Published in final edited form as:
Brain Res. 2010 December 17; 1366: 211–216. doi:10.1016/j.brainres.2010.09.100.

Blockade of the sodium calcium exchanger exhibits anticonvulsant activity in a pilocarpine model of acute seizures in rats

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Abstract

Recent evidence suggests that the sodium calcium exchanger (NCX) may contribute to the etiology of pentylenetetrazol-induced seizures. Here we further investigated the role of NCX in the etiology of seizures by quantifying the effects of KB-R7943 and SN-6, potent inhibitors of the reverse mode of NCX subtypes 3 (NCX3) and 1 (NCX1), respectively, on the occurrence of acute seizures and status epilepticus induced by intraperitoneal administration of pilocarpine, a muscarinic acetylcholine receptor agonist. Pretreatment with KB-R7943 significantly reduced the incidence of pilocarpine-induced seizures and status epilepticus in 22–56% of treated animals. In the remaining animals that exhibited seizures, KB-R7943 pretreatment delayed the onset of seizures and status epilepticus, and reduced seizure severity. Delayed onset of seizures and reduced seizure severity also were seen following pretreatment with SN-6. These findings suggest that altered NCX activity may contribute to the pathophysiology of pilocarpine-induced seizures and status epilepticus.

Keywords

KB-7943; SN-6; status epilepticus

1. Introduction

Epilepsy is a chronic neurological disorder characterized by spontaneous recurrent seizures. Approximately 1–2% of the global population suffers from various epileptic syndromes, and about 30% of patients with epilepsy live with uncontrolled seizures (French 2007). Evidence indicates that temporal lobe epilepsy is the most common type of refractory epilepsy in humans (Semah et al., 1998), and therefore, novel antiepileptic treatments based on new underlying mechanisms for temporal lobe seizures are needed. Experimental evidence indicates that altered levels of intracellular Ca^{2+} in hippocampal CA1 neurons play an important role in the underlying mechanisms of neuronal hyperexcitability that leads to pilocarpine-induced seizures, a validated model of temporal lobe epilepsy (DeLorenzo et al. 2005; Turski et al., 1983). The levels of intracellular Ca^{2+} are highly regulated by Ca^{2+} binding proteins and Ca^{2+} extrusion through transporters/exchangers. One transporter/exchanger of interest is the sodium/calcium exchanger (NCX), a bidirectional membrane ion

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transporter that couples the influx/efflux of Ca^{2+} to the efflux/influx of Na^{+} to regulate levels of intracellular Ca^{2+} in neurons (Blaustein and Lederer, 1999; Annunziato et al., 2004). Under physiological conditions, NCX transports one Ca^{2+} out of the cell and three Na^{+} into the cell. The Ca^{2+} exit is known as the "forward" mode of NCX (Blaustein and Lederer, 1999; Annunziato et al., 2004). However, under certain conditions such as during membrane depolarization, the exchanger can reverse function and transport Na^{+} out of the cell and Ca^{2+} into the cell. The Ca^{2+} entry represents the "reverse" mode of NCX (Baker and McNaughton, 1976; Annunziato et al., 2004). Three different isoforms of NCX including NCX1, NCX2, and NCX3 have been characterized, cloned, and found in the brain, with multiple splice variants of NCX1 and NCX3 (Kofuji et al., 1994; Papa et al., 2003; Quednau et al., 1997). Despite the fact that reverse mode of NCX allows Ca^{2+} entry into the cell and possibly thereby altering Ca^{2+} homeostatic mechanisms, the role of NCX in the pathophysiology of limbic epilepsy remains poorly understood (Keele et al., 2000; Ketelaars et al. 2004). Nevertheless, a recent study reports that genetic deletion of NCX1 confers resistance to pentylenetrazole-induced tonic flexion, indicative of the important role this NCX isotope has in the etiology of these seizures (Saito et al., 2009). Because dysregulation of Ca^{2+} homeostatic mechanisms is an important feature of limbic epileptogenesis and the reverse mode of NCX contributes to Ca^{2+} influx, we sought to determine the extent to which inhibition of the reverse mode of NCX may affect acute pilocarpine-induced seizures and status epilepticus (SE) in rats.

