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REGULATION OF BLOOD-BRAIN BARRIER Na,K,2Cl-COTRANSPORTER THROUGH PHOSPHORYLATION DURING IN VITRO STROKE CONDITIONS AND NICOTINE EXPOSURE[1].

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Brain-to-Blood Potassium Transport with Stroke and Nicotine.

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Abbreviations

BBB, blood-brain barrier
NKCC, Na,K,2Cl-cotransporter
BBMEC, bovine brain microvessel endothelial cells
H/A, hypoxia/aglycemia
N/C, nicotine and cotinine
nAChR, nicotinic acetylcholine receptor
PKC, protein kinase C
ACM, astrocyte conditioned media

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Abstract

Nicotine, a major constituent of tobacco smoke, has important effects on brain recovery after focal ischemia [Wang et al., 1997]. The purpose of this work is to systematically test the effects of nicotine during stroke conditions on blood-brain barrier (BBB) potassium transport, protein expression of the Na,K,2Cl-cotransporter (NKCC) , and cell-signalling pathways that control NKCC activity at the BBB. Confluent bovine brain microvessel endothelial cells (BBMEC) were exposed to both a hypoxic/aglycemic (H/A) environment to model BBB function during stroke conditions and nicotine and cotinine (N/C) to model plasma levels seen in smokers. BBMECs exhibit both Na,K-ATPase and NKCC activity (60 and 34 nmol/min/g, respectively) that contribute to 98% of the K^+ uptake in cultured endothelial cells. An adaptive up regulation of NKCC activity was identified to occur on the basolateral surface of the BBB after *in vitro* stroke conditions. 24 hours of N/C exposure, at doses equivalent to plasma levels of smokers, combined with 6 hours of H/A, reduced NKCC protein expression and total NKCC activity (shown by bumetanide sensitive ^{86}RB uptake) compared to 6 hours of H/A alone ($P<0.01$). Basolateral K^+ transport was found to be modulated by nAChRs expressed at the BBB. NKCC activity on the basolateral surface of the BBB is controlled by an ongoing phosphorylation / dephosphorylation processes. We have identified a potential mechanism in altered BBB response to stroke conditions with prior N/C exposure directly implicating damage to brain-to-blood K^+ transport mediated at the BBB and perhaps neuronal recovery after stroke.

Keywords: blood-brain barrier, stroke, nicotine, Na,K,2Cl-cotransporter, intracellular messengers

Introduction

Endothelial cells, which comprise the blood-brain barrier (BBB), function to segregate the plasma from brain interstitial fluid. Continuous BBB endothelial cells possess tight junctions that limit solute transfer and permit the expression polarity of key ion transport proteins [Reese and Karnovsky 1967, Brightman and Reese 1969]. Proper BBB function is crucial for maintaining brain homeostasis and changes in such can exacerbate damage in a number of neurological disorders. A functional BBB is required to control brain extracellular K^+ homeostasis, which can be crucial for recovery after stroke. To maintain proper neuronal conduction, brain extracellular [K^+] needs to be maintained efficiently constant and low to maintain conduction of action potentials. Several studies have shown that specific brain-to-blood K^+ transport mechanisms exist at the antiluminal surface of the BBB, functioning to keep brain extracellular K^+ constant and low [Bradbury and Stulcova 1970, Keep et al., 1993]. This suggests the importance of specific brain-to-blood K^+ transport mechanisms at the site of the BBB that may be responsible for secretion of K^+ from brain extracellular fluid back into the blood.

Brain K^+ levels are regulated at the BBB mainly by two proteins, the Na,K-ATPase and the Na,K,2Cl-cotransporter (NKCC). The Na,K-ATPase has been proposed to be present at high density in the antiluminal membrane of the BBB [Betz et al., 1980]. The NKCC has been shown to be expressed in bovine [O'Donnell et al., 1995] and rat brain [Vigne et al., 1994] endothelial cells, yet its luminal vs. antiluminal localization is still unknown. It is possible that either of these proteins could be involved in brain-to-blood transport of K^+.

Additionally, cigarette smoking has been associated with an increased risk for stroke [Gill et al., 1989, Hawkins et al., 2002]. Nicotine, a major constituent of tobacco smoke, has important effects on the brain in stroke. Chronic nicotine administration to rats has been shown to enhance focal brain ischemic injury and infarct size in rats using the reversible (1 hr) middle cerebral artery occlusion model [Wang et al., 1997]. Further, the same treatment has been shown to down regulate the expression and function of Na,K-ATPase at the BBB [Wang et al., 1994], and enhance brain edema in stroke, and compromises blood flow in the

periphery of the ischemic core [Wang et al., 1997]. The mechanism(s) by which either nicotine or smoke constituents aggravate brain edema and injury in stroke have not been identified.

When studying the effects of nicotine and the major metabolite, cotinine, on the BBB, one has to investigate the role of nAChRs expression at the BBB. nAChRs belong to a family of ionotropic receptor proteins and are comprised of five subunits that make up a functional receptor containing a central transmembrane cation channel [Lindstrom 2000]. Upon agonist stimulation this cation channel facilitates the inward movement of Ca^{2+} and Na^{+}. Several laboratories have determined that non-neuronal cells can express functional nAChRs [Conti-Fine et al., 2000]. Human keratinocytes, bronchial epithelial, and aortic endothelial cells have all been shown to express functional nAChRs of various subtypes [Grando et al., 1995, Maus et al., 1983, Wang et al., 2001]. [^{3}H]Nicotine binding sites have even been found in preparations of intraparenchymal cerebral microvessels and larger pial vessels from human and pig brains [Kalaria et al., 1994]. Data from our laboratory strongly suggests that BBMEC express the α-3, α-5, α-7, β-2, and β-3 nAChR subunit proteins [Abbruscato et al., 2002]. We were also successful at reversing the BBB effects of nicotine on BBB function with a classical antagonist of nAChRs (bungarotoxin) [Abbruscato et al., 2002]. These findings suggest that nAChRs modulate cellular functions outside of synaptic transmission and could have a role in nicotinic effects on the BBB during ischemia. Non-neuronal cell nAChRs have even been suggested to be involved in tobacco toxicity in tegumental type tissues, such as epithelium and endothelium [Conti-Fine et al., 2000]. In the present studies, we have investigated classical agonist and antagonists of nAChRs to mimic or reverse the effects of nicotine and cotinine (N/C) on BBB mediated K^{+} transport during *in vitro* stroke conditions.

Since our *in vitro* data shows that NKCC is the primary protein involved in removal of K^{+} from the ischemic brain, we have also investigated cell signaling pathways involved in altered BBB K^{+} transport via the cotransporter. In brain endothelial cells, it has been shown that protein kinase C (PKC) inhibition with staurosporine reduces basal activity of NKCC [Vigne et al., 1994]. Also, PKC is believed to be the main operative mechanism involved in NKCC activity since this ion transport protein has been shown to have 10

putative sites for phosphorylation by PKC [Yerby et al., 1997]. Additionally, calyculin A, an inhibitor of protein phosphatases, has been shown to stimulate NKCC activity [Sun and O'Donnell 1996, Vigne et. al. 1994]. We have investigated phosphorylation and dephosphorylation pathways as a potential regulatory mechanism of the Na,K,2Cl-cotranport protein during H/A and/or N/C exposure.

Since 48 hours of hypoxia has been shown to decrease the function of the BBB Na,K,-ATPase and increase the function of the BBB Na,K,2Cl-cotransporter [Kawai et al., 1996] and 14 days of nicotine exposure decreases the BBB expression of the Na,K,-ATPase [Wang et al., 1996], we decided to investigate the combined effects of H/A and N/C exposure on the function and expression of the two major K^+ transport proteins believed to be present at the BBB. We have evaluated the effects of *in vitro* stroke conditions combined with N/C exposure on (1) the ability of the BBB to maintain expression polarity of NKCC, (2) activation of nAChRs expressed on endothelial cells of the BBB, and (3) cell signaling pathways involved in altered NKCC function at the BBB.

Materials and Methods

Cell culturing. The isolation of BBMECs was performed using fresh bovine brains as previously described [Audus and Borchardt 1986, 1987]. Cells were seeded at a cell density of 50,000 cells/cm^2 onto 12-well transwell plates (0.4 μM pore size). Prior experiments [O'Donnell et al., 1995] have shown that adding C6 conditioned medium to pre-confluent endothelial cells provides the most robust Na,K,2Cl-cotransporter activity. Differentiation between luminal and abluminal endothelial surfaces was made based upon addition of C6-conditioned medium to the abluminal chamber 48 hours pre-confluence.

C6 cells were obtained from (American Type Cell Collection, Rockville, MD) #CC1-107 and were cultured in Ham's F-10 with 10% FBS. Astrocyte conditioned media (ACM) was prepared by seeding C6 astrocytes at 40,000 cells/cm^2 and culturing to confluence, then re-feeding with fresh growth media for 48 h. The resultant ACM was passed through a 0.22 mM sterile filter. After 10 days of growth, BBMECs were exposed to conditioned media (applied to the abluminal well) which consisted of a mixture of 45% fresh BBMEC growth media, 45% ACM, and 10% FBS for 48 hours. BBMECs were continuously exposed to media with an osmolality of 280 ± 5 mOsm/L as determined using an automated digital osmometer (Precision Systems Inc, Natick, Massachusetts). At day 12, confluent monolayers of BBMECs were utilized for measurement of K+ uptake or Western blot analysis (both described below).

H/A treatment. Confluent monolayers of BBMECs were exposed to H/A conditions by adding RPMI 1640 (without L-glucose) bubbled with 95% N_2/5% CO_2 at 3 liters / minute for 5 minutes. Hypoxia was induced by placing the cells in a humidified, sealed incubator chamber (Billups-Rothenberg, Del Ma, CA) at 37°C which had been flushed with 95% N_2/5% CO_2. The concentration of oxygen in the atmosphere was maintained at 0% and the PO_2 in the media was below 25 mm Hg. This H/A exposure described above has been used previously to study alterations in BBB properties [Abbruscato and Davis 1999a, 1999b, Abbruscato et al., 2002].

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Nicotine / Cotinine Treatment. Nicotine (100 ng/ml culture media) and cotinine (1000 ng/ml culture media) were added to the luminal (upper chamber) 12, 24, and 48 hours prior to [Rb^{86}] uptake experiments. These doses were chosen to model plasma levels seen in human smokers [Henningfield et al., 1993, Clarke et al., 1994]. Levels of nicotine and cotinine were verified by HPLC analysis after a 48 hour incubation and shown be > 85% intact (data not shown). HPLC identification of nicotine and cotinine in the culture media after 24 hours was accomplished by using the following method. 500 µl culture media was dispensed into a 1.5 ml centrifuge tube containing 10 µl of 2-phenylimidazole (15 ng/µl), 20 µl of 5% antifoam/phenol red solution, 50 µl 30% NH_4OH, and 500 µl of dichloroethane and mixed by gentle inversion for one minute. The solution was centrifuged for 15 minutes using a Beckman microfuge (setting 12). The top layer was then discarded and 400 µl of the clear bottom layer is placed into a 1.5 ml tube and evaporated under N_2 gas until dry. 250 µl of HPLC buffer was added to the tube. A standard curve was generated using culture media extractions of 0, 12.5, 25, 50, 100 and 200 ng/ml of nicotine and cotinine and 150 ng of 2-phenylimidazole as the internal standard.

