

Icilin activates the δ -subunit of the human epithelial Na⁺ channel

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Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; ASIC1a, acid-sensing ion channel 1a; DMSO, dimethyl sulfoxide; ENaC, epithelial Na⁺ channel; hENaC, human epithelial Na⁺ channel; NMDG, *N*-methyl-D-glucamine; TRPA1, transient receptor potential ankyrin-like subfamily 1; TRPM8, transient receptor potential melastatin subfamily 8; TRPV1, transient receptor potential vanilloid subfamily 1.

Abstract

The amiloride-sensitive epithelial Na⁺ channel (ENaC) regulates Na⁺ homeostasis in cells and across epithelia. Four homologous ENaC subunits (α , β , γ , and δ) have been isolated in mammals. The chemical activators acting on ENaC, however, are largely unknown. More recently, we have found that capsazepine activates human ENaC δ (hENaC δ), which is mainly expressed in the brain. In addition, here we show that icilin, which is a tetrahydropyrimidine-2-one derivative unrelated structurally to capsazepine, markedly enhanced the activity of hENaC $\delta\beta\gamma$ heteromultimer expressed in *Xenopus* oocytes. The inward currents at a holding potential of -60 mV in hENaC $\delta\beta\gamma$ -expressing oocytes were increased by the application of icilin in a concentration-dependent manner with an EC₅₀ value of 33 μ M. The icilin-elicited current was mostly abolished by the addition of 100 μ M amiloride or by the removal of external Na⁺. Homomeric hENaC δ was also significantly activated by icilin, whereas hENaC α activity was not affected by icilin and icilin caused a slight inhibition of the hENaC $\alpha\beta\gamma$ current. Furthermore, icilin acted together with protons or capsazepine on hENaC $\delta\beta\gamma$. These findings identify icilin as a novel chemical activator of ENaC δ , providing us with a lead compound for drug development in the degenerin/ENaC superfamily.

Introduction

The degenerin/epithelial Na⁺ channel superfamily has striking functional diversity including Na⁺ homeostasis, acid sensing, peptide-gating, acidosis-evoked nociception, and mechanotransduction (Ugawa et al., 1998, 2002; Alvarez de la Rosa et al., 2000; Kellenberger and Schild, 2002; Welsh et al., 2002). The amiloride-sensitive epithelial Na⁺ channel (ENaC) is an essential control element for Na⁺ transport into cells and across epithelia. Four homologous ENaC subunits (α , β , γ , and δ) have been cloned in mammals (Canessa et al., 1993, 1994; McDonald et al., 1994, 1995; Waldmann et al., 1995). The δ -subunit was originally described to be expressed mainly in the human brain (Waldmann et al., 1995), and we have demonstrated that protons activate ENaC δ (Yamamura et al., 2004a). To explore physiological functions of ENaC δ in the human brain, it is necessary to identify the chemical modulator for ENaC δ . In pharmacological profiles of ENaC δ , it is well published that potassium-sparing diuretics, amiloride and benzamil, inhibit the activities of ENaC δ homomer and the heteromeric complexes with β and γ subunits (Waldmann et al., 1995; Ji et al., 2004). More recently, we have shown that capsazepine, which was originally developed as a competitive antagonist for transient receptor potential vanilloid subfamily 1 (TRPV1) (Bevan et al., 1992; Szallasi et al., 1993; Caterina et al., 1997; Szallasi and Blumberg, 1999), is the first known chemical activator of ENaC δ (Yamamura et al., 2004b).

In this investigation, the effects of icilin, which is a tetrahydropyrimidine-2-one derivative (Wei and Seid, 1983) and is also an activator for transient receptor potential melastatin subfamily 8 (TRPM8) and ankyrin-like subfamily 1 (TRPA1) (McKemy et al., 2002; Story et al., 2003; Andersson et al., 2004; Chuang et al., 2004), were examined on the human ENaC (hENaC) current using electrophysiological analyses in the *Xenopus* oocytes expression system. Here we show that icilin activates hENaC $\delta\beta\gamma$ in a concentration-dependent manner,

and the icilin-evoked current was influenced by amiloride or external Na^+ removal. Icilin activated the hENaC δ homomer as well as hENaC $\delta\beta\gamma$, whereas the hENaC $\alpha\beta\gamma$ current was slightly reduced by icilin. These results indicate that icilin is a novel agonist for ENaC δ .