2. Results

The incidence of pilocarpine-induced motor limbic seizures and SE were first evaluated using two doses of intraperitoneally (i.p.) administered pilocarpine (280 and 380 mg/kg). Although no difference was found in seizure severity between the two groups (280 mg/kg: 4.7±0.3, n=11; 380 mg/kg: 5±0, n=9; H=0.8, P=0.4), the onset of motor limbic seizures and SE was significantly delayed in the 280 mg/kg group (motor limbic seizures: 19±3 min, n=10, F=7.4, P=0.01; SE: 30±1 min, n=10, F=10.4, P=0.005) compared to the 380 mg/kg group (motor limbic seizures: 10±1 min, n=9; SE: 23±2 min, n=9). Furthermore, higher mortality rates were found in the 380 mg/kg group (9/9) compared to those the 280 mg/kg group (1/11; Chi-square: 163, P=0.0001). Therefore, we used the 280 mg/kg dose of pilocarpine for pharmacological studies.

Orally administered (p.o.) pretreatment with KB-R7943 at the doses of 10, 30, and 100 mg/kg significantly suppressed the occurrence of motor limbic seizures in 22% (2/9, Chi-square=5.5; P=0.02), 22% (2/9, Chi-square=5.5; P=0.02), and 33% (3/9, Chi-square=17; P=0.0001) of tested animals, respectively, compared to 91% (10/11) in the control group (Fig. 1A). In the remaining animals that exhibited seizures, KB-R7943 pretreatment significantly (F=4, P=0.02) delayed the onset of motor limbic seizures. This effect was observed at all doses tested (10 mg/kg: 29±3 min, n=7; 30 mg/kg: 31±3 min, n=7; 100 mg/kg: 33±4 min, n=6; P<0.05, Fig. 1B) as compared to the control group (19±2 min, n=10; Fig. 1B). Pretreatment with KB-R7943 also significantly reduced the severity of pilocarpine-induced seizures (H=16, P=0.001); this effect was observed with both 30 mg/kg (3.3±0.3, n=7, P<0.05; Fig. 1C) and 100 mg/kg (2.8±0.3, n=5, P<0.05; Fig. 1C) compared to the control group (4.7±0.3, n=11). Similarly, pretreatment with SN-6 (10 mg/kg; p.o.) also reduced the severity of pilocarpine-induced seizures (control group: 4.7±0.3, n=11; SN-6: 2.8±0.6, n=5; H=7, P=0.01) and delayed the onset of motor limbic seizures (control group: 19±2 min, n=11; SN-6: 34±3 min, n=5; F=10, P=0.01). However, SN-6 (10 mg/kg) pretreatment did not affect the incidence of motor limbic seizures (5/6 compared to the control group 11/12).

In addition to limbic seizures, pilocarpine can also trigger myoclonic seizures reminiscent of brainstem bouncing seizures. Such seizures were seen in 9 of 11 (82%) of tested animals in the control group. Pretreatment with KB-R7943 at all doses tested suppressed the occurrence of myoclonic seizures in 3 of 9 (33%) tested animals (Chi-square=14, P=0.0001; Fig. 2A). KB-R7943 pretreatment also significantly delayed the onset of myoclonic seizures (F=7, P=0.002). This effect was observed at 10 mg/kg (22±3 min, n=6; P<0.05; Fig. 2B) and 30 mg/kg (24±2 min, n=6; P<0.05; Fig. 2B), but not at 100 mg/kg (14±1, n=6; P>0.05; Fig. 2B), compared to the control group (14±1 min, n=9). Pretreatment with SN-6 (10 mg/kg) did not alter the incidence of myoclonic seizures (control group: 9/11; SN-6: 5/6) but significantly delayed the onset of these seizures (control group: 14±1 min, n=9; SN-6: 20±3 min, n=5; F=6; P=0.04).

