Tyrosine kinase and protein kinase C regulate L-type Ca$^{2+}$ current cooperatively in human atrial myocytes

CHRISTOPHE BOIXEL, SOPHIE TESSIER, YVES PANSARD, LOÍC LANG-LAZDUNSKI, JEAN-JACQUES MERCADIER, AND STEPHANE N. HATEM

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Tyrosine protein kinases (TK) are involved in signal transduction mediated by many hormone and growth factor receptors that regulate mitogenesis or cell growth. In addition, these enzymes play an important role in the regulation of ion channels such as L-type Ca$^{2+}$ channels ($I_{Ca}$), which are regulated by TK in various cell types, including pituitary GH3 cells (3), retinal pigment epithelial cells (21), smooth muscle cells (8), and cardiac myocytes (6, 24, 25). TK can regulate $I_{Ca}$ by direct phosphorylation of the α-subunit of Ca$^{2+}$ channels, as is the case of the nonreceptor tyrosine kinases c-Src and focal adhesion kinase in smooth muscle cells (8). The enzymes can also regulate $I_{Ca}$ indirectly by modulating the activity of various signaling pathways such as those resulting in the stimulation of protein kinase C (PKC) or cAMP-dependent protein kinase. For instance, in guinea pig ventricular myocytes, the effect of TK on $I_{Ca}$ results predominantly from regulation, by the enzymes, of the sensitivity of Ca$^{2+}$ channels to β-adrenergic receptor stimulation (6).

In human atrial myocytes, as in those of other species, the L-type Ca$^{2+}$ current is the target of various neurotransmitters, hormones, and therapeutic agents whose effects often involved serine/threonine phosphorylation via activation of cAMP- or cGMP-dependent protein kinases or PKC. However, $I_{Ca}$ regulation by second messengers differs in several aspects between human atrial myocytes and myocytes from other tissues or species, as illustrated by the effects of serotonin (17). We have also reported that phosphodiesterase (PDE) types 2 and 3 participate in the basal PDE activity involved in the regulation of $I_{Ca}$ of human atrial myocytes but not in those of other animal species studied (19, 11).

This species and tissue specificity of $I_{Ca}$ regulation, together with the multiple effects of TK on this current, prompted us to examine whether $I_{Ca}$ in human atrial myocytes is also regulated by the enzymes and, if so, by what mechanism. We examined whole cell patch-clamped myocytes treated with various pharmacological tools modulating not only TK but also serine/threonine kinases. We found that TK regulates $I_{Ca}$ in human atrial myocytes, an effect that appears to involve a cooperation between PKC and TK.

MATERIALS AND METHODS

Cardiac myocyte preparation. Specimens of human right atrial appendages were obtained from 42 patients (15–81 yr of age) undergoing heart surgery for coronary artery insuffi-
ciency (n = 22), mitral valve disease (n = 11), aortic valve disease (n = 7), or congenital heart defect (n = 2). Most patients received a pharmacological treatment that was stopped at least 10 h before surgery (Ca\(^{2+}\)-channel blocker, \(\beta\)-adrenergic antagonist, diuretics, angiotensin-converting enzyme inhibitors, or nitric oxide donor). All patients except one were in sinus rhythm. Myocytes were enzymatically isolated as previously described (5). Briefly, small pieces of atrial appendage were cut up and washed in Ca\(^{2+}\)-free Krebs-Ringer solution containing (in mM) 35 NaCl, 4.75 KCl, 1.19 KH\(_2\)PO\(_4\), 1.6 Na\(_2\)HPO\(_4\), 10 HEPES, 10 glucose, 25 NaHCO\(_3\), 134 saccharose, and 30 2,3-butanedione monoxime (BDM) (pH 7.4 adjusted with NaOH), gassed with 95% O\(_2\)-5% CO\(_2\) and maintained at 37°C. BDM, a compound known to have reversible effects on cardiac cellular electrophysiology (4), was used to prevent tissue injury during cutting (16). Pieces were reincubated in the same solution but without BDM and containing 200 IU/ml collagenase (type IV, Sigma Chemical) and 6 IU/ml protease (type XXIV, Sigma Chemical). After 30 min of digestion, the enzyme solution was replaced by the same solution containing only collagenase (400 IU/ml). Isolated myocytes were incubated at 37°C with continuous gassing with 21% O\(_2\)-5% CO\(_2\) for at least 1 h before use.