HPLC analyses was performed with a 0.46 x 15 cm Inertsil ™ ODS-2 column (MetaChem Technologies Inc., Torrance CA) at a flow rate of 1.0 ml/min at 37°C for 7.5 minutes and then increased to 1.5 for an additional 8 minutes. All samples were autoinjected using a Waters Associates WISP ™ 710B autoinjector. Separations were achieved using an isocratic mobile phase containing (30 mM citric acid, 30 mM monobasic postassium phosphase, 3.65 g/L triethylamine, 0.6 g/L 1-heptanesulfonic acid, and 90 ml/L acetonitrile, pH 4.8). Nicotine, cotinine and 2-phenylimidazole were detected by a Shimadzu SPD-6A UV spectrophotometric detector (259 nm) and peaks were integrated with a Hewlett Packard 3396A integrator.

Measurement of K^+ uptake. All experiments were performed on BBMECs exposed to ACM, since these culturing conditions have been shown to increase BBMEC Na,K,2Cl-cotransporter activity [O'Donnell et al., 1995]. To begin experiments, BBMECs were pre-incubated with a HEPES buffered medium +/- 2 µM ouabain or 20 µM bumetanide for 15 minutes. [Rb^{86}] (0.2 mCi per well) was then added and the cells were

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rotated at 37 °C for 10 minutes. In previous studies, it has been demonstrated that Rb quantitatively substitutes for K in the Na,K,2Cl-cotransport system [Owen and Prastein 1985]. The assay was terminated by rapid triple washing with ice-cold HEPES buffered medium. BBMECs were solubilized with 1% Triton X-100 and the radioactivity present in each extract was determined by a liquid scintillation counter. Protein content was determined by using the detergent-compatible Pierce BCA assay (Pierce, Rockford, IL). K^+ uptake into BBMECs (expressed as nmol/mg of protein/min) was calculated from the ratio of [Rb^{86}] uptake and the K^+ content in the incubation buffer [Kawai et al., 1996]. Bumetanide-insensitive and ouabain-insensitive K^+ uptake were considered Na,K,-ATPase and Na,K,2Cl-cotransporter activity, respectively. For experiments that segrated luminal vs. abluminal activity, both the inhibitor and the [Rb^{86}] were added to either the upper (measuring luminal activity) or lower (measuring abluminal activity) of the transwell. Negligible transport of inhibitors or [Rb^{86}] occurred within the short time course of these experiments.

Second Messenger Experiments. Staurosporine, a general protein kinase inhibitor was incubated at a concentration of 20 nM [Kurihara et al., 2002]. Genistein, a potent protein tyrosine kinase inhibitor was incubated at a concentration of 50 uM [Grando et al., 1995]. PMA, an activator of Protein Kinase C, was incubated at 100 nM [Yang et al., 2001]. Calyculin A, an inhibitor of protein phosphatases 1 and 2A [Ishihara et al., 1989], was incubated at a concentration of 50 nM. All inhibitors and activators were added during both the pre-incubation and [Rb^{86}] uptake time periods.

Western Blotting. Protein was isolated from BBMECs using TRI REAGENT LS (Sigma, St. Louis, MO) at 0.4 ml per 10^2 cm of culture surface. Protein pellets were then air dried and dissolved in 1% SDS. The protein concentration in each tube was determined using the Pierce BCA assay. Exactly 30 ug of protein from each sample and molecular weight markers were separated using a gradient (4-20%), tris-glycine polyacrylamide gel (Novex, San Diego, CA). The protein markers and samples were electrophoretically transferred to a PVDF membrane (Amersham Life Sciences). The PVDF membranes containing the protein samples were then incubated in a blocking buffer (5% non-fat dry milk) overnight.

The antibody used for detection of the Na,K,2Cl-cotransporter was a monoclonal antibody T4, which was developed against the carboxy-terminal 310 aa of the human colonic N,K,2Cl-cotransporter. This antibody was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). The membrane was incubated for 2 hours with the T4 monoclonal antibody at a dilution of 1:200 TBS-TW-20. The membrane was then washed three times in TBS TW-20, then, incubated with anti-mouse IgG-HRP secondary antibody for 2 hours at a dilution of 1:10,000 TBS TW-20.

Antibodies used for specific nAChR subtype immuno-detection (Research and Diagnostic Antibodies, Benicia, CA) were specific to either the amino or carboxy terminal regions (α3 carboxy terminal aa 496-503, α4 amino terminal aa 620-627, α5 carboxy terminal aa 460-468, α7 carboxy terminal aa 493-502, β2 carboxy terminal aa 493-502, β3 carboxy terminal 450-458, and β4 carboxy terminal aa 490-498). The above primary antibodies were incubated at a dilution of 1:200 TBS TW-20 for 2 hours. The membrane was then washed three times in TBS TW-20, then, incubated with anti-rabbit IgG-HRP secondary antibody for 2 hours at a dilution of 1:2,000.

After washing three times with TBS TW-20, the NKCC and nAChR signal was detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Statistical Methods. For all experiments, the data are presented as the mean ± SEM. Statistical analysis of the data was done with the use of one-way analysis of variance (ANOVA) with Newman-Keuls multirange post hoc comparison of the means [Bruning and Kintz 1977].

Results

K^+ uptake after H/A and/or N/C exposure.

Total BBMEC K^+ transport (luminal and abluminal) consists of Na,K,-ATPase (60 nmol/min/mg) and NKCC (34 nmol/min/mg) activity (Figure 1). The activity of these two ion transport proteins contributes to 98% to total BBMEC K^+ uptake. *In vitro* stroke conditions (6 hours of H/A) significantly reduced K^+ uptake due to the Na,K-ATPase ($P<0.05$) and significantly increased K^+ uptake due to NKCC ($P<0.01$). 24 hour exposure to nicotine (100 ng/ml) and cotinine (1000 ng/ml) significantly reduced the amount of H/A stimulated BBMEC K^+ uptake due to NKCC ($P<0.01$). Control experiments were also performed to show the effects of removing either Na or Cl from the preincubation and assay buffer on NKCC activity measured as oubain-insensitive K^+ influx. We replaced Na and Cl isotonically with choline and gluconate, respectively. In either case, negligible oubain-insensitive K^+ influx was observed, ensuring that we are measuring cotransporter activity (Figure 2).

Experiments were also designed to separate luminal from abluminal NKCC activity (Figure 3 A-C). K^+ uptake due to NKCC was on average 54% greater on the abluminal side of the endothelial cell compared to the luminal side. Only abluminal BBMEC K^+ was sensitive to either N/C exposure and/or H/A. These observations occur at all N/C exposure (12, 24, 48 hours).

We have also identified (Figure 4) that nicotine alone (100ng/ml for 24 hours) reduced hypoxic/aglycemic induced Na,K,2Cl-cotransporter activity ($P<0.01$) on the abluminal sides of BBMECs to a comparable level as seen with the combination of nicotine and cotinine for 24 hrs. Additionally, cotinine had no effect on H/A induced abluminal Na,K,2Cl-cotransporter activity. We also tested different doses of N/C and determined that only a dose equivalent to plasma levels of smokers resulted in a significant decrease ($P<0.01$) in H/A induced NKCC activity and doses below that had no significant effects.

Western Blotting of NKCC and nAChR subunits.

The monoclonal antibody T4, which was developed against the carboxy-terminal 310 aa of the human colonic N,K,2Cl-cotransporter recognized a protein in cultured BBMECS and rat kidney microsomes that

was ~ 145 kDa (Figure 5). A statistically significant ($P<0.01$) increase in immunoreactivity was observed in the 145 kDa protein band after 6 hours H/A treatment. Similar to the [Rb^{86}] experiments, 24 hour N/C exposure significantly ($P<0.01$) attenuated the H/A induction of NKCC protein expression.

Western blot analysis was also used to determine the dose-response effects of N/C on nAChR subunit expression (Figure 6). The polyclonal antibody recognized a protein in BBMECs that was 55 kDa for the α3, α5, α7, β2, and β3 nAChR subunit experiments. The 55 kDa protein band was not recognized when using the α4 and β4 subunit specific antibody (data not shown). A dose dependent decrease in nAChR subunit expression was observed after a 24 hour N/C exposure with concentrations of $1 \times 10^{2-5}$ ng/ml for nicotine and $1 \times 10^{3-6}$ ng/ml for cotinine (lanes 3-6). For all subunits tested, lane 7 represents an additional exposure of BBMECs to nicotine (100 ng/ml) and cotinine (1000 ng/ml), a concentration equivalent to plasma levels in smokers (same as lane 3).

Pharmacological activation and inhibition of BBMEC nAChRs.

BBMECs are sensitive to classical agonists and antagonists of nAChRs. Figure 7 clearly demonstrates that epibatidine, a potent agonist for nAChR mimics the actions of nicotine by attenuating the increased abluminal K^+ uptake induced by 6H H/A at concentrations of 1 nM ($P<0.05$), 10 nM ($P<0.01$), and 100 nM ($P<0.01$). Additional experiments confirmed that the noncompetitive antagonist mecamylamine and competitive antagonist bungarotoxin (BGT) reverse the effects of N/C on H/A induction of abluminal NKCC activity at concentrations 100 uM ($P<0.05$) and 500 uM ($P<0.01$) for mecamylamine and 1 nm ($P<0.05$) and 10 nm ($P<0.01$) for BGT.

Second messenger signaling controlling NKCC activity.

We studied the possible cell-signaling pathways responsible for N/C induced down regulation of abluminal Na,K,2Cl-cotransporter activity during stroke conditions. We have determined (Figure 8) that both basal and stroke (6H H/A) induced Na,K,2Cl-cotransporter activity is controlled by protein kinase C, since 20 nM staurosporine inhibits and 100 nM PMA induces cotransporter activity compared to each respective control experiment. Interestingly, tyrosine kinase also controls basal Na,K,2Cl-cotransporter activity, but not

stroke (6H H/A) induced activity, as evidenced by genistein inhibition only during normal conditions. Additionally, combining both insults (6 hours H/A with 24 hours of N/C) resulted in an expected decrease in abluminal Na,K,2Cl-cotransporter activity. This double insult was not inhibited by either 20 nM staurosporine or 50 nM genistein, suggesting that PKC or tyrosine kinase is not involved in nicotine induced attenuation of cotransporter activity during H/A induction. Yet, 50 nM calyculin A was found to prevent nicotine's effects of attenuating cotransporter activity during H/A. This suggests that nicotine effects are mediated through the protein phosphatase pathway and this mechanism could prevent PKC induction of Na,K,2Cl-cotransporter activity at the abluminal side of the BBB during stroke conditions.