Motor limbic seizures progressively developed into SE in 30±3 min after pilocarpine administration in 91% (10/11) of tested animals in the control group. Pretreatment with KB-R7943 at the doses of 10, 30, and 100 mg/kg significantly suppressed the occurrence of pilocarpine-induced SE in 22% (2/9, Chi-square:5, P=0.02; Fig. 3A), 33% (3/9, Chi-square: 17, P=0.0001; Fig. 3A), and 56% (5/9; Chi-square:48, P=0.0001; Fig. 3A) of tested rats, respectively. In the remaining rats in which continuous seizures were not suppressed, KB-R7943 pretreatment significantly (F=3; P=0.04) delayed the onset of SE; an effect that was only observed at 10 mg/kg (38±2 min, n=7; P<0.05) compared to the control group (30±1 min, n=10; Fig. 3B). No significant increase in seizure latency was observed at the doses of 30 mg/kg (33±3 min, n=6) and 100 mg/kg (33±3 min, n=4) compared to the control group (30±1 min, n=10; Fig. 3B). SN-6 pretreatment also significantly (F=83, P=0.0001) delayed the onset of SE (control group: 30±1 min, n=10; SN-6: 48±2 min, n=5) but did not alter its incidence (control group: 10/11; SN-6: 5/6).

3. Discussion

In the present study, we used a relatively low dose of pilocarpine to induce seizures because higher doses (300–380 mg/kg) can cause a massive seizure-induced brain damage syndrome (Fabene et al., 2003, 2007; Fujikawa, 1996; Marchi et al., 2007). The main finding of this study is that blockade of the reverse mode of NCX significantly reduced the incidence of acute pilocarpine-induced seizures and SE. In the remaining animals that exhibited seizures, inhibition of the reverse mode of NCX reduced seizure severity and delayed the onset of motor limbic seizures, myoclonus, and SE. These findings suggest that inhibition of the reverse mode of NCX may have anticonvulsant activity in an acute model of pilocarpine-induced seizures and SE.

Evidence shows that levels of KB-R7943 plasma concentration at 5, 15 and 100 minutes following its intravenous administration were 31, 15, and 0.6 nmol/L, respectively, (Miyata et al. 2002). These findings suggested that KB-R7943 is rapidly cleared from the plasma and its brain penetration may be excellent. Interestingly, brain concentration of 2-[4-[2,5-difluorophenyl)methoxyl]-phenoxyl]-5-ethoxyaniline (SEA400), another NCX inhibitor was 3 and 7 μM after its intravenous injection at the doses of 1 and 3 mg/kg, respectively (Matsuda et al., 2001). Such brain concentration of KB-R7943 and SN-6 also may be found following their administration.

Although KB-R7943 and SN-6 are potent inhibitors of the three NCX isotopes, evidence indicates that these compounds preferentially block NCX3 and NCX1, respectively, indicative of the important role of these NCX isotopes as molecular targets for seizure suppression in the acute pilocarpine model (Iwamoto and Shigekawa, 1998; Iwamoto 2004). The role of NCX in the pathophysiology of epilepsy remains poorly understood. Nevertheless, a recent report indicates genetic deletion of NCX1 subtype suppresses the

expression of pentylenetetrazol-induced tonic flexion (Saito et al., 2009). Consistent with these studies, we found that inhibition of NCX3 and NCX1 significantly reduced the incidence of acute pilocarpine-induced seizures and SE. The underlying mechanisms of how blockade of the reverse mode of NCX by KB-R7943 and SN-6 suppresses pilocarpine-induced seizures and SE are not yet fully understood. Although KB-R7943 and SN-6 are potent inhibitors of the reverse mode of NCX, multiple lines of evidence indicate they also block (to some degree) various voltage- and ligand-gated channels, including L-type Ca^{2+} channels, N-methyl-D-aspartate (NMDA) receptors, canonical transient receptor potential channels (TRPCs), and nicotinic acetylcholine receptors (Birinyi et al., 2005; Kraft 2007; Ouardouz et al., 2005; Pintado et al., 2000; Sobolevsky and Khodorov, 1999). Evidence shows that nimodipine, a potent blocker of L-type Ca^{2+} channels that can also block T-channels, suppresses pilocarpine-induced seizures and SE (Akaike et al., 1989; Becker et al., 2008; Takahashi and Akaike, 1991; Su et al., 2002). Multiple lines of evidence indicate that MK-801, a noncompetitive NMDA receptor blocker as well as clinically used anticonvulsant phenytoin and carbamazepine thought to block voltage-gated Na^{+} channels channels, failed to prevent pilocarpine-induced seizures (Leite and Cavalheiro, 1995; Ormandy et al., 1989; Turski et al. 1989). Evidence also shows that KB-R7934 potently blocks the TRPCs that also play an important role in Ca^{2+} homeostasis in the brain (Kraft, 2007). However, the role of TRPCs in the pathophysiology of epilepsy remains unknown. Nicotine can also induce seizures in rodents; however, the role nicotinic receptors play in pilocarpine epileptogenesis remains unknown (Newman et al., 2001). Thus, KB-R7943 or SN-6 pretreatment likely suppresses the occurrence of pilocarpine-induced seizures and SE via inhibition of the reverse mode of NCX.