Materials and Methods

Molecular biology

All experiments were approved by the Ethics Committee of the Nagoya City University Graduate School of Medical Sciences and were conducted in accordance with the Declaration of Helsinki. The full-length hENaC α (GenBank accession numbers X76180), hENaC β (X87159), hENaC γ (U48937), and hENaC δ (U38254) were isolated from human skin (for α , β , and γ subunits) or brain (for δ subunit) cDNA, as described previously (Yamamura et al., 2004b).

Xenopus oocyte electrophysiology

Electrophysiological studies in *Xenopus* oocytes, using a two-electrode voltage clamp technique, were performed as described previously (Yamamura et al., 2004a, 2004b). In brief, cRNA transcript(s) (1 ng for homomeric channel or each 0.01 ng for co-expression) was injected into *Xenopus* oocytes, whereas the control oocytes were injected with an equal volume of diethyl dicarbonate-treated water, described as native throughout. After injection, oocytes were incubated at 20 °C in a recording solution supplemented with 20 units/ml penicillin G, 20 μ g/ml streptomycin, and either 10 (for hENaC α and $\alpha\beta\gamma$) or 100 μ M (for hENaC δ and $\delta\beta\gamma$) amiloride for 24-48 h before electrophysiological recordings. The recording solution had an ionic composition of 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES. The pH of the solution was adjusted to 7.5 with NaOH. The external Na⁺-free solution was prepared by replacement of 96 mM Na⁺ with the equivalent *N*-methyl-D-glucamine (NMDG). All electrophysiological recordings were performed at a holding potential of -60 mV. The current-voltage relationships were measured using a ramp protocol from -100 to 50 mV for 30 s. The recording chamber was continuously perfused with solution at a flow rate of 5 ml/min. All electrophysiological

experiments were carried out at room temperature (25 ± 1 °C).

Drugs

Pharmacological reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Icilin was dissolved in dimethyl sulfoxide (DMSO) at the concentration of 100 mM as a stock solution. It was confirmed that up to 1 % of DMSO did not affect the oocyte currents.

Statistics

Pooled data are shown as the mean \pm s.e. Statistical significance between two groups and among groups was determined by Student's *t*-test and Scheffé's test after one-way analysis of variance, respectively. Significant difference is expressed in the figures (** or ###, $p < 0.01$). The data of the relationship between icilin concentrations and current responses were fitted using the following equation after normalization by the current amplitude in the absence of icilin (see Figs. 4B and 5): $I_{\text{Icilin}}/I_{\text{Control}} = (I_{\text{max}}/I_{\text{Control}}) / \{1 + (K_d/[I_{\text{Icilin}}])^n\}$, where I_{max} is the maximum amplitude of the icilin-evoked current, K_d is the apparent dissociation constant of icilin, $[I_{\text{Icilin}}]$ is the concentration of icilin, and n is the Hill coefficient.

Results

Activation of hENaC $\delta\beta\gamma$ current by icilin

The effects of icilin, a class of compounds known as tetrahydropyrimidine-2-one derivatives (Wei and Seid, 1983), on the hENaC $\delta\beta\gamma$ current were examined using a two-electrode voltage clamp technique in the *Xenopus* oocyte expression system. When the hENaC $\delta\beta\gamma$ heteromultimer was expressed in *Xenopus* oocytes, an inward current was induced at a holding potential of -60 mV, and the current was mostly blocked by 100 μ M amiloride (Fig. 1A). The mean amplitude of the amiloride-sensitive current in hENaC $\delta\beta\gamma$ -expressing oocytes was 575 ± 27 nA ($n=29$, $p<0.01$ vs. native of 3 ± 1 nA, $n=6$). In hENaC $\delta\beta\gamma$ -injected oocytes, the application of 100 μ M icilin was markedly increased in the inward current (1.79 ± 0.04 -fold, $n=23$, $p<0.01$; Fig. 1C). The current increase by icilin was recovered to the resting level by the removal of icilin in all hENaC $\delta\beta\gamma$ -expressing oocytes tested ($n=15$). After washout for a few minutes, re-administration of icilin caused current activation with a similar amplitude and kinetics to the first challenge ($n=6$). The current-voltage relationship showed that the application of 100 μ M icilin potentiated channel activity at all voltages examined in hENaC $\delta\beta\gamma$ -expressed oocytes ($n=5$; Fig. 1B). On the other hand, in native oocytes, the application of 100 μ M icilin did not induce any current (1.01 ± 0.01 -fold, $p>0.05$, $n=6$).