Current measurements. Currents were recorded with the patch-clamp technique in the whole cell configuration using borosilicate glass pipettes with a tip resistance of 1–2 M\(\Omega\) connected to the input stage of a patch-clamp amplifier (Axoclamp 200A, Axon Instruments). Currents filtered at 5 kHz were digitized by a Labmaster (Lab Rac, Scientific Solution) and stored on the hard disk of a personal computer. Data were acquired and analyzed using a program written for our laboratory (Acqis, G. Sadock). Resistance in series was compensated for to obtain the fastest capacity transient current. Membrane capacitance was calculated using the fit of the capacity transient decay. Current recording was performed 1 min after the patch was broken to obtain a steady-state intracellular dialysis, and the average duration of the experiments was around 20 min. Rundown of \(I_{\text{Ca}}\) usually occurred 10 min after the patch was broken. Experiments were carried out at room temperature (22–24°C).

Solutions and reagents. The compositions of the standard solutions used were as follows (in mM): normal Tyrode solution, 136 NaCl, 5.4 KCl, 2 CaCl\(_2\), 10 glucose, 1.06 MgCl\(_2\), 0.33 NaH\(_2\)PO\(_4\), and 10 HEPES; pH adjusted to 7.4 with NaOH; \(I_{\text{Ca}}\) recording solution, normal Tyrode solution with NaCl replaced by tetraethylammonium; and pipette solution, 130 CsCl, 2 MgCl\(_2\), 10 HEPES, 15 EGTA, 10 glucose, and 3 MgATP, pH adjusted with 7.2 with CsOH. A multibarrel system allowed exchange of the fluid solution bathing the myocyte within 2 s. Genistein, lavendustin A, tyrphostin 23, and acetylcholine were dissolved in distilled water and kept as stock solutions at \(-20°C\); staurosporine, \(4\alpha\)-phorbol 12,13-didecanoate (\(4\alpha\)-PDD), and phorbol 12-myristate 13-acetate (PMA) were dissolved in ethanol and stored as stock solutions at \(-20°C\). Isoproterenol was diluted in the Tyrode solution.

Fig. 1. A: superimposition of L-type Ca\(^{2+}\) current (\(I_{\text{Ca}}\)) traces elicited by a test potential from −60 to 0 mV recorded with Tyrode external solution (○, control) and with same solution containing 50 µM genistein (●). B: time course of changes in \(I_{\text{Ca}}\) on genistein exposure, where x-axis indicates time from beginning of current recording. Cell capacitance, 70 pF. C: current density-voltage relationships of \(I_{\text{Ca}}\) recorded in control conditions and on genistein exposure. Each point is average current density in 10 cells. *P < 0.05; **P < 0.01 vs. control. D: concentration-dependent effect of genistein on \(I_{\text{Ca}}\). Each point is mean from 5–15 cells.
The protein kinase inhibitor was directly dissolved in pipette solution. In some experiments, myocytes were preincubated with the PKC inhibitor calphostin C, which, to be activated, required that myocytes were maintained for 30 min under ultraviolet light. All drugs were purchased from Sigma Chemical, except for isoproterenol (Sanofi Winthrop, France).

Data analysis. Depolarizing voltage pulses were delivered at 0.1 Hz. The amplitude of peak \( I_{\text{Ca}} \) was measured as the difference between the amplitude of the peak inward current and that recorded at the end of the 350-ms test pulses. Concentration-response curves were fitted as follows: \( E = E_{\text{max}}[D]/([D] + EC_{50}) \), where \( E \) is the percentage change in \( I_{\text{Ca}} \), \( E_{\text{max}} \) is the maximal response induced by the drug, and \([D]\) is the concentration of genistein, to estimate the \( EC_{50} \).

Statistical analysis. Values are expressed as means ± SE; \( n \) indicates the number of experiments. Paired Student's t-test was used to determine the statistical significance of differences between means obtained before and after the effects of a given drug. One-way ANOVA was used to determine the statistical significance of differences between means obtained under different experimental conditions. \( P \) values < 0.05 were considered significant.