In conclusion, blockade of the reverse mode of NCX has anticonvulsant potential against pilocarpine-induced acute seizures. Understanding how inhibition of the reverse mode of NCX affects neuronal hyperexcitability that leads to seizures can provide a framework for the development of clinically useful NCX blockers.

4. Experimental Procedures

Sprague-Dawley rats (male, 150–200g, Taconic, Germantown NY) were used. All experiments were performed with the approval of the Georgetown Animal Care and Use Committee. Effort was made to minimize the number of animals used as well as animal suffering. Pilocarpine hydrochloride (280 or 380 mg/kg, Sigma Chemical, St. Louis, MO) was dissolved in 0.9% saline and i.p. injected to induce seizures. Methyl scopolamine (1 mg/kg in 0.9% saline) was i.p. administered 30 min before pilocarpine administration to minimize the peripheral effects of pilocarpine (Turski et al., 1983). Rats were closely observed for the occurrence of limbic seizures and SE during 120 min following pilocarpine administration. The convulsive behavior of seizures were classified as follows (Racine 1972, modified): stage 0, no response; stage 1, mouth and facial automatisms; stage 2, myoclonus (clonic seizures while the animal is lying on its belly); stage 3, bilateral forelimb clonic seizures without rearing; stage 4, forelimb clonic seizures and rearing; stage 5, rearing and loss of posture. SE was defined as an uninterrupted seizure class 3–5 for 30 min. The pilocarpine model was chosen because seizures induced by this method exhibit a gradual development over a period of about 10 min as compared to seizures initiated by electrical stimulation, characterized by an abrupt onset. Inhibitors of the reverse mode of NCX including 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate (KB-R7943; 10, 30, and 100 mg/kg; Tocris, Ellisville, MO) and SN-6 (2-[[4-[(4-Nitrophenyl)methoxy]phenyl]methyl]-4-thiazoli dinecarboxylic acid ethyl ester; 10 mg/kg; Tocris, Ellisville, MO) were dissolved in sterile water, filtered, and administered 90 min before pilocarpine injections. KB-R7934 and SN-6 were given orally by gastric intubation in a volume of 0.2 ml/100 g body weight using an 18-gauge stainless steel feeding needle with

round tips (ball diameter 3 mm). The initial dose of 10 mg/kg KB-R7943 was based on previous *in vivo* pharmacological studies (Blokin et al., 2008). Subsequent doses of KB-R7943 were progressively increased by ~3-fold until significant protection against pilocarpine-induced seizures was observed. To validate the effects of KB-R7943, we also tested and quantified the effects of SN-6 (10 mg/kg), another potent blocker of NCX. The 90 min time interval was the most effective in our preliminary tests (data not shown). Animals that did not display class 1 seizures within the 2-hour observation period were considered protected. Time intervals from the end of pilocarpine injection to the appearance of the first episode of forelimb clonic seizures, myoclonus, and SE were recorded, and referred to as limbic seizure latency, myoclonus latency, and SE latency, respectively. For each animal, the seizure severity score and seizure latency were recorded. For each group, the occurrence of myoclonus, limbic seizures, and SE were recorded. Data analysis of seizure latency and SE were performed using one-way ANOVA with Dunn's post hoc test. Before using ANOVA, data were submitted to the normality test (Shapiro-Wilk test) and the test for homogeneity of variances (Levene's test). Comparison of seizure severity scores was performed with the Kruskal-Wallis rank test and Dunn's post hoc test. The incidence of seizures and SE was analyzed using the Chi-square test. Significance level was set at $P<0.05$. Data are presented as mean ± S.E.M.

Acknowledgments

This publication was made possible by Public Health Service grant NS047193 (PN) and Short-Term Education Program for Underrepresented Persons (YM) from the National Institutes of Health (NIH). Contents are the responsibility of the authors and do not necessarily represent the official views of NIH.