It was examined whether the activation of the inward current by icilin was mediated through either ENaC δ alone or the accessory β or γ subunit. In homomeric hENaC δ -expressing oocytes, the application of 100 μ M icilin significantly elicited an inward current (1.22 ± 0.03 -fold, $n=5$, $p<0.01$; Fig. 1C), as well as in hENaC $\delta\beta\gamma$ -injected oocytes. Furthermore, we tested whether icilin was effective on another ENaC core unit, the α -subunit,

in *Xenopus* oocytes. In contrast to hENaC δ - and $\delta\beta\gamma$ -injected oocytes, the application of 100 μ M icilin had no effect on the inward currents in hENaC α -expressing oocytes (0.99 ± 0.01 -fold, $n=8$, $p>0.05$), and the hENaC $\alpha\beta\gamma$ current was significantly inhibited by 100 μ M icilin (0.96 ± 0.01 -fold, $n=9$, $p<0.01$).

Effects of amiloride on icilin-induced current

To confirm whether the icilin-induced current originated from the ENaC $\delta\beta\gamma$ expression, the effects of amiloride, an inhibitor of ENaC, on the inward current in the presence of icilin were examined. The 100 μ M icilin-induced current in hENaC $\delta\beta\gamma$ -injected oocytes (1421 ± 76 nA, $n=6$) was dramatically inhibited by the addition of 100 μ M amiloride (85 ± 3 % decrease, $n=6$, $p<0.01$) and, moreover, significantly reduced the current to 209 ± 23 nA ($n=6$, $p<0.01$ vs. the initial resting current of 785 ± 55 nA; Fig. 2). On the other hand, in native oocytes, the current amplitudes after the application of 100 μ M icilin in the absence and presence of 100 μ M amiloride (72 ± 1 nA and 69 ± 1 nA, respectively, $n=6$) were not significant to the initial resting current (70 ± 1 nA, $n=6$, $p>0.05$).

Effects of external Na⁺ removal on icilin-evoked current

In turn, the effects of external Na⁺ removal were analyzed in hENaC $\delta\beta\gamma$ -expressing oocytes. The Na⁺-free solution was prepared by the substitution of 96 mM Na⁺ in recording solution with the equivalent NMDG. In the absence of external Na⁺, the amiloride-sensitive current was significantly small (14 ± 4 nA, $n=5$, $p<0.01$ vs. before the removal of external Na⁺; Fig. 3). The current increase by the application of 100 μ M icilin was significantly reduced by removal of external Na⁺ (39 ± 14 nA, $n=5$, $p<0.01$ vs. 96 mM [Na⁺]_o of 599 ± 23 nA, $n=5$).

Dose-dependence of current activation by icilin

The concentration dependency of the icilin-induced current was examined in hENaC $\delta\beta\gamma$ -expressed oocytes. In hENaC $\delta\beta\gamma$ -expressed oocytes, changing the concentration of icilin in the range of 1 to 1000 μM showed that the inward current was significantly increased by icilin at a concentration of 10 μM and more ($n=5$, $p<0.01$ vs. control of 743 ± 66 nA), and the enhancement was in a concentration-dependent manner (1547 ± 143 nA at 1000 μM , $n=5$; Fig. 4). The EC_{50} value of icilin on the inward currents was 33 ± 1 μM , and the Hill coefficient was 1.3 ± 0.1 ($n=5$).

