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Activation of endogenous c-Src or a related tyrosine kinase by intracellular (pY)EEI peptide increases voltage-operated calcium channel currents in rabbit ear artery cells

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Abstract The effect of activation of endogenous c-Src tyrosine kinase by (pY)EEI peptide was examined on voltage-operated calcium channel (VOC) currents in arterial smooth muscle cells. In single rabbit ear artery cells intracellular application of (pY)EEI peptide increased calcium channel currents. Inactive, non-phosphorylated YEEI peptide had no effect on currents. Peptide-A, a 21 amino acid inhibitor of c-Src inhibited currents and prevented the effect of (pY)EEI peptide on calcium channel currents. These results indicate that activation of intrinsic c-Src increases VOC and support a role for c-Src in the regulation of VOC in vascular smooth muscle cells.

Key words: Voltage-operated calcium channel; Vascular smooth muscle; c-Src tyrosine kinase; Tyrosine phosphorylation

1. Introduction

Voltage-operated calcium channels (VOC) are a major route of calcium entry into the vascular smooth muscle cell [1]. Recently it has been proposed that tyrosine phosphorylation by receptor and non-receptor tyrosine kinases may be an important mechanism by which VOCs are regulated in vascular smooth muscle [2,3]. The product of the cellular src protooncogene is a non-receptor tyrosine kinase of 60 kDa known as c-Src that is widely distributed in many cells including smooth muscle cells and is the cellular counterpart of the product of the Rous sarcoma virus oncogene (v-src) [4]. c-Src is found in association with the cytoskeleton [5] and concentrated around cellular attachment sites to the extracellular matrix, such as focal adhesions [6]. We have shown previously that intracellular application of human c-Src increased VOC currents in rabbit ear artery cells [3] indicating that exogenous c-Src could modulate channel function. Peptide A, an inhibitor of c-Src, inhibited the effects of c-Src and also reduced VOC currents in the absence of exogenous c-Src [3] suggesting that endogenous c-Src may regulate the availability of VOC in these cells. The finding of high levels of c-Src in smooth muscle [7] is consistent with this proposal.

The activity of cellular c-Src is regulated by a variety of stimuli through tyrosine phosphorylation and dephosphorylation reactions of c-Src itself. An important tyrosine residue found in the kinase domain of the c-Src molecule is Tyr^{416}, the phosphorylation of which is essential for activation of c-Src [8]. A conserved carboxyl-terminal tyrosine residue, Tyr^{527}, is also important for regulation, phosphorylation of which results in inhibition of c-Src enzyme activity [9,10]. Such phosphorylation-dependent inhibition appears to be a general feature of members of the Src family tyrosine kinases such as Lck and Fyn [11,12], and is catalysed by another tyrosine kinase known as C-terminal Src kinase (CSK), which is not known to catalyse other tyrosine phosphorylation reactions [13]. Also essential for this negative regulatory mechanism is the non-catalytic N-terminal Src homology 2 (SH2) domain of the c-Src molecule [14] and also the SH3 domain [15]. The inhibition is due to an intramolecular reaction in which the Tyr^{527}-phosphorylated tail of c-Src associates with the SH2 domain of the same c-Src molecule in a 'closed' (inactive) conformation [16], inhibiting the catalytic domain and preventing interactions with other proteins [17]. However, this intramolecular association is a relatively weak interaction with only moderate affinity and specificity [18]. Therefore it is predicted that a protein with greater affinity to the Src SH2 domain than the c-Src tail, such as an activated growth factor receptor, would repel the c-Src tail from the SH2 domain and cause activation of c-Src [16].

c-Src SH2 domain is composed of approximately 100 amino acids and has the ability to bind specifically and with high affinity to tyrosine phosphorylated proteins [19]. The amino acid sequence pTyr-Glu-Glu-Ile ((pY)EEI) is known to bind with optimal affinity to Src SH2 domain [20]. A peptide containing this sequence has been shown to increase c-Src activity severalfold in vitro probably by competing with the c-Src tail [18] and represents a means of activating endogenous cellular c-Src.

