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Rapid report

Epidermal growth factor increases i_f in rabbit SA node cells by activating a tyrosine kinase

Ji-Ying Wu, Hangang Yu, Ira S. Cohen *

Department of Physiology and Biophysics, Health Sciences Center, SUNY at Stony Brook, Stony Brook, NY 11794-8661, USA

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Abstract

Our previous results have demonstrated that tyrosine kinase inhibition reduces i_f in rabbit SA node myocytes, suggesting that tyrosine kinases regulate i_f. One receptor tyrosine kinase the EGF receptor kinase is known to increase heart rate. To determine if this action is mediated through changes in i_f, we examined the effect of epidermal growth factor (EGF) on i_f with the permeabilized patch-clamp technique. 0.1 μM EGF increased i_f amplitude in response to single-step hyperpolarizations in the diastolic range of potentials. This increase was 20 ± 3%, $n = 11$ at −75 mV. This effect is caused by activating a tyrosine kinase because 50 μM genistein, a tyrosine kinase inhibitor, eliminated this EGF action. A two-step pulse protocol showed that maximal i_f conductance was increased by EGF. We further examined this conductance change by constructing the activation curve. The maximal i_f conductance was increased by 23% with no change in midpoint, $V_{1/2}$, control = −74 ± 2 mV, $V_{1/2}$ EGF = −74 ± 1 mV. Thus EGF acts via a tyrosine kinase to increase maximal i_f conductance with no change in the voltage dependence of activation. These results suggest that EGF effects on i_f contribute to the positive chronotropic effect of EGF on SA node.

Keywords: Tyrosine kinase; Epidermal growth factor; Genistein; i_f

Protein kinases play important roles in intracellular signalling pathways [1]. Serine/threonine kinases phosphorylate and modulate transmembrane ionic channels in heart [1–3]. Recent studies indicate that tyrosine kinases also play a crucial role in modulation of neurotransmitter receptors and transmembrane ionic channels [4]. Although tyrosine kinases are associated with relatively long-term cellular processes like proliferation, differentiation and metabolism [5,6], activation of tyrosine kinases also contribute to a wide variety of cellular functions [7] including ion channel modulation [8].

In a previous study [9], we have shown that tyrosine kinase inhibition reduces i_f in rabbit SA node myocytes. Our results strongly suggest that tyrosine phosphorylation can be an important component of i_f regulation. However, these results did not demonstrate whether the tyrosine kinase activity is controlled by a ligand which binds to a so-called receptor tyrosine kinase increasing its activity or whether regulators exist in the cytosol. To attempt to discriminate between these two alternatives, we examined the effect of epidermal growth factor (EGF) on i_f. The reasons we selected EGF for our study were (1) the EGF receptor is the best understood growth factor receptor protein tyrosine kinase [7], and (2) EGF is known to produce positive chronotropic effects in

* Corresponding author. Fax: +1-516-444-3432;
E-mail: icohen@physiology.pnb.sunysb.edu

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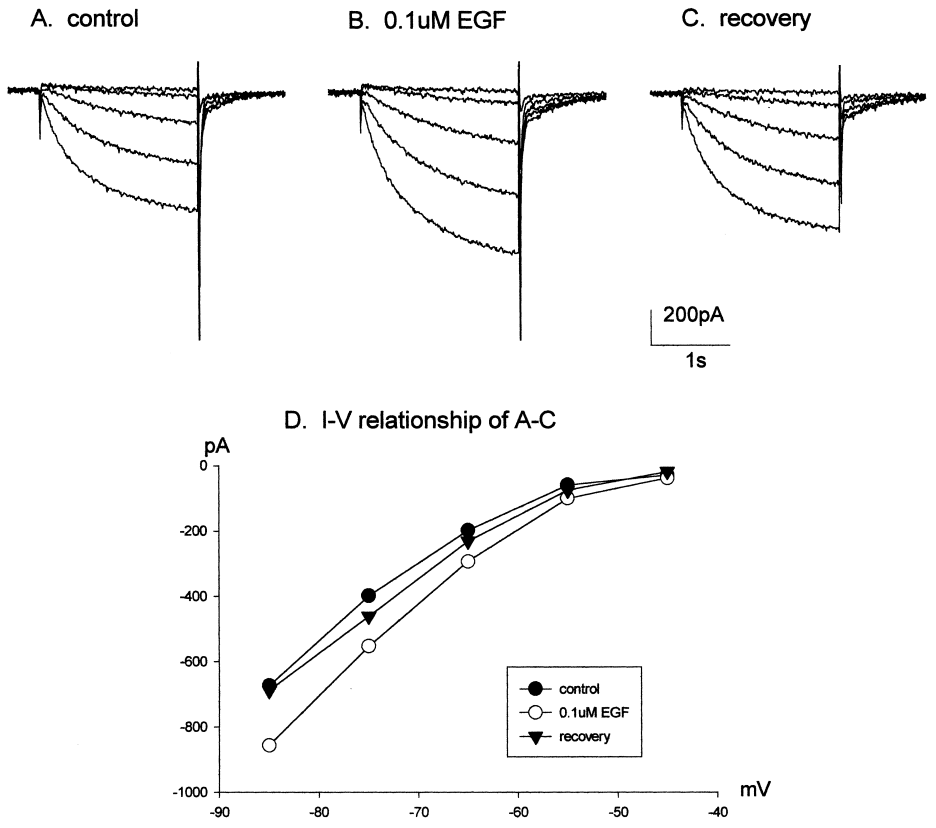