Discussion

We measured K^+ uptake (using $^{86}RB^+$ as a tracer for K^+) through a combination of both ouabain (2 μM) sensitive K^+ uptake (Na,K,ATPase) and bumetanide (20 μM) sensitive K^+ uptake (NKCC). Six hours of H/A, an *in vitro* model of stroke [Abbruscato and Davis 1999a, 1999b], caused a 62% decrease in Na,K,ATPase activity and a 91% increase in NKCC activity (Figure 1) These results suggest that increased BBB NKCC activity after H/A conditions may provide an additional mechanism for the removal of excess brain K^+, specifically by the antiluminal NKCC which shuttles K^+ from the brain extracellular fluid to the blood. Similar results have been made in rat brain endothelial utilizing longer periods of hypoxia without controlling glucose levels (24 hours) [Kawai et al., 1996]. We also determined the effects of incubating confluent BBMECs with nicotine (100 ng/ml) and cotinine (1000 ng/ml) on H/A induction of cotransporter activity. It was observed that N/C exposure, at levels equivalent to average plasma levels of smokers [Abbruscato et al., 2002], attenuated the H/A induction of NKCC function. These results are interesting in light of the fact that pre-exposure to N/C could effect the ability of the ischemic brain to efflux K^+ ions back into the blood during the re-establishment of neuronal conduction. Additional experiments were conducted to determine the polarity (apical vs. basolateral) expression of NKCC at the BBB. When BBMECs were cultured with ACM 48 hours pre-confluence, NKCC activity was determined to be greater on the abluminal side of the BBB. Abluminal NKCC was also significantly decreased with 12, 24, and 48 hour N/C exposure combined with 6 hours H/A exposure compared to 6 hours H/A exposure alone (Figure 3 A-C), whereas luminal NKCC activity was not changed. These experiments provide evidence that abluminal BBB K^+ transport mechanisms during H/A are modulated N/C exposure and this could influence ischemic brain K^+ homeostasis in crucial times of neuronal recover after stroke.

More specific experiments were also designed to determine the independent effects of nicotine and cotinine on abluminal NKCC activity. We determined (Figure 4) that nicotine alone (100ng/ml for 24 hours) reduced H/A induced NKCC activity on the abluminal sides of BBMECs to a comparable level as seen with the combination of N/C for 24 hrs. Additionally, cotinine had no effect on H/A induced NKCC activity. We

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also tested different doses of the combination of N/C and determined that only a dose equivalent to plasma levels of smokers resulted in a significant decrease in H/A induced NKCC activity (100 ng/ml nicotine and 1000ng/ml cotinine) and doses below that had no significant effects (Figure 4). This suggests that drug therapies utilizing nicotine at lower doses have no effects on BBB NKCC activity.

We observed a correlation when comparing [Rb^{86}] uptake data to the BBMEC protein expression of NKCC subjected to the same experimental paradigm described in Figures 1 and 3. H/A conditions induced NKCC protein expression which was reversed with 24 hour exposure to N/C (Figure 5). Utilizing the T4 monoclonal antibody specific for the carboxy terminal portion (MET-902-to Ser-1212) of the human colonic T84 epithelial Na,K-2Cl-cotransporter, we see specific immunoreactivty with the fully glycosylated version of NKCC1 (145 kDa) and faint immunoreactivity for a low molecular weight, nonglycosylated form in both BBMECs and rat kidney control. These results confirm that the loss in NKCC function is most likely due to a reduction in total NKCC protein with the conditions tested in these studies.

An obvious target for nicotine action at the BBB is nAChRs expressed on endothelial cells of the cerebrovasculature. Previous studies have shown that BBMECs express α-3, α-5, α-7, β-2, β-3 nAChR subunit protein and do not express α-4 and β-4 nAChR subunit protein (Abbruscato et al., 2002). Additionally, N/C exposure was found to reduce α-7 and β-2 nAChR subunit protein expression in a time dependent manner. In the present study we determined the effects of exposing BBMECs to increasing doses of N/C on nAChR subunit protein expression. In all cases, we observed a dose dependent decrease in α-3,-5,-7 and β-2,-3 nAChR subunit protein (Figure 6). Interestingly, in all cases we observed this dose-dependent decrease in nAChR subunit protein expression occurred at a dose equivalent to plasma levels of smokers (100 ng/ml nicotine and 1000 ng/ml cotinine, Lane 3 in Figue 6). Future *in vivo* experiments are required to determine the effects of chronic exposure to N/C on brain microvascular nAChR expression.

N/C modulation of abluminal NKCC function during H/A was found to be both mimicked and reversed by classical agonist and antagonists of nAChRs. We incubated BBMECs with epibatidine, a potent natural agonist of nAChRs, and reversed the effect of H/A on abluminal NKCC activity to a level compared

to the N/C exposure (Figure 7). Additionally, we could antagonize the effects of N/C on H/A induced NKCC activity with both bungarotoxin (competitive antagonist) and mecamylamine (noncompetitive antagonist), suggesting a nAChR mediated response (Figure 7). These experiments suggest that functional nAChRs are present on BBMECs and they are activated at nicotine levels equivalent to smokers (0.1-1 μM).

The activity of NKCC protein is known to be modulated by pharmacological agents and conditions that change the phosphorylation state of the cotransporter. In general, conditions that promote phosphorylation of the NKCC activate the protein [Lytle and Forbush 1992, Lytle and Forbush 1996, Payne et al., 2001]. We examined the modulation of BBMEC NKCC activity by reagents known to affect the phosphorylation state and activity of NKCC (Figure 8). Our data supports the hypothesis that basal NKCC activity on the abluminal side of BBB is regulated by protein kinases, since staurosporine pre-treatment resulted in a statistically significant reduction in cotransporter activity ($P<0.05$). Additionally, basal NKCC activity at the abluminal side of the BBB is also be controlled by protein tyrosine kinases, since genistein reversed the normal activity of this cotransporter to statistically significant level ($P<0.05$). Calyculin A, a protein phosphatase inhibitor known to lead to increased phosphorylation and activation of NKCC [Lytle and Forbush 1996], markedly stimulated abluminal NKCC activity in BBMECs compared to control levels ($P<0.01$). These results suggest that protein phosphatases must be involved in controlling the activity of NKCC on the abluminal side of the BBB during basal conditions. Interestingly, we also observed that H/A induction of abluminal NKCC activity in BBMECs was sensitive ($P<0.01$) to treatment with 20 nM staurosporine, a general protein kinase inhibitor, but not sensitive to treatment with 50 μM genistein, an inhibitor of protein tyrosine kinase, suggesting that protein tyrosine kinase is not involved in H/A induction of abluminal NKCC activity. In all cases, PMA, a general PKC activator, augmented NKCC activity, suggesting a role for PKC. Since PMA does not stimulate all PKC isoforms and the inhibitors used in these studies block a broad range of kinases, future experiments will test the role of conventional, novel, and atypical PKC isoforms on regulation of NKCC activity.

When testing conditions of both H/A and N/C exposure we observed that only 50 nM calyculin A was able to increase abluminal cotransporter activity (no effects by modulating protein kinase, PKC or protein tyrosine kinase). These results suggest that nicotine effects are mediated through the protein phosphatase pathway and this mechanism could prevent PKC induction of NKCC activity on the abluminal side of the BBB during stroke conditions (Figure 9). Currently, our studies suggest that nAChR activation coupled to hypoxia / aglycemic (right side of schematic) could increase brain endothelial cell calcium levels to a point whereby the cytoplasmic pool of PKC is depleted (1). Additionally, nicotine may also activate protein phosphatase, which could either dephosphorylate PKC (2) and prevent translocation to the membrane for activity, or dephosphorylate the Na,K,2Cl-cotransporter directly (3), thus inhibiting its function. In the future, we plan to identify the specific PKC and protein phosphatase isoforms responsible for these effects and directly measure the phosphorylation state of BBMEC NKCC after stroke conditions with and without prior nicotine exposure. A better understanding of the mechanisms that regulate expression and activity of this key carrier protein after the above insults potentially could lead to approaches that will protect the CNS from neurological damage associated with nicotine, smoke constituent, and/or ischemic insults. These experiments may provide important information about the long term effects of nicotinic therapeutics that currently are under investigation for treatments of Alzheimer's and Parkinson's Disease.

We have determined that basal level activity of the NKCC is maintained by ongoing phosphorylation / dephosphorylation processes. Activation and inhibitions via phosphorylation may be dependent on the phosphorylation of AA sites on the cotransporter that result in either activation or inhibition of activity during H/A conditions or N/C exposure. Additionally, constitutively active kinases may exist that phosphorylate the cotransporter to maintain basal activity.

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Footnotes

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Figure Legends

1. Effects of H/A and/or N/C exposure on total K^+ uptake (luminal and abluminal) into BBMECs cultured with ACM. Bumetanide (20μM)-insensitive and ouabain (2μM)-insensitive K^+ uptake (using ^{86}Rb as a replacement for K^+) are expressed as Na,K-ATPase and Na,K,2Cl-cotransporter activity, respectively. Data represent mean ± SEM of six independent determinations. (****** denotes significance of P<0.01 and *****denotes significance of P<0.05 using one-way ANOVA and Newman-Keuls post hoc analysis).

2. Na- and Cl-dependance of BBMEC K^+ uptake. Oubain (2μM)-insensitive K^+ uptake (using ^{86}Rb as a replacement for K^+) is expressed as Na,K,2Cl-cotransporter activity. Cells assayed in Na- or Cl-free media were both preincubated and assayed in HEPES-buffered MEM in which Na was isosmotically replaced with choline and Cl isosmotically replaced with gluconate. Data represent mean ± SEM of six independent determinations. (****** denotes significance of P<0.01 using one-way ANOVA and Newman-Keuls post hoc analysis).

3 (A-C). Contribution of abluminal and luminal Na,K,2Cl-cotransporter to total K^+ uptake into BBMECs after H/A and/or N/C exposure. Ouabain(2μM)-insensitive K^+ uptake is considered Na,K,2Cl-cotransporter activity. (A-C) represent contribution of the luminal and abluminal membranes to total BBMEC K^+ uptake after 12, 24 and 48 hours of nicotine (100 ng/ml) and cotinine (1000 ng/ml), respectively.. Data represent mean ± SEM of six independent determinations. (** denotes significance of P<0.01 compared to normal conditions and ‡‡ denotes significance of P<0.01

compared to 6 H H/A treatment, using one-way ANOVA and Newman-Keuls post hoc analysis).

4. Effects of nicotine or cotinine and sub-therapeutic doses of N/C on H/A induction of abluminal Na,K,2Cl-cotransporter activity. Na,K,2Cl-cotransporter activity is expressed as ouabain(2μM)-insensitive K^+ uptake, using ^{86}Rb as a replacement for K^+. Data represent mean ± SEM of six independent determinations. **P<0.01 significantly different from normal conditions using one-way ANOVA and Newman-Keuls post hoc analysis.