Abbreviations

NMDA	N-methyl-D-aspartate
NCX	sodium calcium exchanger
SE	status epilepticus
TRPCs	transient receptor potential channels

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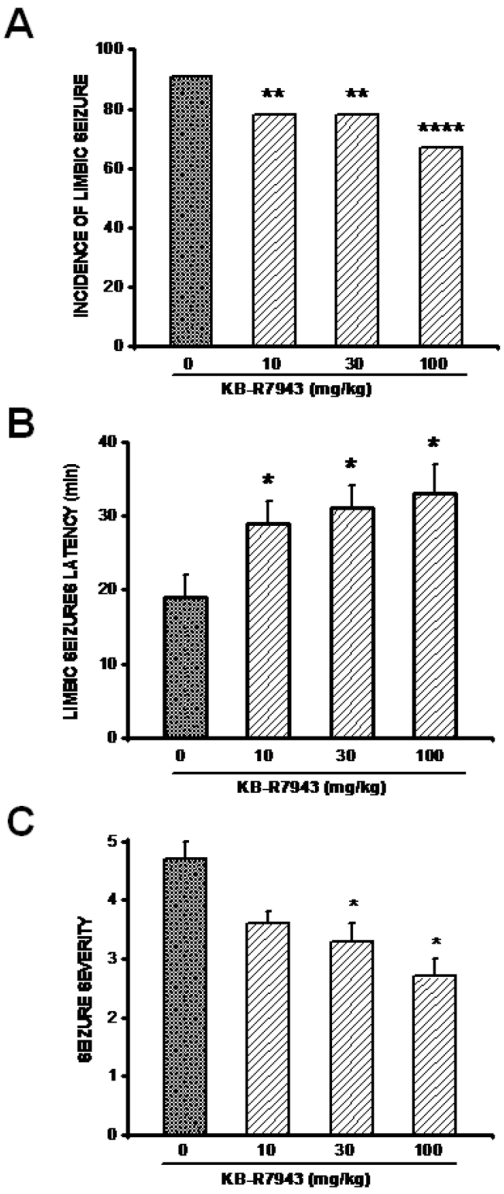

Figure 1.

KB-R7943 pretreatment alters the expression of pilocarpine-induced seizures. **A.** KB-R7943 pretreatment (10, 30, or 100 mg/kg; p.o.) reduced the incidence of motor limbic seizures. **B.** KB-R7943 pretreatment (10, 30, or 100 mg/kg; p.o.) delayed the onset of motor limbic seizures. **C.** KB-R7943 pretreatment (10 or 30 mg/kg; p.o.) reduced the severity of pilocarpine-induced seizures. Data represent mean ± S.E.M. *P<0.05, **P<0.01, ****P<0.0001.