Fig. 1. The effects of EGF on i_f in rabbit sinus node myocytes studied with the permeabilized patch technique. The cell was held at −35 mV and hyperpolarized in 10-mV increments to a maximum value of −75 mV. (A) i_f recorded in control solution; (B) currents recorded in the presence of 0.1 μM EGF; (C) washout EGF. (D) The *I–V* relationship for A to C.

cultured ventricular myocytes [10] and in isolated perfused rat hearts [11].

Isolated rabbit SA node myocytes were prepared as previously described by standard techniques [12]. Tyrode solution contained (mM): NaCl, 137.7; NaOH, 2.3; $MgCl_2$, 1; glucose, 10; Hepes, 5; KCl, 5.4; $CaCl_2$, 1.8; $MnCl_2$, 2 and $CdCl_2$, 0.2; pH 7.4. Pipette solution contained (mM): NaCl, 6; potassium aspartate, 130; $MgCl_2$, 2; $CaCl_2$, 5; EGTA, 11; Na_2-ATP, 2; Na-GTP, 0.1; Hepes, 10; pH 7.2 (titrated with 3 M KOH). The amphotericin B perforated patch technique was used to prevent 'rundown' of i_f. The pipette was filled with pipette solution containing amphotericin B (240 μg/ml) which was prepared just before the experiments [13]. The pipette resistance when filled with pipette solution was 6–8 MΩ. The isolated cells were placed in a temperature-controlled lucite bath (35 ± 0.5°C). An Axopatch 1D amplifier was employed.

Epidermal growth factor (EGF) and genistein were purchased from Sigma Chemical (St. Louis, MO, USA) and were added to the control solution in the concentrations indicated below. Genistein was dissolved in DMSO and diluted 2000 times in Tyrode solution.

Our previous observations demonstrated that tyrosine phosphorylation modulates i_f [9]. Here we examine the effect of EGF, which activates the intrinsic protein tyrosine kinase activity of its receptor [14], on i_f current.

Fig. 1 shows records of i_f in control solution, in the presence of 0.1 μM EGF and after washout. We employed a single hyperpolarizing voltage step of 1.5 to 3 s in duration from a holding potential of −35 mV to a maximum of −125 mV in 10-mV increments. The increase in i_f amplitude was observed about 5–10 min after the addition of 0.1 μM EGF (Fig. 1B), and this effect was reversible (Fig. 1C). In a total of 11 experiments EGF increased i_f by an average of 20 ± 3% at −75 mV (mean ± S.E.M.).