5. Western blot analyses indicate alterations in Na,K,2Cl-cotransporter (NKCC) expression in BBMECs exposed to N/C and/or H/A conditions. Exposures consisted of either 6 hours H/A and/or nicotine (100 ng/ml) and cotinine (1000 ng/ml) for 24 hours. The T4 monoclonal antibody is specific for the carboxy terminal 310 aa of the human colonic Na,K,2Cl-cotransporter. Exactly 30 ug of protein was loaded per well as determined by a Pierce BCA protein assay. Protein sample and molecular weight markers were separated on a 4-20 % SDS / polyacrylamide gradient gel. Six hours of H/A exposure significantly (**P < 0.01) increased the % relative content of NKCC compared to control, and the combination of 6 H H/A and 24 H N/C significantly(##P < 0.01) reduced the expression of NKCC compared to 6 H H/A alone. N = 4 monolayers/treatment and statistical significance determined using one-way ANOVA and Newman-Keuls post hoc analysis. *Inset*, representative blot image showing mobility of the BBMEC Na,K,2Cl-cotransporter is 145 kDa which is equivalent to that detected in rat kidney microsomes.

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6. Western blot analyses indicate alterations in nAChR subunit protein in BBMECs incubated with nicotine and cotinine. Exposures consisted of control (lane 1), 10 ng/ml nicotine and 100 ng/ml cotinine (lane 2), 100 ng/ml nicotine and 1000 ng/ml cotinine (lane 3), 1000 ng/ml nicotine and 10,000 ng/ml cotinine (lane 4), 10,000 ng/ml nicotine and 100,000 (lanes 5), 100,000 ng/ml nicotine and 1,000,000 ng/ml cotinine (lane 6) and plasma equivalents to smokers (100 ng/ml nicotine and 1000 ng/ml cotinine, lane 7). The specific nAChR subunit antibodies consistently recognized a protein in cultured BBMECs that was approximately 55 kDa for the α3, α5, α7, β2, and β3 nAChR subunit experiments. Exactly 30 ug of protein was loaded per well as determined by a Pierce BCA protein assay. Protein sample and molecular weight markers were separated on a 4-20 % SDS / polyacrylamide gradient gel. Statistically significant reductions in nAChR subunit expression were observed for all subunits detected, yet the N/C concentration required for this reduction varied. N = 4-6 monolayers / treatment and **P<0.01 and *P<0.05 compared to control using one-way ANOVA and Newman-Keuls post hoc analysis.. *Inset*, representative blot images showing the mobility of all nAChR subunit proteins is 55 kD and this protein band was not recognized when using the α4 and β4 subunit specific antibody (data not shown).

7. Effects of a nAChR agonist and antagonists on BBMEC K^+ uptake after 6H H/A. Na,K,2Cl-cotransporter activity is expressed as ouabain(2μM)-insensitive K^+ uptake, using ^{86}Rb as a replacement for K^+. Epibatidine acts as a potent nAChR agonist and mecamylamine is a non-competitive antagonist and bungarotoxine is a competitive antagonist. Data represent mean ± SEM of 8 independent determinations. **p<0.01 significantly different from normal conditions, ##p<0.01 and #P<0.05 significantly

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different from 6H H/A, and ‡‡p<0.01 and ‡p<0.05 significantly different from 6H H/A and 24H N/C all using one-way ANOVA and Newman-Keuls post hoc analysis..

8. Regulation of abluminal Na,K,2Cl-cotransport by intracellular messengers. Staurosporine, a general protein kinase inhibitor was incubated at a concentration of 20 nM [21]. Genistein, a potent protein tyrosine kinase inhibitor was incubated at a concentration of 50 uM. PMA, an activator of Protein Kinase C, was incubated at 100 nM. Calyculin A, an inhibitor of protein phosphatases 1 and 2A, was incubated at a concentration of 50 nM. All inhibitors and activators were added during both the pre-incubation and [^{86}Rb] uptake time periods. Data represent mean ± SEM of 6 independent determinations. (****** denotes significance of P<0.01 and *****denotes significance of P<0.05 compared to their respective control conditions using one-way ANOVA and Newman-Keuls post hoc analysis).

9. Cell signaling mechanisms involving ion transport during stroke with and without nicotine exposure. Our studies suggest that luminal nAChR activation coupled to hypoxia / aglycemic (right side of schematic) could increase brain endothelial cell calcium levels to a point whereby the cytoplasmic pool of PKC is depleted **(1)**. Additionally, nicotine may also activate protein phosphatase, which could either dephosphorylate PKC **(2)** and prevent translocation to the membrane for activity, or dephosphorylate the Na,K,2Cl-cotransporter directly **(3)**, thus inhibiting its function.

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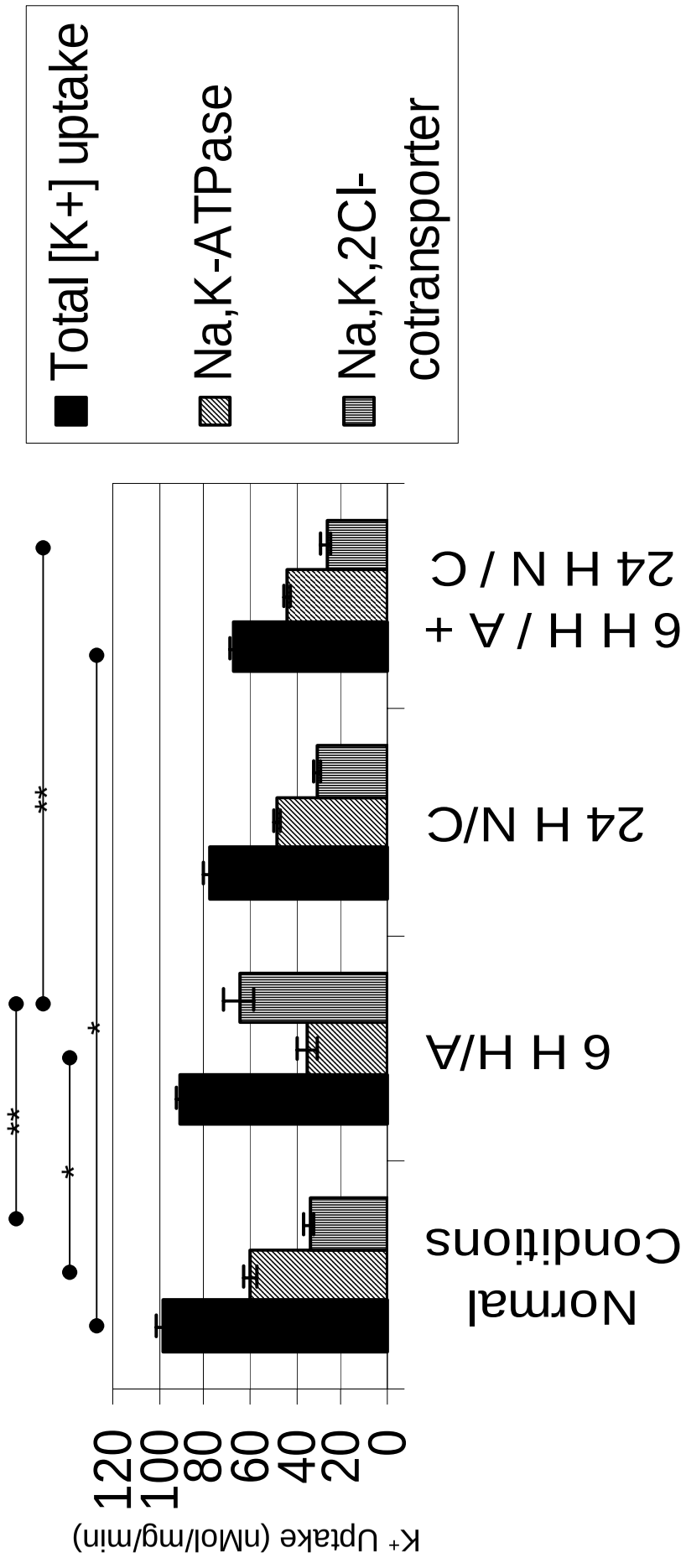

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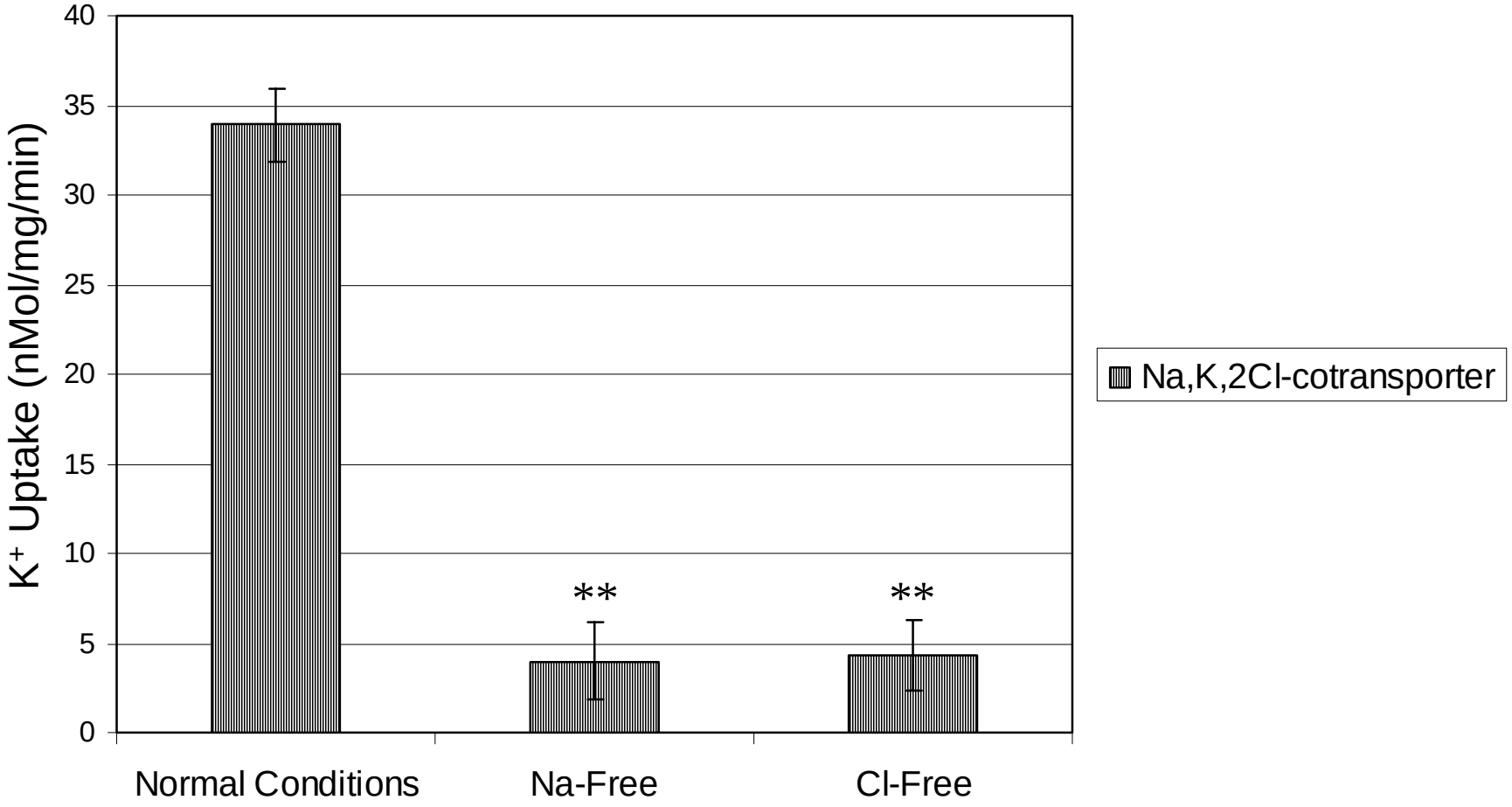