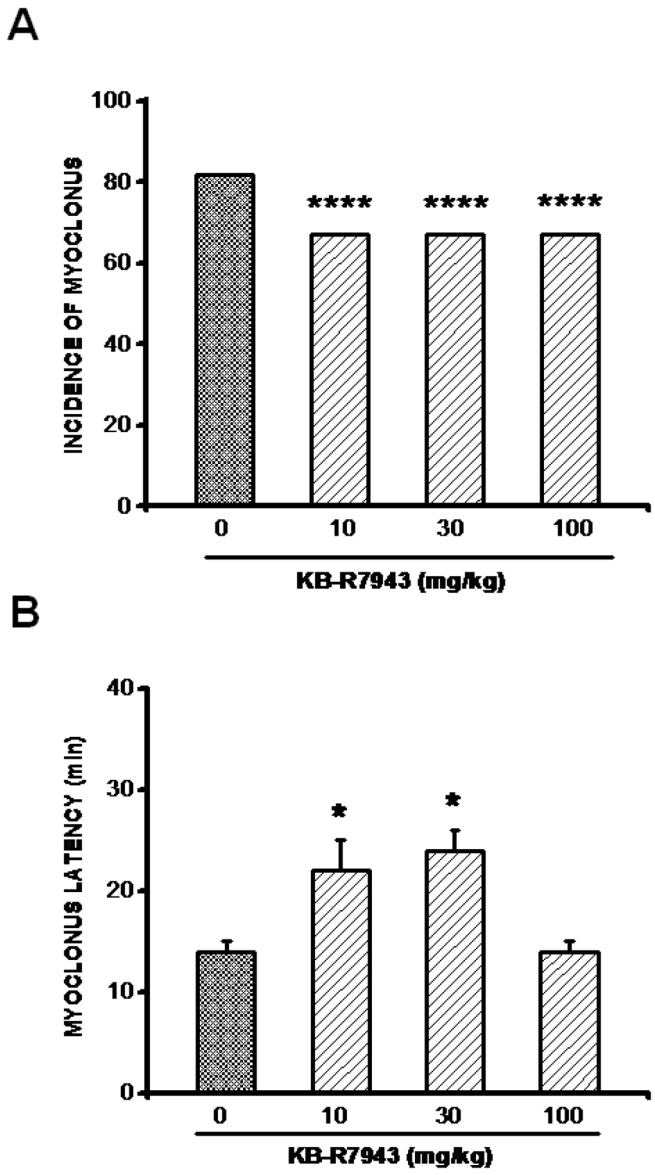

Figure 2.

Effects of various doses of KB-R7943 on the expression of pilocarpine-induced myoclonic seizures. **A.** KB-R7943 pretreatment (10, 30, or 100 mg/kg; p.o.) reduced the incidence of myoclonic seizures. **B.** KB-R7943 pretreatment (10 or 30 mg/kg; p.o.) delayed the onset of pilocarpine-induced myoclonic seizures. Data represent mean ± S.E.M. *P<0.05, ****P<0.0001.

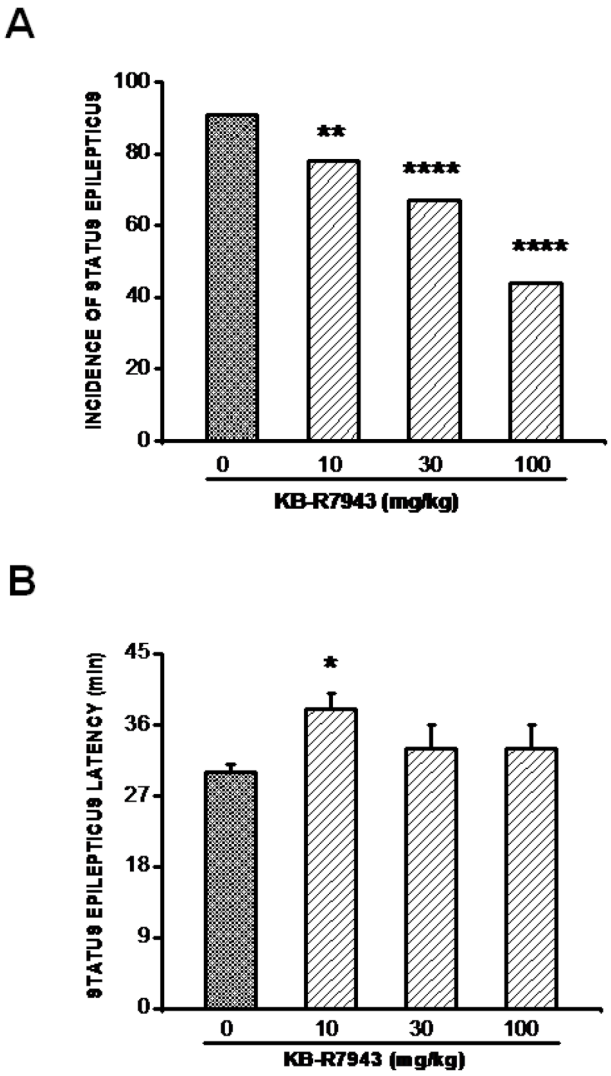

Figure 3.

KB-R7943 pretreatment alters the expression of pilocarpine-induced status epilepticus (SE). **A.** KB-R7943 pretreatment (10, 30, or 100 mg/kg; p.o.) reduced the incidence of SE. **B.** KB-R7943 pretreatment (10 mg/kg; p.o.) delayed the onset of pilocarpine-induced SE. Data represent mean ± S.E.M. *P<0.05, **P<0.01, ****P<0.0001.