Sensitization by protons and capsazepine on icilin-activated hENaC $\delta\beta\gamma$ current

We reported previously that ENaC $\delta\beta\gamma$ activity was enhanced by external protons or capsazepine in the *Xenopus* oocyte expression system (Yamamura et al., 2004a, 2004b). Therefore, the effects of icilin on the weak acidification of pH 7.0 medium, which was the subthreshold concentration of protons on hENaC $\delta\beta\gamma$ current (by 23 ± 2 nA, $n=5$), were examined in hENaC $\delta\beta\gamma$ -expressed oocytes (Fig. 5A). The decrease in external pH from 7.5 to 7.0 caused a left shift of the dose-response curve for icilin on the inward current to the EC_{50} value of 16 ± 2 μM ($n=5$, $p<0.01$ vs. pH 7.5) and Hill coefficient of 1.2 ± 0.1 . The maximum response of the icilin-induced current during the exposure to pH 7.0 (by 871 ± 144 nA, $n=5$) was not statistically significant to that in pH 7.5 medium (by 804 ± 110 nA, $n=5$, $p>0.05$). Alternatively, the effects of icilin in the presence of 1 μM capsazepine, which by itself had a very small effect on hENaC $\delta\beta\gamma$ current (by 120 ± 6 nA, $n=5$), were examined in hENaC $\delta\beta\gamma$ -injected oocytes (Fig. 5B). The dose-response curve for icilin on the inward currents was shifted to the left with an EC_{50} value of 12 ± 1 μM ($n=5$, $p<0.01$) and Hill coefficient of 1.2 ± 0.1 . The maximum amplitude of the icilin-evoked current in the presence

of 1 μ M capsazepine (by 873 ± 44 nA, n=5) was not statistically significant compared with that in the absence of capsazepine ($p > 0.05$).

Discussion

Amiloride-sensitive ENaCs, members of the degenerin/ENaC superfamily, regulate essential control elements for Na⁺ homeostasis in cells and across epithelia. Since the ENaC $\alpha\beta\gamma$ complex is expressed mainly in epithelia such as the kidney, lung, and colon to play a pathophysiological role, the physiological and pharmacological characterization has been well documented (Alvarez de la Rosa et al., 2000; Kellenberger and Schild, 2002). Alternatively, the physiological function of the δ -subunit has not yet been identified. More recently, we have shown that ENaC δ is widely distributed throughout the brain and is activated by protons, indicating that it may act as a pH sensor in the human brain (Yamamura et al., 2004a). In addition, we have demonstrated that capsazepine is the first chemical agonist for ENaC δ (Yamamura et al., 2004b). In this investigation, we have found that the application of icilin, which is a tetrahydropyrimidine-2-one derivative unrelated structurally to capsazepine, activates hENaC $\delta\beta\gamma$ in a concentration-dependent manner, and the enhancement is sensitive to amiloride.

When the heteromultimeric hENaC $\delta\beta\gamma$ complex was expressed in *Xenopus* oocytes, the application of icilin at a concentration of 10 μ M and more was markedly increased by an inward current. Since the icilin-induced current was significantly abolished by the addition of 100 μ M amiloride, an inhibitor of ENaC, or by the removal of external Na⁺ in hENaC $\delta\beta\gamma$ -expressing oocytes, and icilin-elicited currents were not observed in native oocytes, the icilin-evoked currents originated from the ENaC $\delta\beta\gamma$ expression. The icilin-induced current was maintained at a steady level during icilin exposure in hENaC $\delta\beta\gamma$ -expressed oocytes (~5 min; see Fig. 2A). After washout for a few minutes, the sequential challenge of icilin causes current activation to the same extent as the first trial in hENaC $\delta\beta\gamma$ -expressing oocytes. These results indicate that icilin did not induce sensitization

and desensitization to the activity of hENaC $\delta\beta\gamma$ under these conditions, similarly to protons and capsaizepine, which are also activators of ENaC δ (Yamamura et al., 2004a, 2004b; Ji and Benos, 2004). In addition to the enhancement actions by icilin on TRPM8 and TRPA1 at micromolar concentrations (McKemy et al., 2002; Story et al., 2003; Andersson et al., 2004; Chuang et al., 2004), in this investigation, we have clarified that icilin activates hENaC $\delta\beta\gamma$ with an EC₅₀ of 33 μ M.