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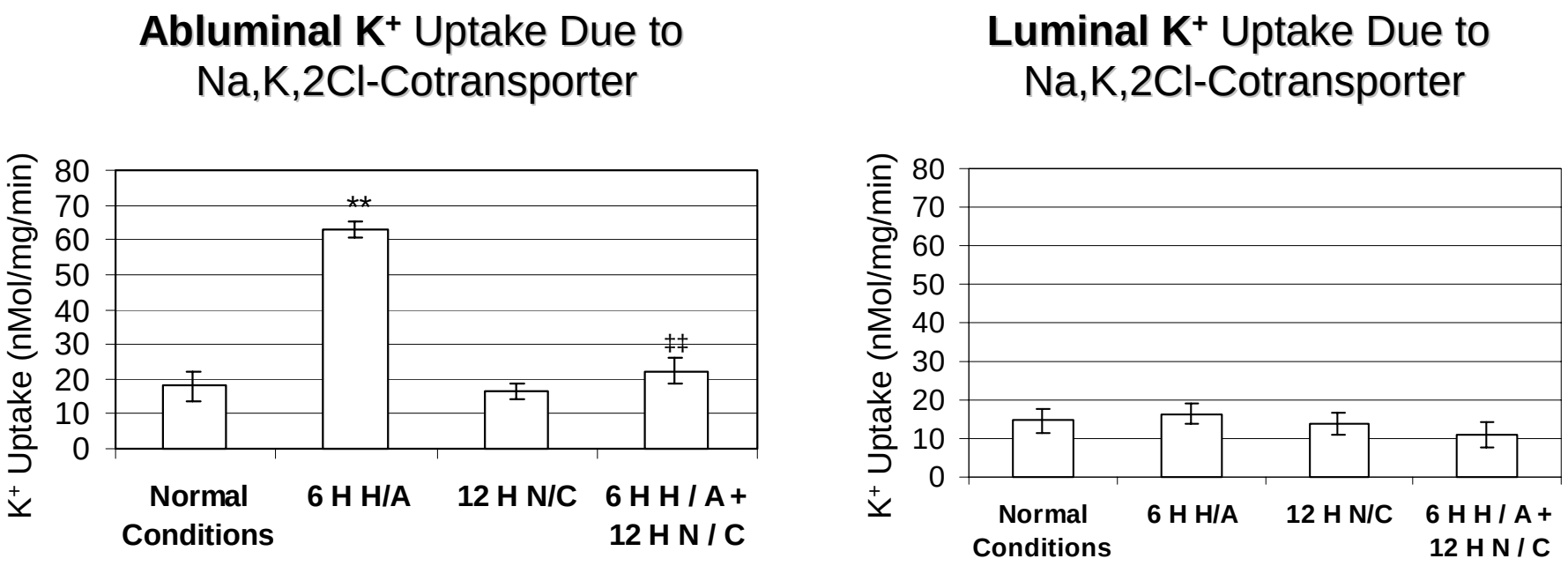

12 hour nicotine (100ng/ml) and cotinine (1000ng/ml)

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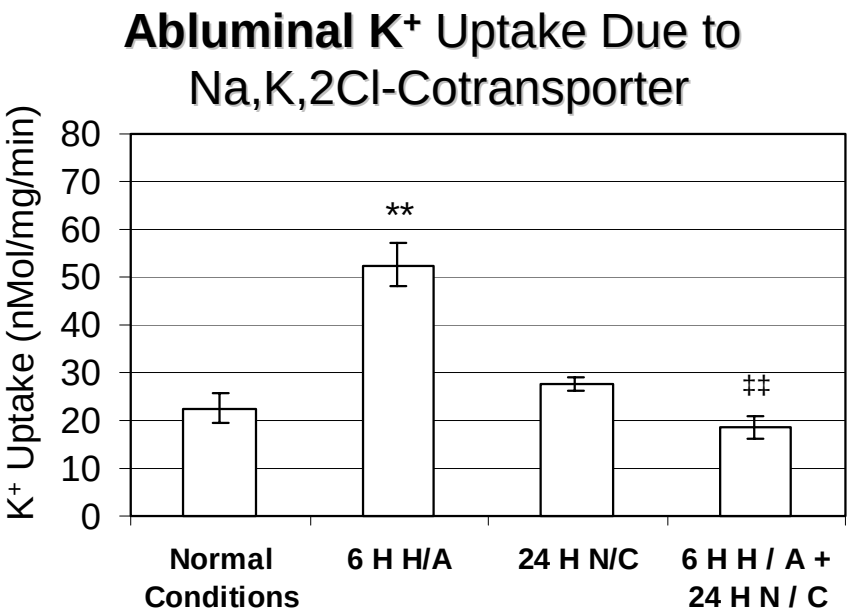

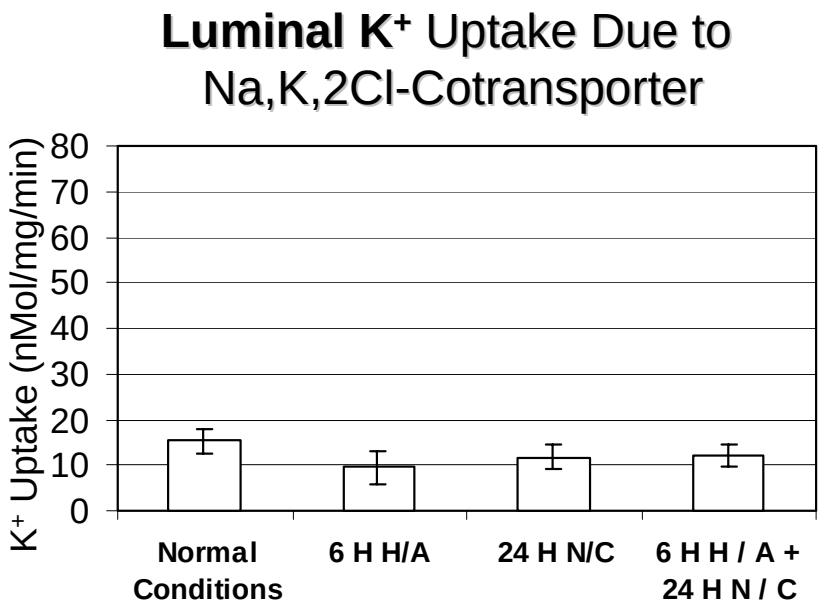

24 hour nicotine (100ng/ml) and cotinine (1000ng/ml)

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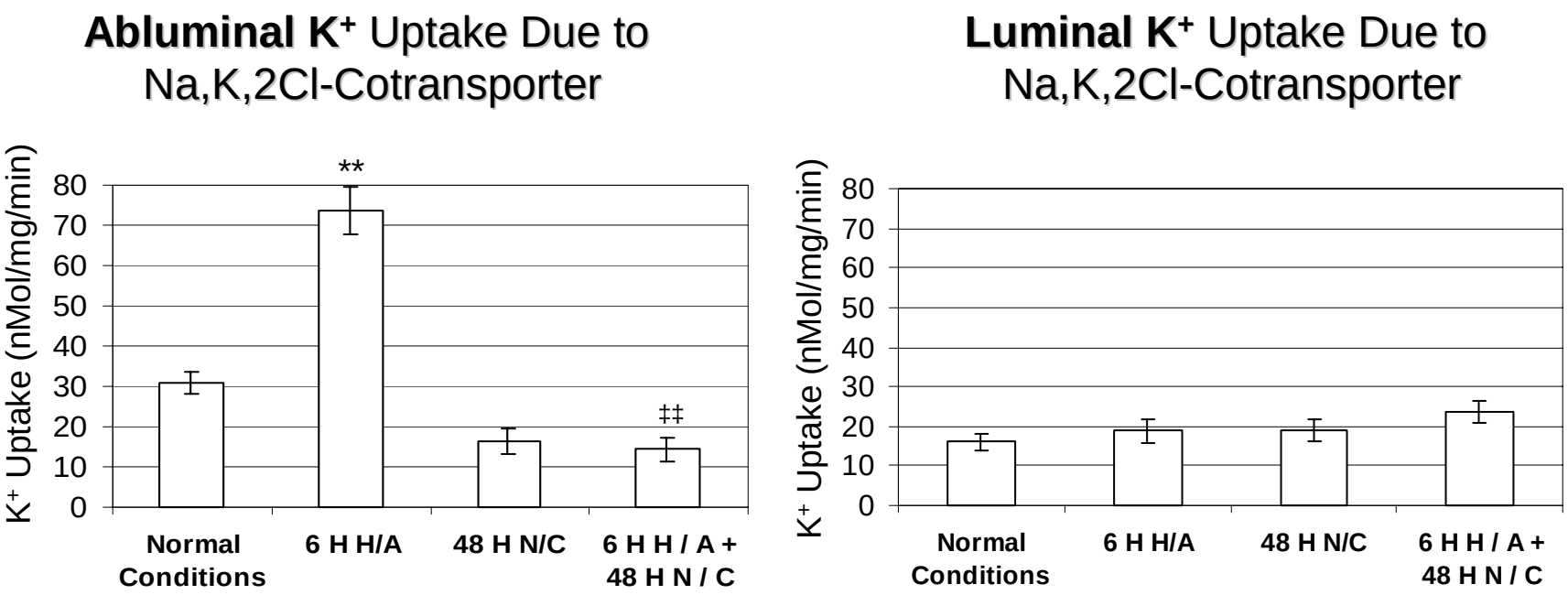

48 hour nicotine (100ng/ml) and cotinine (1000ng/ml)