Therefore the objective of the present study was to further investigate the role of endogenous c-Src in regulating VOC in arterial smooth muscle cells using the c-Src activating peptide (pY)EEI.

2. Materials and methods

Single smooth muscle cells were freshly dispersed from rabbit ear arteries by an enzymatic method [21]. Short (2–3 mm) segments of artery were incubated for 50 min in a modified physiological salt solution (PSS) containing (mM): NaCl 130, KCl 6, $CaCl_2$ 0.01, $MgCl_2$ 1.2, glucose 14, and HEPES 10.7 buffered to pH 7.2 with NaOH, 2 mg/ml bovine serum albumin, 1 mg/ml collagenase (130 U mg^{-1}), 0.5 mg/ml papain (15 U mg^{-1}) and 5 mM dithiothreitol. Cells were dispersed by mild agitation and resuspended after centrifugation in normal PSS containing 1.7 mM $CaCl_2$. Cells were stored on cover slips at 4°C and used within 4–6 h. Internal pipette solution contained (mM): NaCl 126, $MgCl_2$ 1.2, EGTA 2, MgATP 2, TEA 10, and HEPES 11 buffered to pH 7.2 with NaOH. Patch pipettes were fabricated from borosilicate glass and had a resistance of 3–5 MΩ.

Calcium channel currents were measured by the whole cell config-

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uration of the patch clamp technique [22] using a List EPC-7 amplifier and a Labmaster A/D interface board with commercially available software (Pclamp 5.5, Axon Instruments, CA, USA) on a IBM-compatible PC. The experiments were carried out in a 'high barium' solution ($BaCl_2$ 110 mM, HEPES 10 mM and buffered to pH 7.2 with TEA-OH) to increase the size of the inward current and to minimise calcium-dependent inactivation of currents [23]. Data were recorded on-line, or on digital audio tape using a DAT recorder (Biologic, France), and analysed off-line after analogue-to-digital conversion. The currents were digitally filtered at 2 kHz and leak currents were subtracted digitally, using average values of steady leakage currents elicited by a 10 mV hyperpolarising pulse [23]. All recordings were made at room temperature (22–25°C).

1 mM stock solutions of (pY)EEI (active) and YEEI (inactive) peptides were made up in distilled water. Stock solution of peptide A (400 μM) was made up in ethanol. The final concentration of ethanol used (1%) has been previously shown to have no effect on VOC [3]. All peptides were included in the intracellular pipette solution. A final concentration in excess of the reported EC_{50} values were used for peptide A and (pY)EEI peptides. To minimise batch to batch variations, the effects of (pY)EEI, YEEI and peptide A were compared in cells obtained from the same isolates.

2.1. Data analysis

To investigate the effect of (pY)EEI on the voltage dependence of activation and inactivation, activation and inactivation curves were calculated. The activation curve was derived from the current-voltage relationships. Conductance (g) was calculated from the equation: $g = I_{Ba}/(E_m - E_{rev})$, where I_{Ba} is the peak current elicited by depolarising test pulses to various test potentials (E_m). E_{rev} is the reversal potential estimated from the current-voltage curves. The conductance data was fitted to a Boltzman function: $g = 1/[1+\exp\{(E_m - V_h)/k\}]$, where V_h is the potential required for half activation and k is the slope factor.

The steady-state inactivation curve was obtained from a double-pulse protocol. The data were also fitted to a Boltzman equation: $I = [I_{max}/1+\exp\{E_m - V_h\}/k] + I_{non}$, where I is the peak current at any potential, I_{max} is the maximum peak current evoked by such a step, V_h is the potential at which current is half inactivated, k is a slope factor and I_{non} is the non-inactivating fraction of current. Data were fitted by non-linear regression analysing using Microsoft Excel.

Data are presented as means ± S.E.M of (n) observations. Comparisons of data were made using a Student's t-test for single paired or unpaired comparisons as appropriate. $P < 0.05$ was considered statistically significant.