The sensitivity to icilin was increased in a weak acidic medium of pH 7.0. Protons activated the hENaC δ current, but pH 7.0 is the subthreshold concentration of protons in the hENaC $\delta\beta\gamma$ current (Yamamura et al., 2004a, 2004b; Ji and Benos, 2004). At this proton concentration, the dose-response for icilin on the inward currents was enhanced, indicating that the effect of icilin was sensitized by the addition of protons. Moreover, the concentration dependency of hENaC $\delta\beta\gamma$ for icilin was shifted to the left by the lower concentration of capsaizepine (1 μ M), which by itself had a very small effect on hENaC $\delta\beta\gamma$ current (Yamamura et al., 2004b). Similar shifts in sensitivity by the mixture of two different ligands were observed: protons/capsaicin, capsaicin/2-aminoethoxydiphenyl (2-APB), and protons/2-APB in TRPV1 (Caterina et al., 1997; Hu et al., 2004) as well as protons/capsaizepine in ENaC $\delta\beta\gamma$ (Yamamura et al., 2004b). These results indicate that icilin acts synergistically with protons or capsaizepine, activating factors of ENaC δ . As the structural similarity between icilin (1-(2-hydroxyphenyl)-4-(3-nitrophenyl)-1,2,3,6-tetrahydropyrimidin-2-one) and capsaizepine (N-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide) appeared to be low, it is still unknown whether the mechanism of channel activation by icilin is similar to that by protons or capsaizepine. Interestingly, icilin, capsaizepine, and protons all activate ENaC δ but all partially inhibit ENaC α (Chalfant et al., 1999; Ji and Benos, 2004; Yamamura et al., 2004a, 2004b), which

may be a key point for elucidating the mechanism underlying channel modulation. These findings provide useful information for drug development in the degenerin/ENaC superfamily.

Recently, acid-sensing ion channel 1a (ASIC1a) in the central nervous system has been implicated in long-term potentiation, suggesting that minute fluxes in synaptic pH may activate a proton-sensitive channel to enhance synaptic plasticity, learning, and memory (Bianchi and Driscoll, 2002; Wemmie et al., 2002). This raises the possibility that ENaC δ could also play a role in learning and memory in the human brain (Yamamura et al., 2004a). Interestingly, the expressed sequence tag and genome project databases show that an ENaC δ gene has been found only in humans and chimpanzees (GenBank accession numbers U38254 and O46547, respectively), and there is no evidence of the orthologues in rats and mice. The corresponding genomic assignments of ENaC δ were identified on human chromosome 1p36.3-p36.2 (Waldmann et al., 1996). As the chemical agonists strongly influencing ENaC δ have been poorly investigated, icilin and capsaizepine are potentially powerful tools for the electrophysiological analysis of ENaC δ and the elucidation of the clinical ENaC δ function in humans.

In conclusion, we found that icilin acts on ENaC δ , and causes the activation of ENaC δ , indicating that icilin is a novel activator of ENaC δ . In addition to the physiological function of ENaC δ as a pH sensor in the human brain (Yamamura et al., 2004a), this finding provides a starting point for a number of exciting follow-up investigations into the physiological and pathological roles of ENaC δ *in vitro* and *in vivo* in humans.

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Footnotes

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Figure legends

Fig. 1. Activation of hENaC $\delta\beta\gamma$ current by icilin

Whole-cell currents were recorded at a holding potential of -60 mV in the *Xenopus* oocyte expression system using a two-electrode voltage clamp technique. **A**, a hENaC $\delta\beta\gamma$ -expressed oocyte possessed a larger inward current than a native oocyte. The larger current in a hENaC $\delta\beta\gamma$ -expressing oocyte was mostly inhibited by 100 μ M amiloride (*Ami*). In a hENaC $\delta\beta\gamma$ oocyte, the application of 100 μ M icilin enhanced the inward current, and this current increase was recovered by the removal of icilin. After washout for a few minutes, a re-administration of icilin caused similar current activation. Note that neither the application of icilin nor amiloride induced any current in native oocytes. **B**, the current-voltage relationships in the absence and presence of 100 μ M icilin in native and hENaC $\delta\beta\gamma$ -injected oocytes are shown. Icilin stimulus enhanced hENaC $\delta\beta\gamma$ activity at all voltages examined. **C**, the effects of 100 μ M icilin on homomeric and heteromeric hENaCs expressed in *Xenopus* oocytes are summarized. The icilin-induced current was normalized by the current amplitude in the absence of icilin. The number of oocytes used is in parentheses. The statistical significance of the difference is expressed as $p < 0.01$ (**). vs. control.