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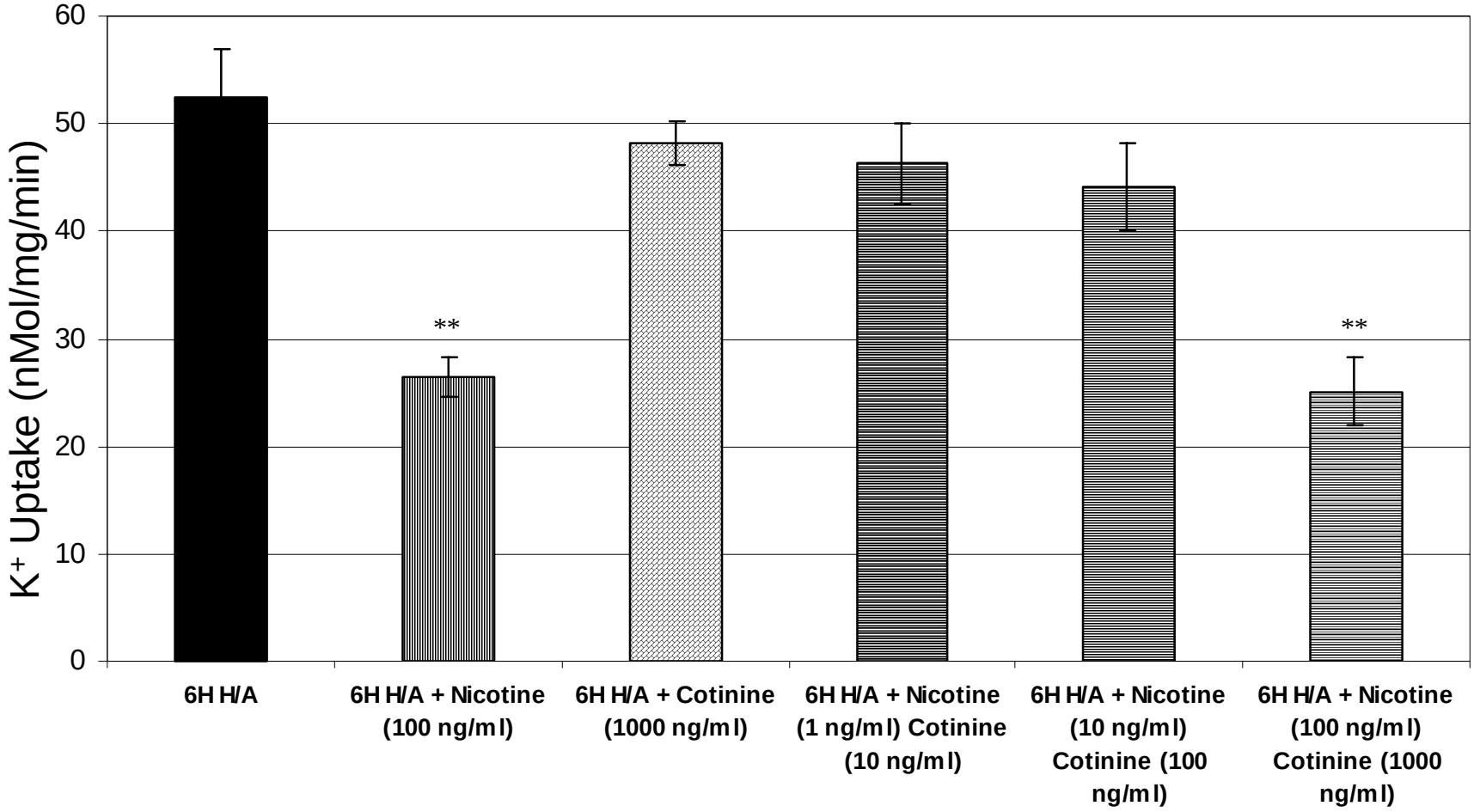

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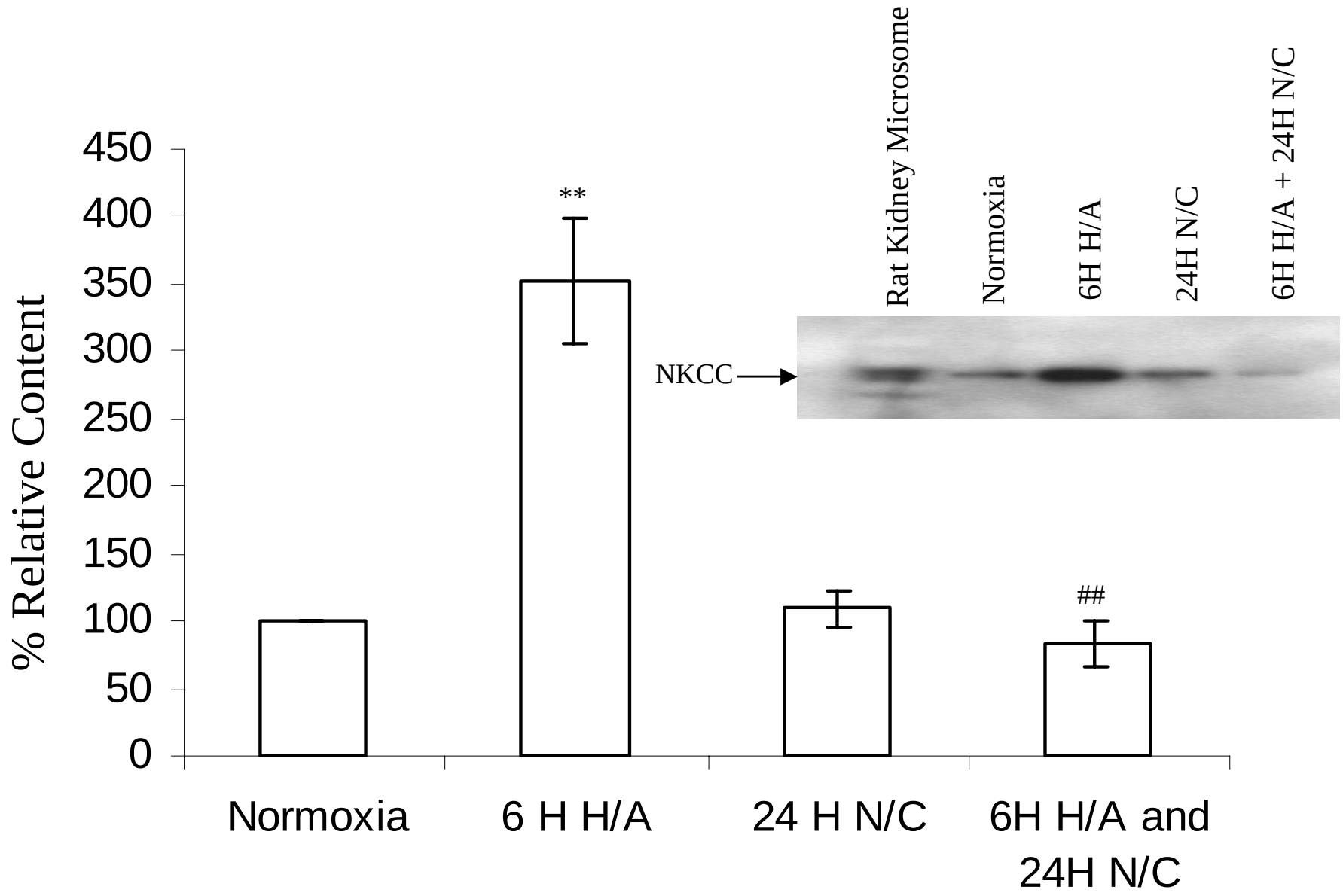

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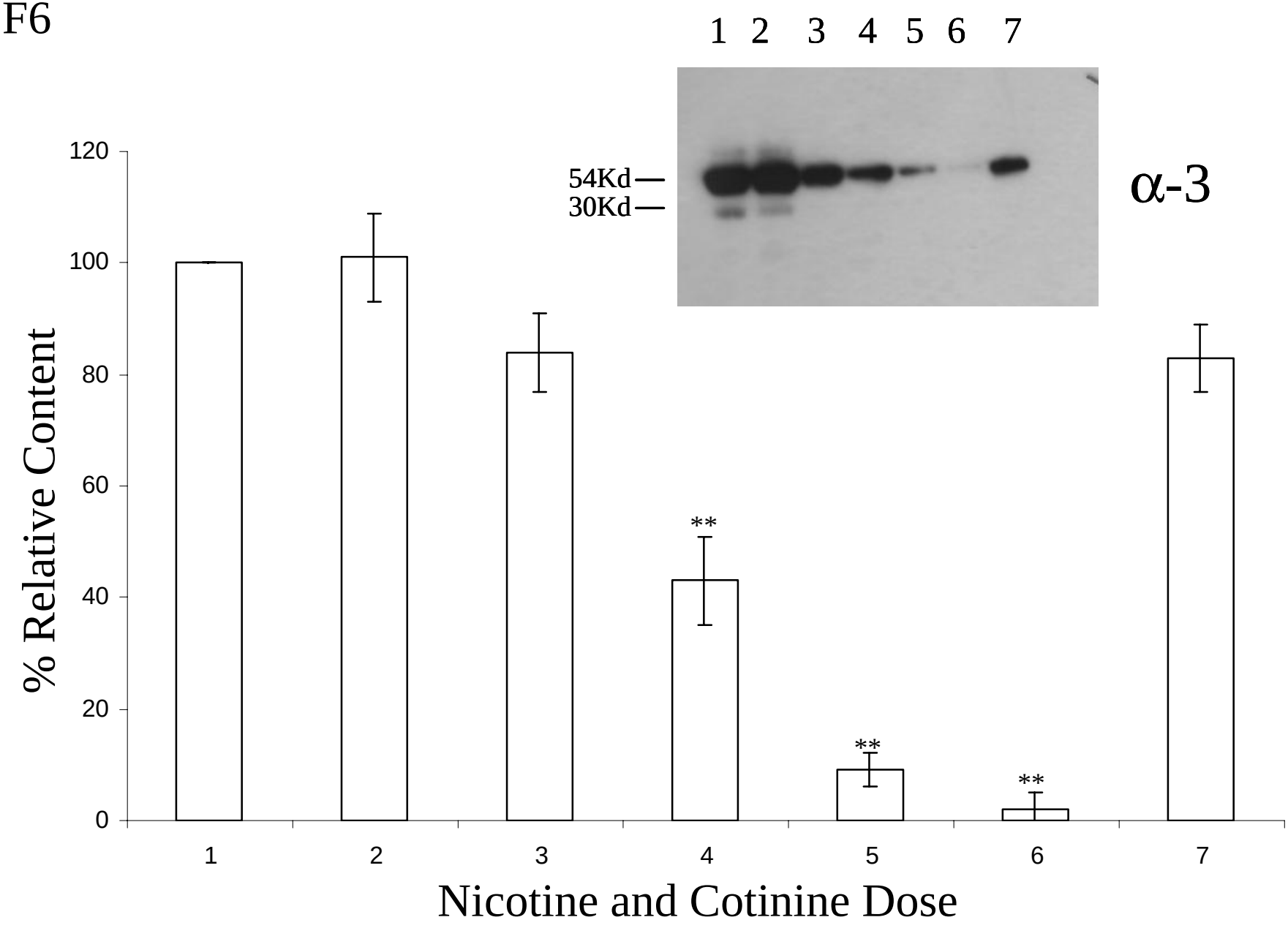