2.2. Drugs and chemicals

Bovine serum albumin (essentially fatty acid free) and papain (Sigma, Dorset, UK), collagenase (Worthington, Reading, UK), peptide A (VAPSDSIQAEEWYFGKITRRE) (Novabiochem, Nottingham, UK), (pY)EEI peptide (EPQY(PO_3H_2)EEIPIYL) and YEEI (LC Laboratories, UK), all other chemicals from Sigma, Dorset, UK.

3. Results

In cells voltage clamped at a holding potential of −60 mV, the inclusion of (pY)EEI peptide (100 μM) in the pipette solution progressively increased calcium channel currents evoked every 1 s by a 20 ms step to +10 mV, from 58 ± 8 pA to reach a steady level of 90 ± 14 pA at around 2–3 min. This corresponds to a 55.1 ± 14% increase in current ($n = 6$, $P \leq 0.007$, Figs. 1 and 4). The increase in current was observed to begin within 1–2 min following establishing whole cell mode with a pipette solution containing (pY)EEI (Fig. 1). When peptide A (100 μM) was included in the pipette solution together with (pY)EEI peptide, (pY)EEI failed to increase calcium currents and the current was reduced by 21 ± 2% ($n = 5$, $P = 0.05$, Figs. 1 and 4). As we have previously reported, addition of peptide A alone also reduced currents (Fig. 4). The effects of (pY)EEI and peptide A together and peptide A alone did not differ significantly. Control YEEI peptide (non-phosphorylated) did not have any significant effect on the VOC currents when included in the pipette solution ($n = 5$, Figs. 1 and 4).

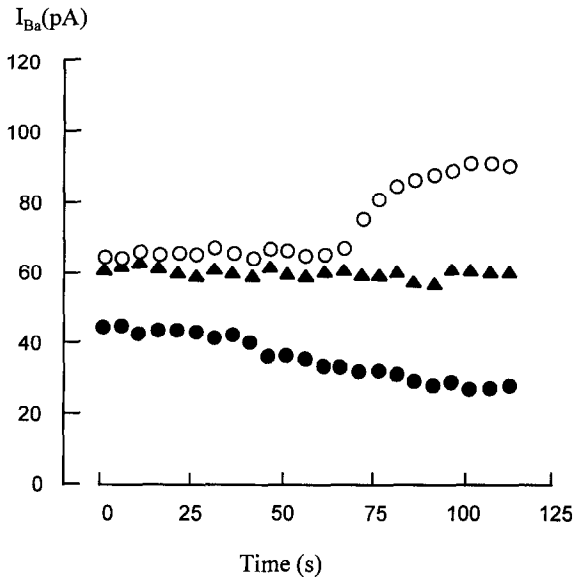

Fig. 1. Effect of activation of endogenous c-Src by (pY)EEI peptide on calcium channel currents. Peptides were contained in the intracellular pipette solution and calcium channel currents measured using whole cell voltage clamp techniques in a single ear artery cell with 110 mM Ba^{2+} as the charge carrier. Currents were evoked every 1 s following achieving whole cell mode using a 20 ms depolarising step to +10 mV from a holding potential of −60 mV. The figure shows calcium channel currents recorded from individual cells following establishment of whole cell mode. (○) (pY)EEI peptide 100 μM; (▲) YEEI peptide 100 μM; (●) (pY)EEI 100 μM in the presence of peptide A (100 μM). Results are representative of 5–8 similar experiments.

(pY)EEI peptide had no significant effect on the voltage dependence of the activation curve (Fig. 2). V_h was shifted negligibly from 7.6 ± 2 mV (control) to 9.6 ± 3 mV ((pY)EEI) ($n = 4$, $P = 0.4$). Slope = −11.3 ± 1 (control) and −8.1 ± 1 ((pY)EEI). Similarly (pY)EEI did not affect the voltage dependence of the inactivation curve significantly (Fig. 3); V_h was −18.2 ± 2 mV in control and −16.1 ± 6 mV following (pY)EEI ($n = 4$, $P = 0.3$) and slope = −13.7 ± 3 (control) and −10.7 ± 1 ((pY)EEI).

