elliot, P. J., Conner, E. M., Brand, W., Wong, H. L., Pien, C., Mazzola, L. M., Destree, A., Parent, L., and Adams, J. (1999) *Methods Enzymol.* **300,** 345–363
44. West, A. E., Chen, W. G., Dalva, M. B., Dolmetch, R. E., Kornhauser, J. M., Shaywitz, A. J., Takasu, M. A., Tao, X., and Greenberg, M. E. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 11024–11031
45. West, A. E., Griffith, E. C., and Greenberg, M. E. (2002) *Nat. Rev. Neurosci.* **3,** 921–931
46. Fernando, K. C., and Barritt, G. J. (1994) *Biochim. Biophys. Acta* **1222,** 383–389
47. Merritt, J. E., Armstrong, W. P., Benham, C. D., Hallam, T. J., Jacob, R., Jaxa-Chamiec, A., Leigh, B. K., McCarthy, S. A., Moores, K. E., and Rink, T. J. (1990) *Biochem. J.* **271,** 515–522
48. Mason, M., Mayer, B., and Hymel, L. J. (1993) *Am. J. Physiol.* **264,** C564–C662
49. Stein, T. D., and Johnson, J. A. (2002) *J. Neurosci.* **22,** 7380–7388
50. Kaltschmidt, B., Uherek, M., Volk, B., Baeuerle, P. A., and Kaltschmidt, C. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 2642–2647
51. Miyakawa-Naito, A., Uhlen, P., Lal, M., Aizman, O., Mikoshiba, K., Brismar, H., Zelenin, S., and Aperia, A. (2003) *J. Biol. Chem.* **278,** 50355–50361
52. Berridge, M. J. (1993) *Nature* **361,** 315–325
53. Tomsig, J. L., Sohma, H., and Creutz, C. E. (2004) *Biochem. J.* **378,** 1089–1094
54. Camandola, S., Cutler, R. G., Gary, D. S., Milhavet, O., and Mattson, M. P. (2005) *J. Biol. Chem.* **280,** 22287–22296

Nuclear Factor-κB Activated by Capacitative Ca^{2+} Entry Enhances Muscarinic Receptor-mediated Soluble Amyloid Precursor Protein (sAPP α) Release in SH-SY5Y Cells

Shinkyu Choi, Jin Hyoung Kim, Eun-Jihn Roh, Moon-Jeong Ko, Ji-Eun Jung and Hwa-Jung Kim

J. Biol. Chem. 2006, 281:12722-12728.
doi: 10.1074/jbc.M601018200 originally published online February 20, 2006