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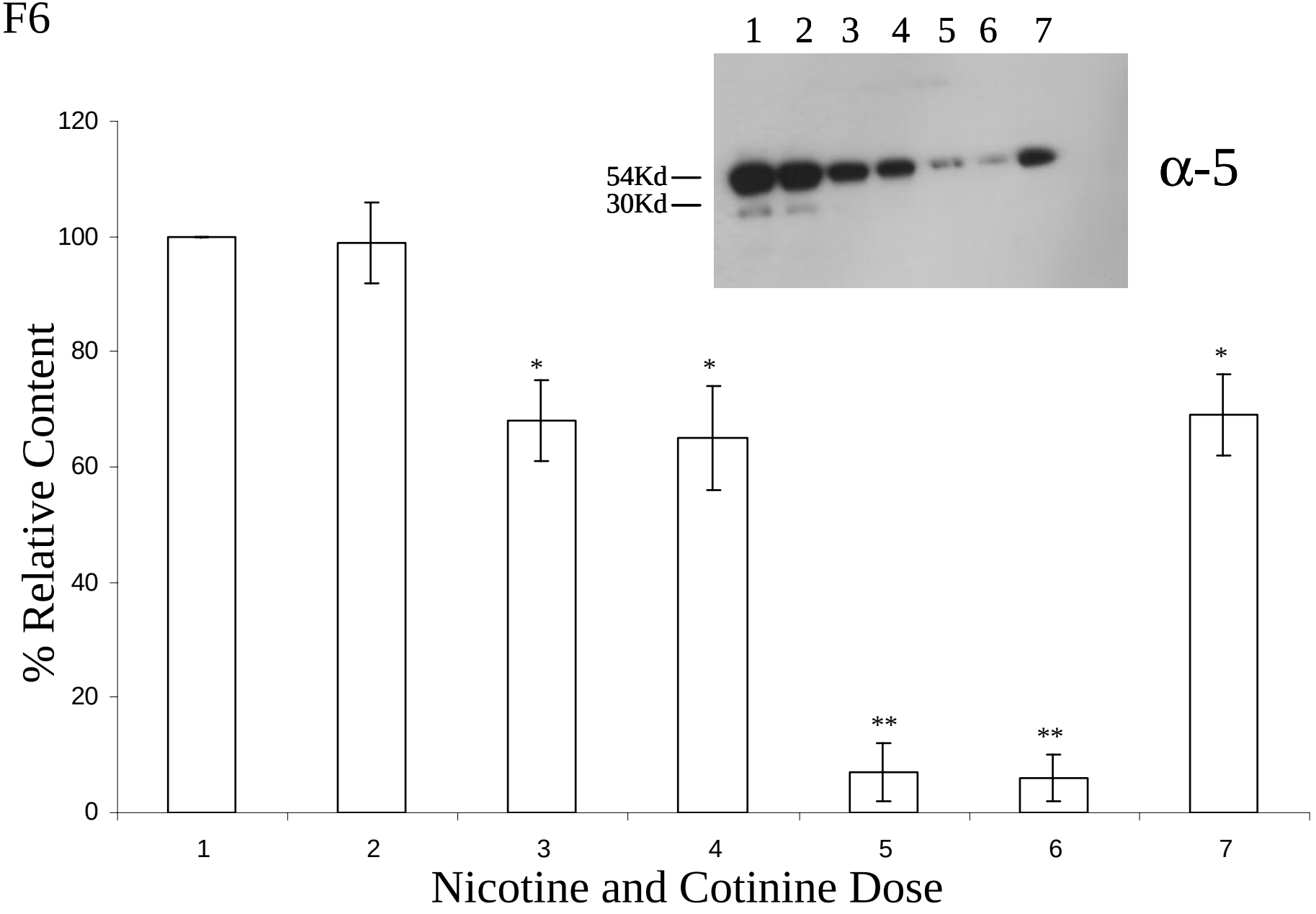

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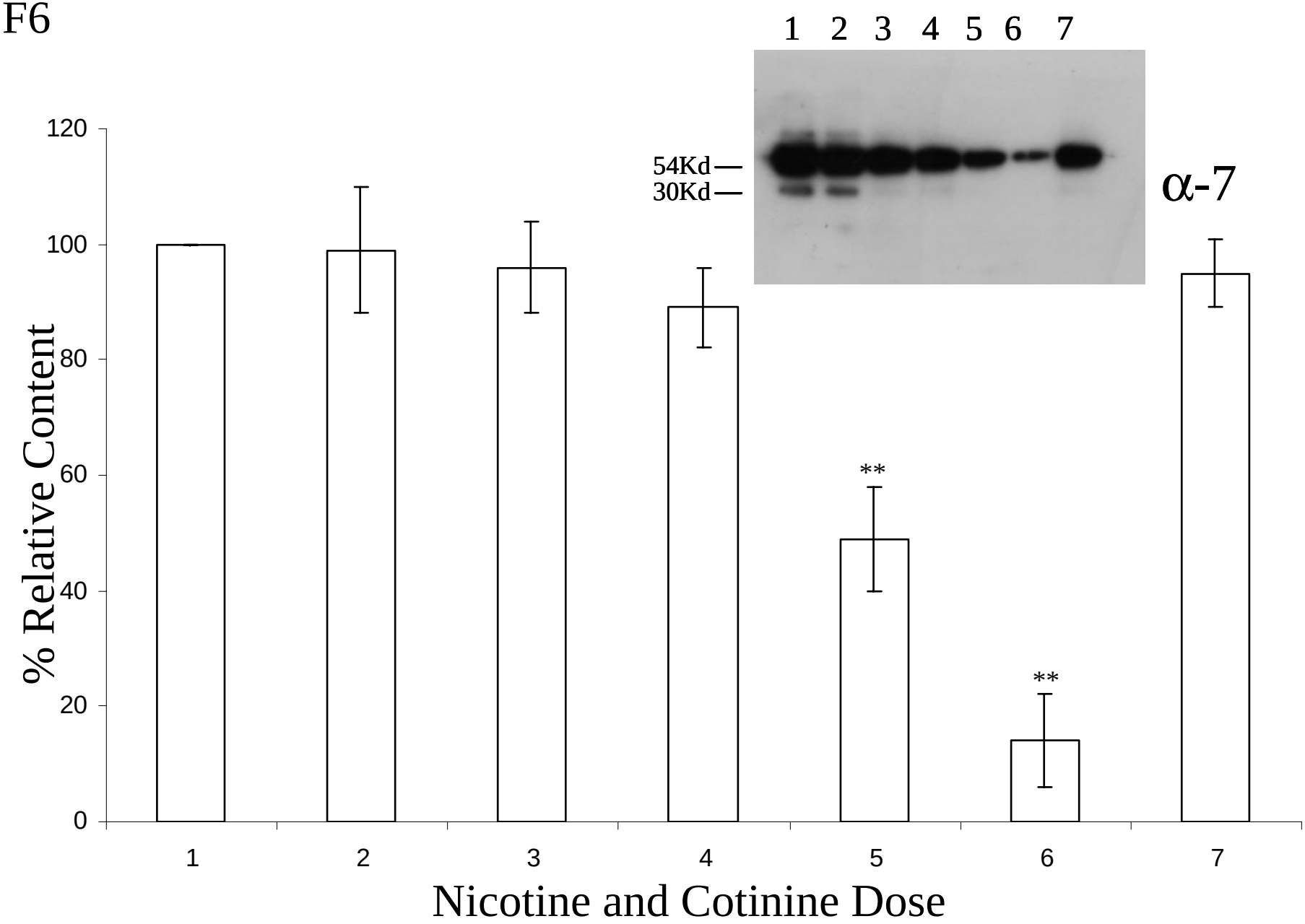

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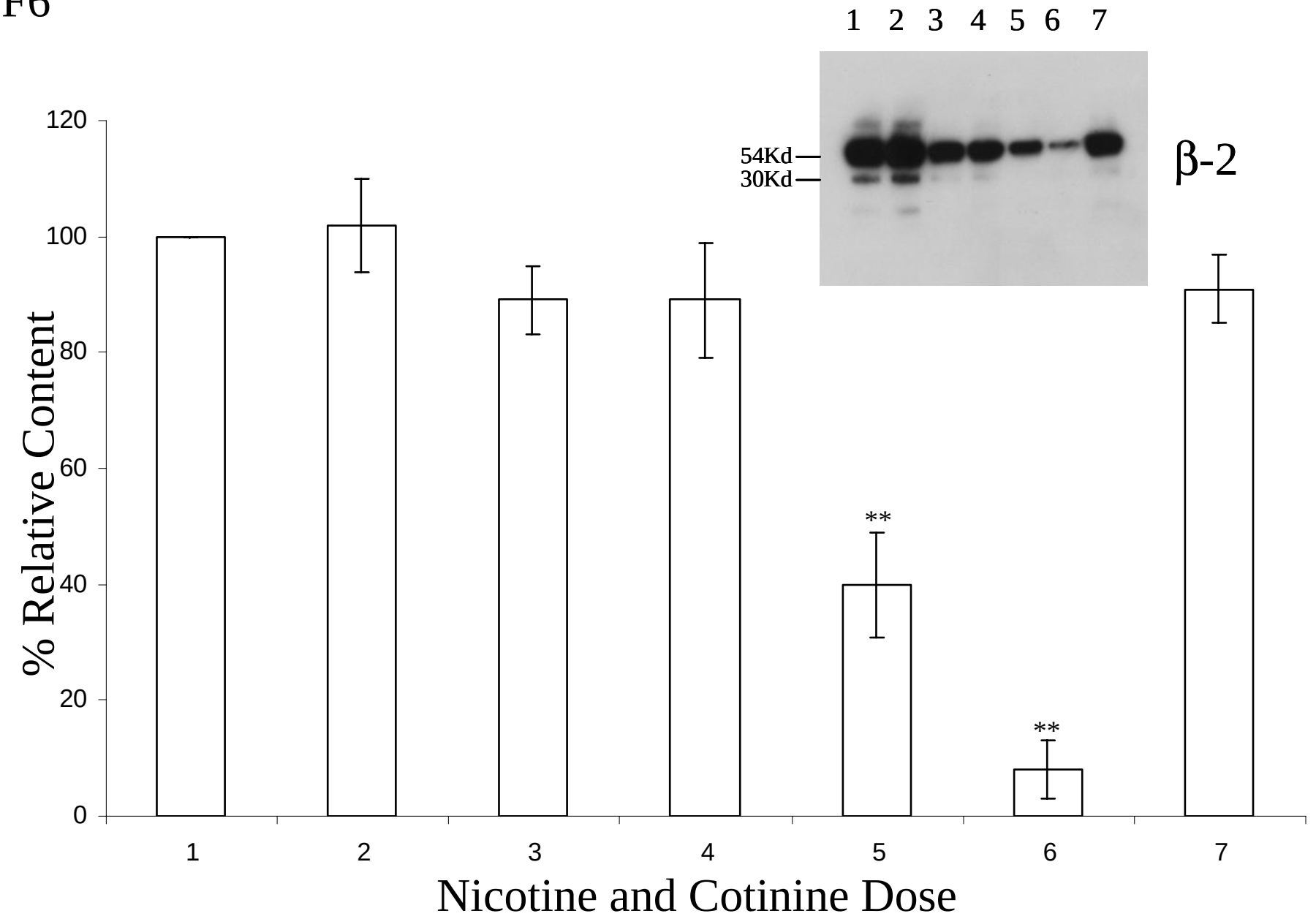