4. Discussion

Voltage-operated calcium channels are the major route of calcium entry into vascular smooth muscle cells and are involved in vascular contraction [1], cell migration and proliferation [24]. It is increasingly recognised that tyrosine phosphorylation by protein tyrosine kinases is important in regulating VOC in different cell types [25–27], and in vascular smooth muscle cells activation of tyrosine kinases, by platelet-derived growth factor (PDGF) receptor [2] or c-Src [3], increases calcium channel currents. It has been previously proposed that an endogenous tyrosine kinase may also have a physiological role in maintaining VOC availability under resting conditions since selective low molecular weight inhibitors of tyrosine kinases reduced VOC currents [25]. c-Src was considered a likely candidate for this role since it is found in large amounts in smooth muscle cells [7] including rabbit ear artery

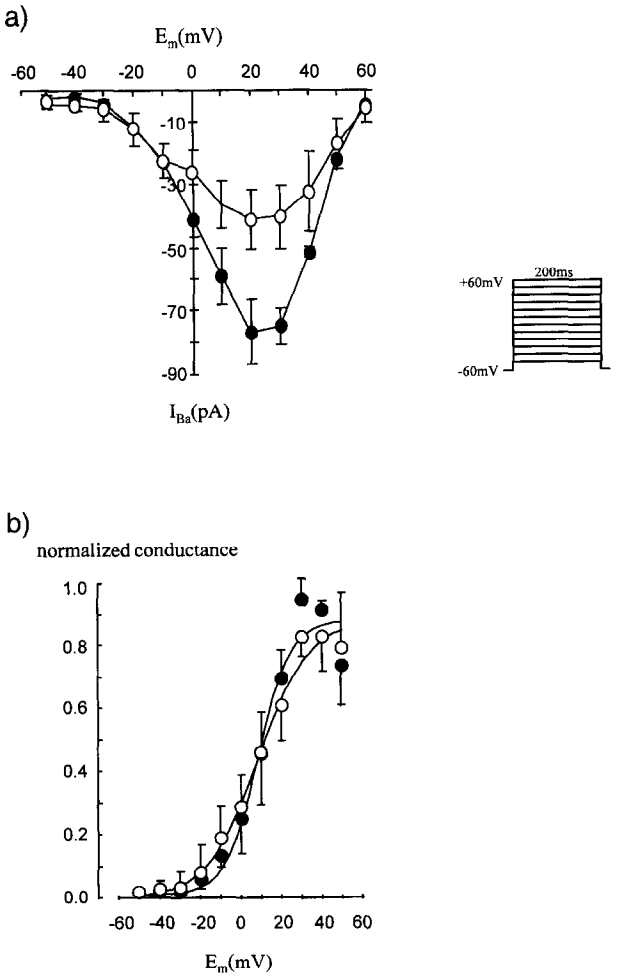

Fig. 2. The effect of (pY)EEI peptide on the activation of voltage-dependent calcium channels. a: The mean current-voltage (IV) relationship of calcium channel currents. IV relationships were derived from a holding potential of −60 mV by depolarising to various test potentials as shown in the protocol (inset). IV data were measured at least 3–4 min after establishment of whole cell mode when the size of the calcium channel currents had stabilised. (○) Control cells, (●) cells with intracellularly applied (pY)EEI (100 μM). b: Activation curve for calcium channels showing normalised conductance as a function of test potential (E_m). Conductance data were calculated as described in Section 2. The average reversal potential for calcium channels was not changed in the absence (62 ± 2 mV) or presence (61 ± 1 mV) of (pY)EEI. Data were fitted to a Boltzman function as described in Section 2. (○) Control cells, (●) cells with intracellularly applied (pY)EEI (100 μM). Data are means ± S.E.M. of 4 observations.

cells (unpublished data) and may be activated by cell-matrix interactions [28]. In the present study we have provided confirmatory evidence supporting such a role for endogenous c-Src in regulating VOC in vascular smooth muscle cells.