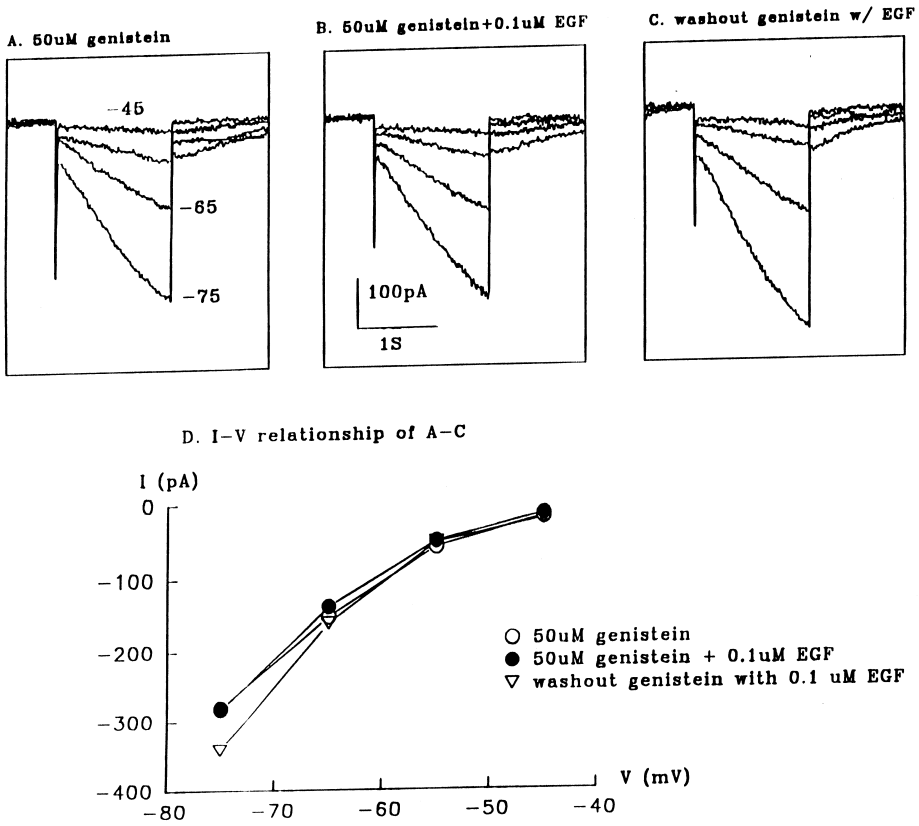

Fig. 2. Effect of 0.1 μM EGF on i_f in the presence of genistein. The holding potential was −35 mV, the voltage steps were −45 mV to −75 mV in 10-mV increments. (A) In the presence of 50 μM genistein; (B) 0.1 μM EGF plus 50 μM genistein; (C) washout of genistein with EGF-containing solution. (D) The *I–V* relationship for A to C.

In order to determine whether the increase in i_f produced by EGF was due to activation of a tyrosine kinase, the tyrosine kinase inhibitor, genistein (50 μM), was employed. Fig. 2 provides one example from these experiments. In the presence of 50 μM genistein, EGF decreased i_f at −75 mV by 7±4% ($n=4$). On washout of genistein, i_f was increased by 0.1 μM EGF (Fig. 2C). i_f at −75 mV was 32±1%, $n=3$, larger after washout of genistein. These data suggest that the action of EGF on i_f was mediated through the activation of a tyrosine kinase.

To determine if the increase of i_f amplitude by EGF is caused by a change in i_f conductance (as indicated by tyrosine kinase inhibition in our previous study [9]), a two-step pulse protocol was employed. The holding potential was −35 mV, a first hyperpolarizing pulse was applied to −75 mV which is near the middle of the i_f activation range, a second hyperpolarizing pulse was then applied to −110 mV near the top of the activation curve. We can see from Fig. 3 that addition of 0.1 μM EGF increased i_f in response to both hyperpolarizing clamp pulses. Similar results were observed in a total of five experiments. These data suggest an increase in maximal i_f conductance but do not rule out a concomitant positive shift of i_f activation. In order to further investigate this question we constructed activation curves for i_f. We hyperpolarized to various potentials within the activation range and recorded the tail currents on return to the holding potential (see Fig. 4). We plotted the amplitude of the tail currents against the test potential. The results from four cells are shown in Fig. 4. The control data were normalized to a maximal conductance of unity. The data was fit to a Boltzmann two-state model. The half-activation voltages were −74±2 in control and −74±1 in EGF. The slope factor was 11.2±1.3 mV in control and 10.7±0.7 mV in EGF. The conductance was 1.23 times the control conductance in EGF.

We also examined the kinetics of activation in control and in the presence of EGF at voltages between −65 mV and −105 mV. No change in the time constant was observed at any test potential consistent with the absence of a shift in the voltage dependence of activation.

The present study demonstrates for the first time that EGF activates i_f through a tyrosine kinase in rabbit SA node myocytes.

The evidence to date strongly suggests that tyrosine phosphorylation is an important component of ion channel modulation [8]. EGF is one of the receptor tyrosine kinases which is known to act by binding to its receptor and increasing its intrinsic receptor protein tyrosine kinase activity, resulting in phosphorylation of a number of cellular proteins [7,15,16]. The activity of this receptor tyrosine kinase can be inhibited by genistein, one of the specific tyrosine kinase inhibitors [17].

Several reports have shown that genistein's ability to inhibit responses to growth factors correlates with a decrease in the level of tyrosine phosphorylation [18]. Our observations indicate that the increase in amplitude of i_f induced by EGF requires activation of its tyrosine kinase because cells pretreated with 50 μM genistein exhibited no i_f increase on exposure to EGF. Since EGF binds to its receptor to exert its biological actions, the tyrosine kinase activated by EGF is most likely its receptor protein tyrosine kinase. These results are consistent with observations that EGF produces positive chronotropic effects in cultured ventricular myocytes (1.6 μM) [10] and in isolated perfused rat hearts (15 nM) [11] by activating the EGF receptor tyrosine kinase.