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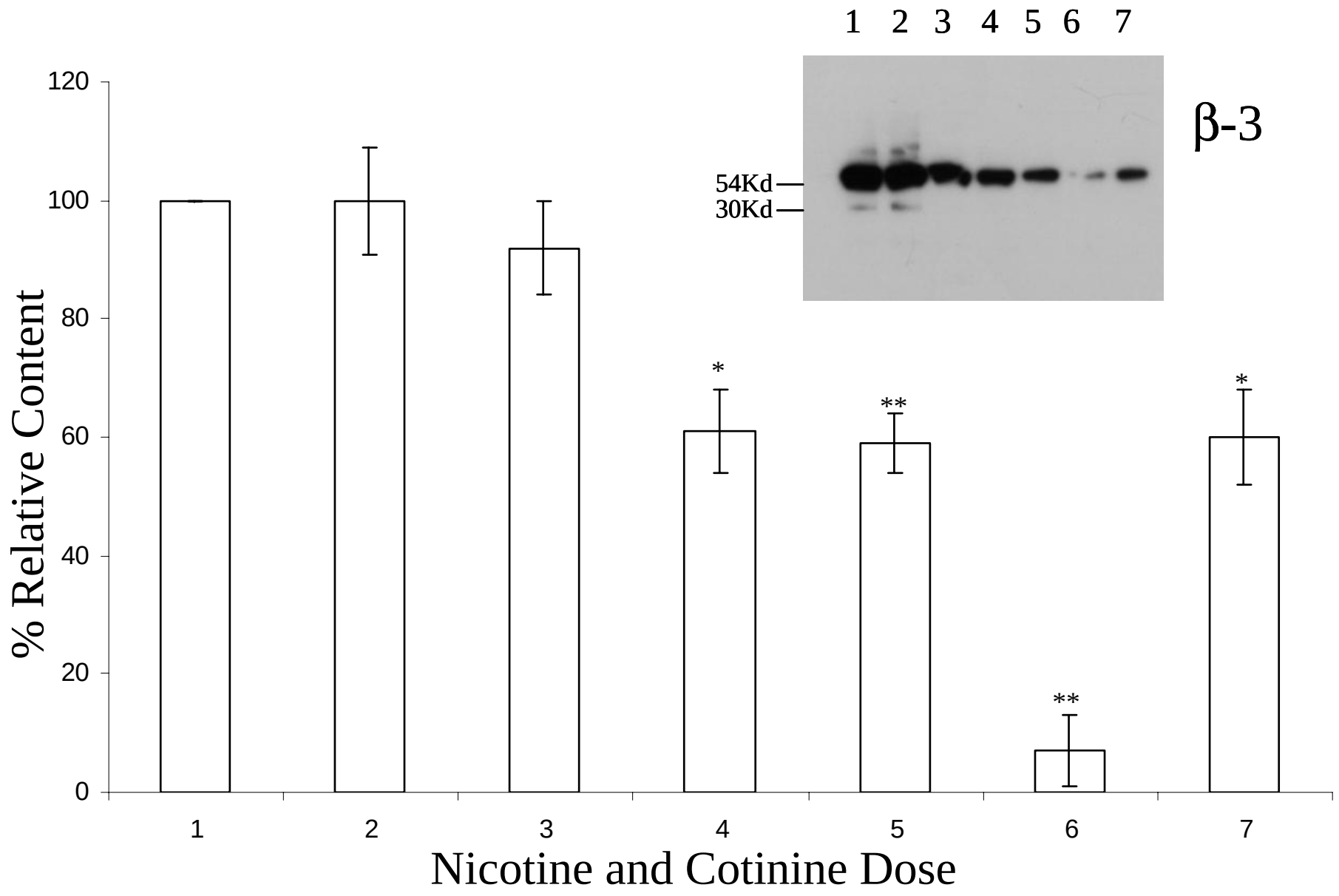

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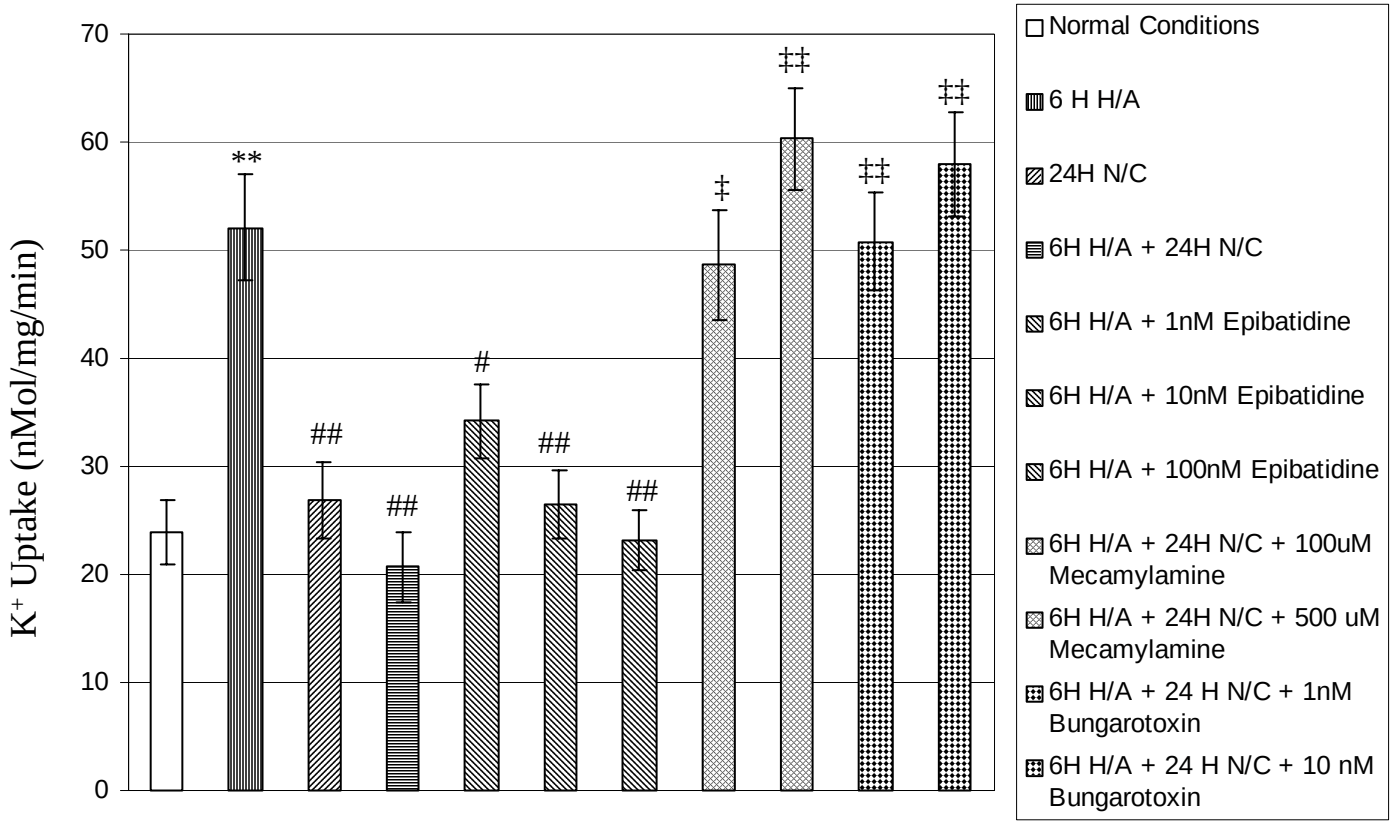

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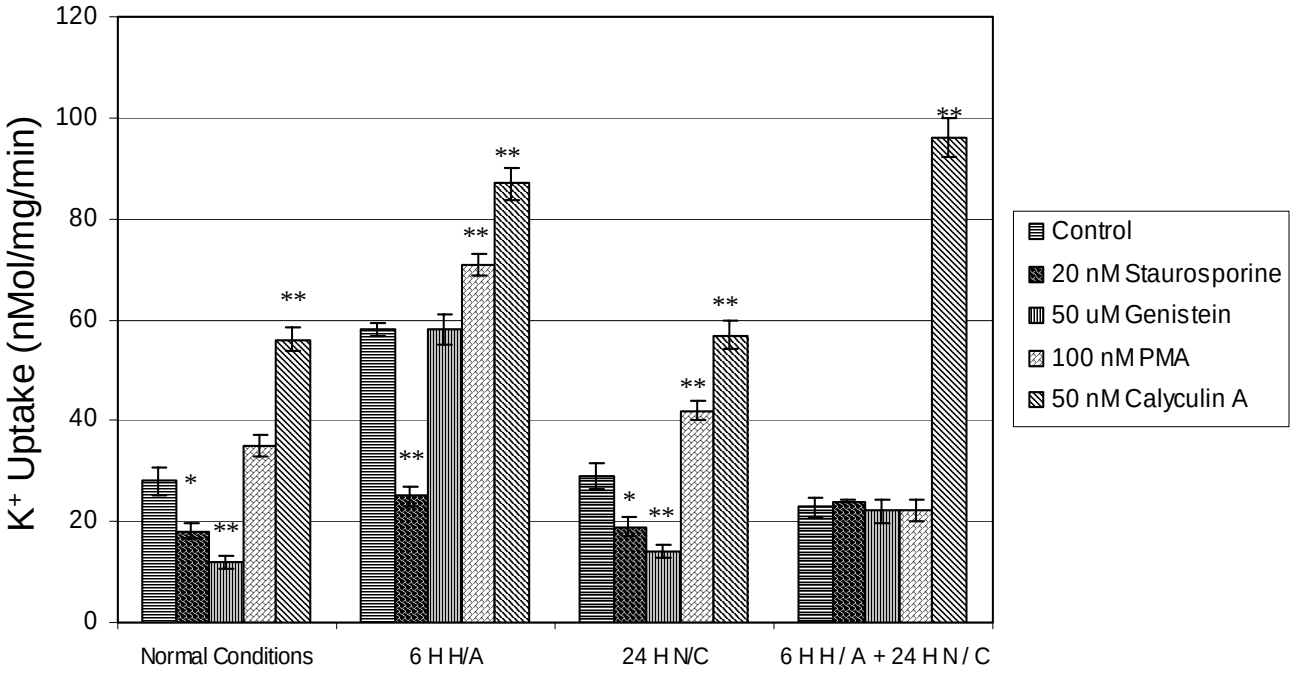

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