The activity of endogenous c-Src is regulated in part through an intramolecular interaction between the tyrosine phosphorylated tail and the SH2 domain of c-Src [16]. Peptides containing the sequence Glu-Glu-Ile (EEI) bind to the Src SH2 domain with about 40-fold greater affinity than the Tyr^{527}-phosphorylated c-Src tail sequence [29] which is composed of the amino acids (pY)Gln-Pro-Gly ((pY)QPG). (pY)EEI peptide is an artificial amino acid sequence derived from the hamster polyoma virus middle T antigen [30], consisting of 11 amino acids. (pY)EEI has been shown to inhibit binding of the PDGF receptor to c-Src and the related tyrosine kinase Fyn by competing with the activated (tyrosine phosphorylated) PDGF receptor for binding to the SH2 domains of these enzymes [31]. (pY)EEI has also been shown to increase the activity of c-Src in vitro in NIH3T3 cells several-fold [18]. In our study (pY)EEI peptide increased the amplitude of VOC currents presumably by activating c-Src or a related endogenous tyrosine kinase. Although the amplitude of current was increased the estimated activation or inactivation curves for calcium channel currents were not affected by the application of (pY)EEI suggesting that c-Src may modulate the open probability of calcium channels rather than their voltage dependence of activation or inactivation. Further single channel studies will be necessary to confirm this hypothesis.

The negative regulatory mechanism induced by the intramolecular reaction between the tyrosine-phosphorylated tail and SH2 domain of c-Src is strictly dependent on phosphorylation of c-Src Tyr^{527}, as substitution of this residue by phe-

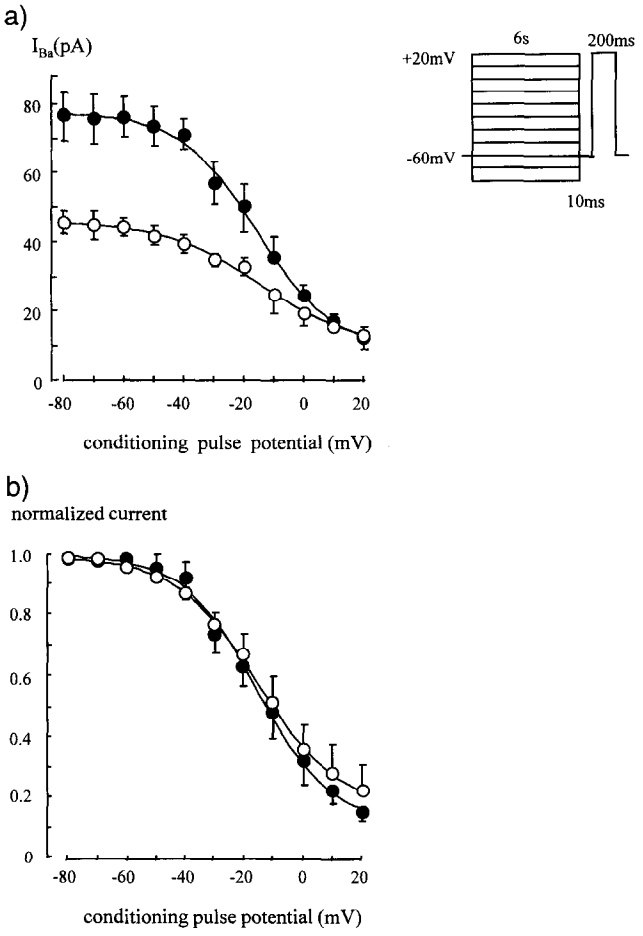

Fig. 3. The effect of (pY)EEI peptide on the steady-state inactivation of calcium channel currents. Cells were held at various conditioning voltages for 6 s to achieve inactivation, then held for 10 ms at the holding potential of −60 mV before currents were evoked by a 20 ms depolarizing step to +20 mV as shown in the voltage protocol (inset). Mean I_{Ba} (a), or I_{Ba} normalized with respect to I_{Ba} evoked following a −80 mV conditioning potential (b), is plotted against the conditioning potential. Inactivation data were measured at least 5–6 min after establishment of whole cell mode when the magnitude of I_{Ba} had stabilized. (○) Control cells, (●) cells dialysed with (pY)EEI (100 μM). Data are means ± S.E.M. of 4 observations.