Fig. 2. Effects of amiloride on icilin-induced current

The effects of amiloride on the inward current evoked by icilin were examined in *Xenopus* oocytes. **A**, a typical current trace in a hENaC $\delta\beta\gamma$ -expressing oocyte in response to icilin and amiloride (*Ami*) is represented. The current increase by 100 μ M icilin was dramatically influenced by the addition of 100 μ M amiloride. **B**, the effects of 100 μ M icilin in native (*open*) or hENaC $\delta\beta\gamma$ -expressed (*closed*) oocytes in the absence and presence of 100 μ M amiloride are summarized. Neither the application of icilin nor amiloride induced any

current in native oocytes. Experimental data were obtained from 6 oocytes for each. The statistical significance of the difference is expressed as $p < 0.01$ vs. control (**) and $p < 0.01$ vs. icilin alone (##).

Fig. 3. Effects of external Na^+ removal on icilin-evoked current

The effects of external Na^+ removal on the inward current elicited by icilin were examined in *Xenopus* oocytes. **A**, a typical current trace in a hENaC $\delta\beta\gamma$ -injected oocyte in response to icilin and amiloride (*Ami*) in the absence and presence of external Na^+ is shown. The external Na^+ -free was prepared by the substitution of 96 mM Na^+ in the recording solution with the equivalent NMDG. In the absence of external Na^+ , the current amplitude evoked by 100 μM icilin was smaller than that in the presence of external Na^+ . **B**, the effects of 100 μM icilin in hENaC $\delta\beta\gamma$ -expressed oocytes in the absence (*hatched*) and presence (*closed*) of external Na^+ are summarized. Experimental data were obtained from 5 oocytes for each. The statistical significance of the difference is expressed as $p < 0.01$ vs. 96 mM $[\text{Na}^+]_o$ (**).

Fig. 4. Dose-dependence of current activation by icilin

The dependency on icilin concentration of the hENaC $\delta\beta\gamma$ current was analyzed in *Xenopus* oocytes. **A**, a typical current trace of icilin responsiveness from 1 to 1000 μM in a hENaC $\delta\beta\gamma$ -injected oocyte is represented. The activation of an inward current by icilin was observed in a concentration-dependent manner. **B**, icilin sensitivity in hENaC $\delta\beta\gamma$ currents is plotted. The icilin-induced current was normalized by the current amplitude in the absence of icilin. The inward current was significantly potentiated by icilin at a concentration of 10 μM and more in hENaC $\delta\beta\gamma$ -expressed oocytes ($p < 0.01$). The EC_{50} value for icilin on the inward currents was 33 ± 1 μM , and the Hill coefficient was 1.3 ± 0.1 . Experimental data were obtained from 5 oocytes.

Fig. 5. Sensitization by protons and capsazepine on icilin-activated hENaC $\delta\beta\gamma$ current

The effects of protons and capsazepine on the icilin-activated hENaC $\delta\beta\gamma$ current in *Xenopus* oocytes were examined. **A**, in hENaC $\delta\beta\gamma$ -injected oocytes, the decrease in pH from 7.5 to 7.0, which is the subthreshold concentration of protons on the hENaC $\delta\beta\gamma$ current, caused a left shift of the dose-response curve for icilin on the inward current to an EC₅₀ value of 16±2 μM and Hill coefficient of 1.2±0.1. **B**, in the presence of 1 μM capsazepine, which by itself had a very small effect on hENaC $\delta\beta\gamma$ current, the dose-response curve for icilin on inward currents was shifted to the left with an EC₅₀ value of 12±1 μM and Hill coefficient of 1.2±0.1. The icilin-induced current was normalized by the current amplitude in the absence of icilin. The dotted line indicates a dose-response curve of icilin alone on the inward current obtained from Fig. 4B. Experimental data were obtained from 5 oocytes for each.