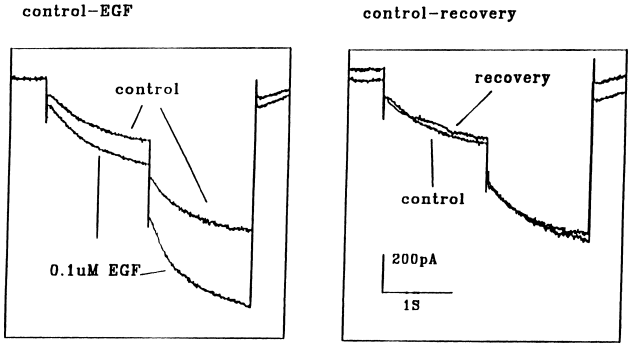

Fig. 3. EGF alters i_f conductance. A two-step pulse protocol was used: the holding potential was −35 mV, the first pulse was to −75 mV which was near the middle of the i_f activation curve, the second pulse was to −110 mV which was near the top of the activation curve. (Left) Currents recorded in control and in solution containing 0.1 μM EGF. (Right) Washout of EGF resulted in recovery of i_f near control levels.

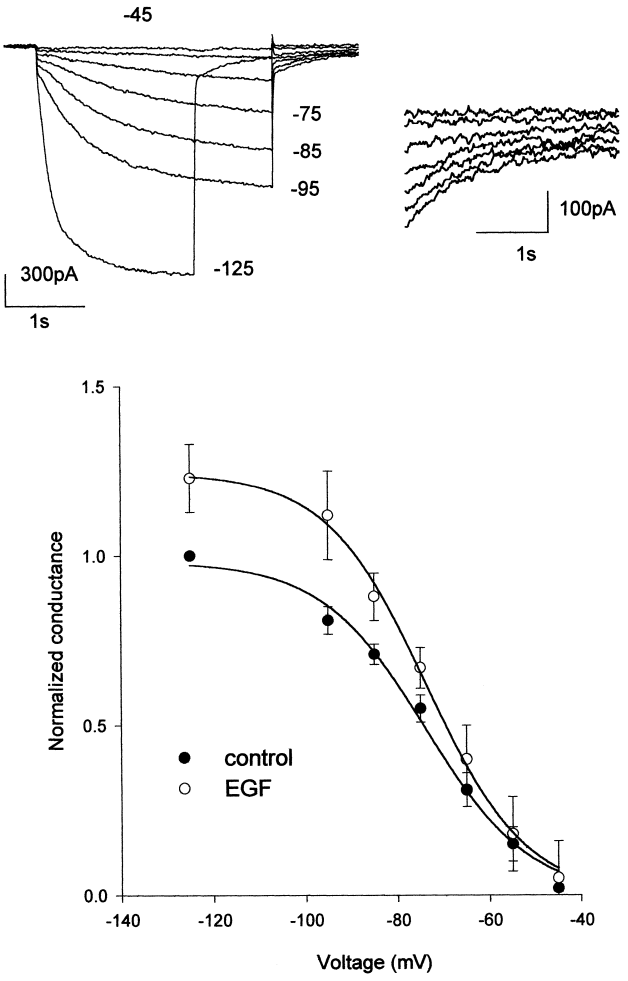

Fig. 4. i_f activation curves in control and in the presence of 0.1 μM EGF. (Upper) i_f currents were elicited upon hyperpolarization to various voltages from a holding potential of −35 mV then followed by deactivating pulses to −35 mV. (Lower) Normalized conductances of control and EGF obtained from the tail current amplitudes shown in the inset of the upper panel. The data were fit to a Boltzmann two-state model as in [24].

Our results also provide conclusive data indicating the mechanism of EGF stimulation. Two different experimental approaches (construction of activation curves and the two-pulse protocol) suggested the same conclusion that the effect of EGF is primarily on maximal i_f conductance. These data are consistent with previous work from our laboratory which demonstrated that tyrosine kinase inhibition reduces i_f conductance.

Recently the molecular correlate of i_f was identified [19–21]. The family of i_f ion channel subunits was called HCN (for hyperpolarization-activated cyclic nucleotide gated). There are four members of this family, three of which are present in SA node [22]. The initial cloning was achieved by homology to src [23], suggesting the potential importance of tyrosine phosphorylation. Given our previous study with tyrosine kinase inhibition [9] and the present results with EGF, it is clear that tyrosine kinases regulate i_f. However, much remains uncertain about the second-messenger pathways involved. The intervening steps in this regulation remain fruitful areas for future investigation.

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