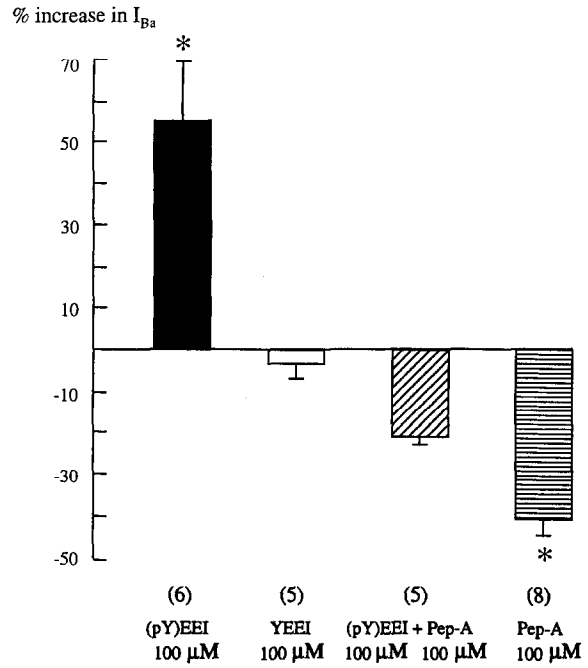

Fig. 4. Effects of (pY)EEI, YEEI peptides and peptide A on voltage-operated calcium channel currents and the effect of peptide A on the response to (pY)EEI peptide. Figure shows % increase in peak current induced by the peptides (calculated as % increase in current measured at 3–4 min following establishment of whole cell mode when currents had reached a steady level compared with currents measured 10 s after establishment of whole cell mode). Columns represent means ± S.E.M. of (n) observations. Currents were evoked by a 20 ms depolarization to +10 mV from a holding potential of −60 mV. Peptides were included in the pipette solution. The % increase in currents by peptides was analysed using a Student's t-test for paired data ($^*P < 0.05$). The effects of peptide A alone (column 4) and peptide A in the presence of (pY)EEI (column 3) were compared using an unpaired t-test and did not differ significantly.

nylalanine or dephosphorylation of Tyr^{527} results in activation of c-Src kinase [32,33]. In vitro studies have shown that the dephosphorylated YEEI peptide was unable to increase c-Src activity [18]. Similarly in our study dephosphorylated YEEI peptide had no effect on calcium channel currents.

Peptide A is a synthetic peptide containing 21 amino acids which corresponds to the non-catalytic domain (residues 131–157) of v-Src tyrosine kinase and is known to inhibit the activity of c-Src [34]. It has no inhibitory effects on serine or threonine kinases although it may also inhibit the epidermal growth factor receptor tyrosine kinase [34]. Peptide A inhibited VOC currents when applied intracellularly and also inhibited the calcium channel activating effect of exogenous c-Src [3]. Application of peptide A together with (pY)EEI abolished the effect of (pY)EEI peptide suggesting that (pY)EEI increases VOC currents by activating endogenous c-Src.

In conclusion these results show that in rabbit ear artery cells activation of c-Src or a closely related tyrosine kinase increases, and that inhibition of this kinase reduces, voltage-operated calcium channel currents. In view of these findings we propose that a c-Src-like non-receptor tyrosine kinase plays an important role in regulating VOC in vascular smooth muscle cells under resting as well as under growth factor-activated conditions.

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