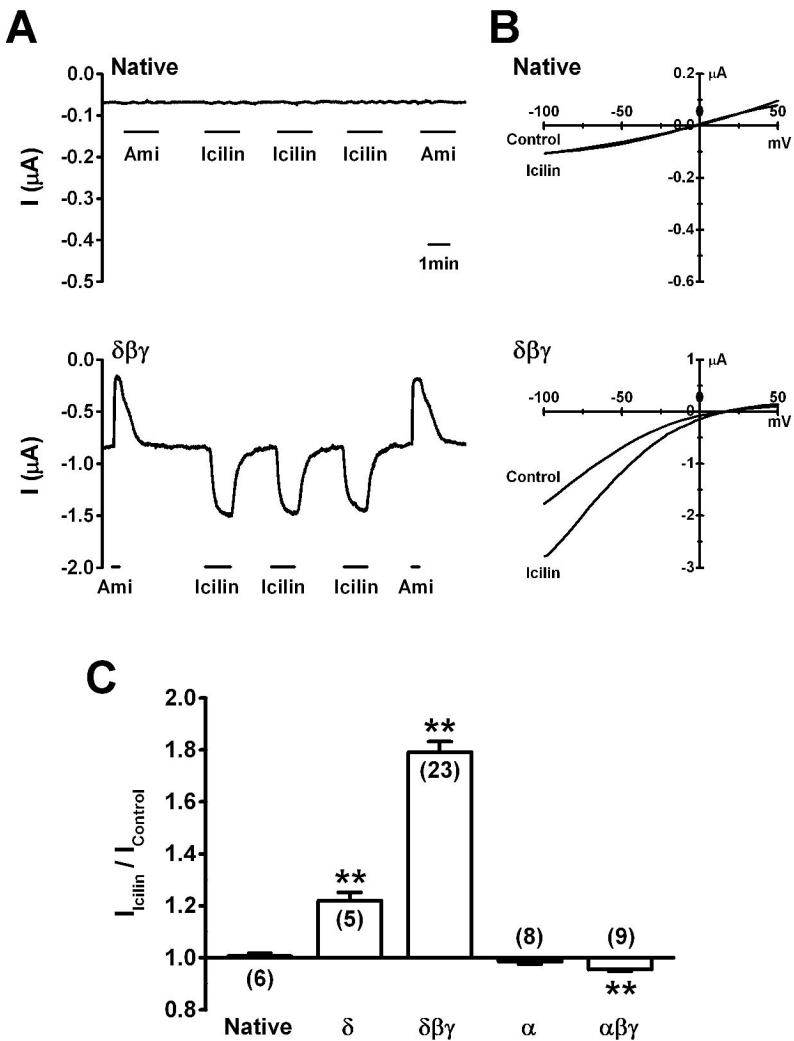


Figure 1

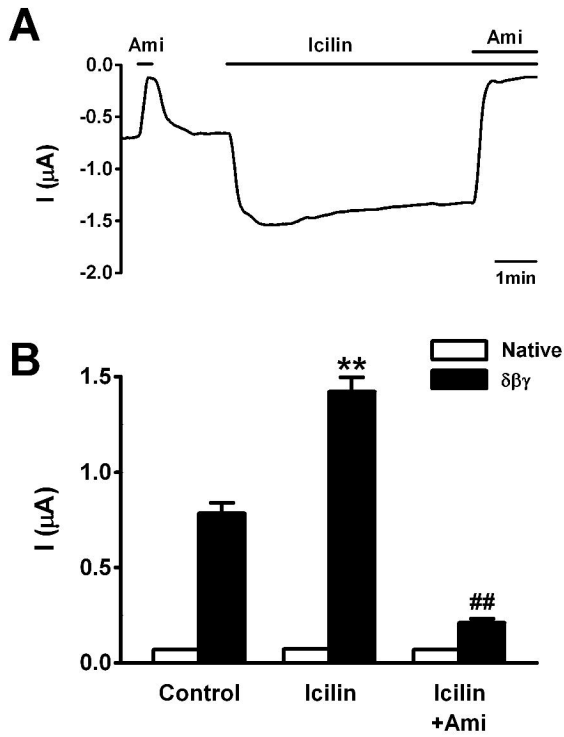


Figure 2

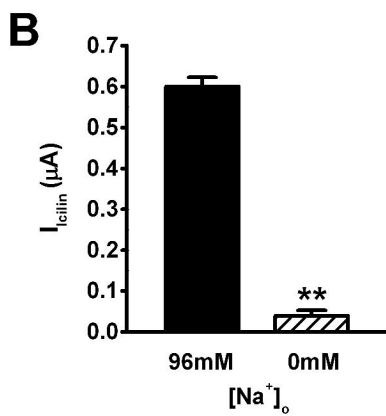
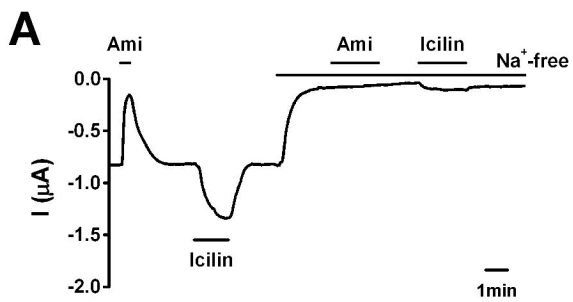


Figure 3

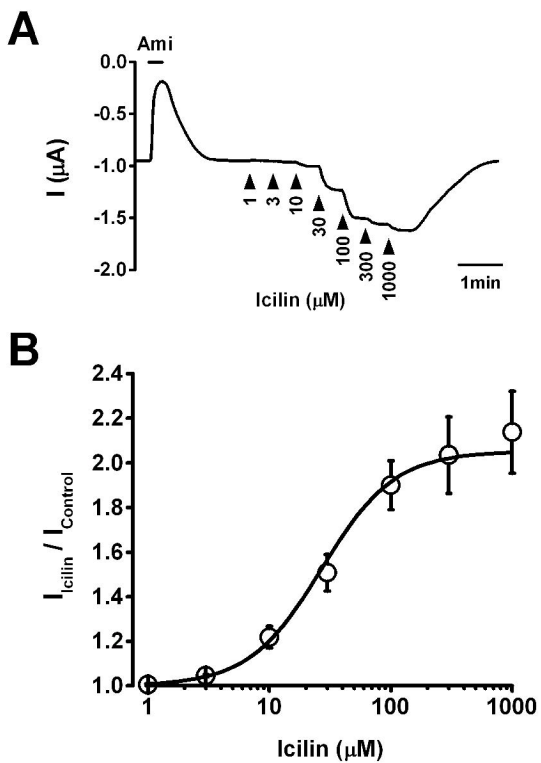


Figure 4

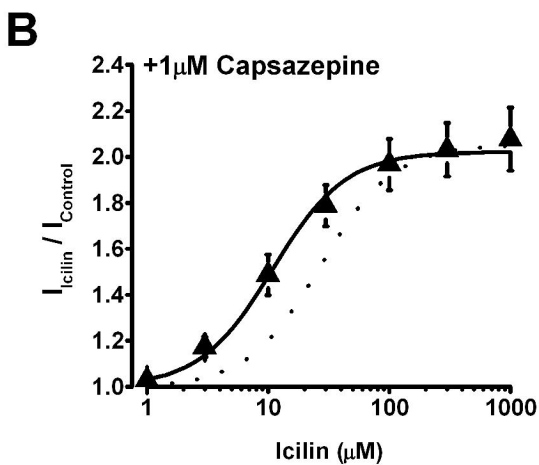
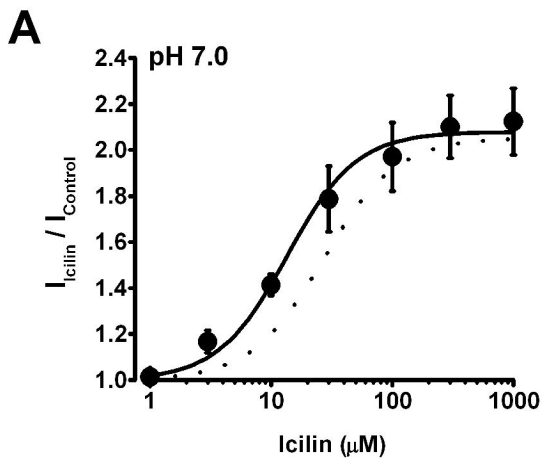


Figure 5