RESULTS

TK inhibitors stimulate \( I_{\text{Ca}} \) in human atrial myocytes. Figure 1A shows an example of the effects of external application of 50 \( \mu \)M genistein on the \( I_{\text{Ca}} \) elicited by 350-ms depolarizing test pulses from −60 mV to 0 mV. In the majority of cells studied (84%, \( n = 53 \)), genistein stimulated \( I_{\text{Ca}} \) (132 ± 18%, \( n = 28 \), \( P < 0.001 \)), an effect that was sometimes preceded by a slight inhibition of the current. In the remaining 16% of myocytes (\( n = 9 \)), genistein inhibited \( I_{\text{Ca}} \) or had no significant effect. There was no clear relationship between the source patient’s clinical data and the effects of genistein. The stimulatory effect of genistein developed slowly, reaching steady-state after ~1 min (Fig. 1B), and was not reversible on drug washout during the time of the experiments. The increase in \( I_{\text{Ca}} \) on genistein application was associated with a shift in the current-voltage relationship toward a negative potential of ~10 mV (Fig. 1C). The \( EC_{50} \) of genistein on the \( I_{\text{Ca}} \) was 53 \( \mu \)M, a value close to the \( IC_{50} \) of genistein on TK (Fig. 1D) (2).

At low concentration (<20 \( \mu \)M), genistein only inhibited \( I_{\text{Ca}} \). Lavendustin A and tyrphostin 23, other TK inhibitors structurally distinct from genistein (14), also stimulated \( I_{\text{Ca}} \) and shifted the current voltage-relationship leftward (116 ± 18%, \( n = 5 \), \( P < 0.05 \); 60 ± 6%, \( n = 23 \), \( P < 0.001 \), respectively) (Fig. 2). The smaller increase of \( I_{\text{Ca}} \) on tyrphostin exposure may reflect the fact that this compound is more specific for distinct TK compared with the broad-spectrum TK inhibitors genistein and lavendustin A (14, 18). It should be noted that in our experimental conditions, low concentrations (<0.01%) of DMSO altered the human atrial myocyte \( I_{\text{Ca}} \) and thus ruled out experiments with the inactive genistein analog daidzen, a compound solely soluble in DMSO.

Effects of genistein on \( I_{\text{Ca}} \) are independent of cAMP. The slowly developing stimulatory effects of TK inhibitors on \( I_{\text{Ca}} \), which were accompanied by a leftward shift in the current-voltage relationship, suggested that these compounds affected the phosphorylation status of Ca\(^{2+}\) channels. However, the observation that 1 \( \mu \)M isoproterenol still caused an enhancement of \( I_{\text{Ca}} \) prestimulated by TK inhibitors (83 ± 23%, \( n = 8 \), \( P < 0.01 \)) argues against the involvement of a cAMP-dependent regulatory pathway (Fig. 3, A and B). In addition, acetylcholine (10\(^{-6}\) M), a muscarinic agonist that reversed the stimulatory effect of isoproterenol on \( I_{\text{Ca}} \), did

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![Image](https://via.placeholder.com/150.png?text=Fig. 2. Superimposition of \( I_{\text{Ca}} \) traces recorded in control conditions and in presence of 50 \( \mu \)M lavendustin A (A) or 50 \( \mu \)M tyrphostin 23 (B). Cell capacitances, 83 and 62 pF in A and B, respectively. C: percent change in \( I_{\text{Ca}} \) in response to genistein, lavendustin A, and tyrphostin 23 exposure. *P < 0.05; ***P < 0.001 vs. control.)
not prevent genistein-induced enhancement of the current, indicating that the effect of the TK inhibitor was independent of adenyl cyclase activity (n = 13) (Fig. 3C). Finally, in cells dialyzed with an internal solution containing the cAMP-dependent protein kinase inhibitor at a concentration of 20 µM, genistein, contrary to isoproterenol, stimulated I_{Ca}(130 ± 19%, n = 5, P < 0.05) (Fig. 3D).

PKC-dependent effects of genistein. We next examined whether PKC and TK regulated I_{Ca} cooperatively. As shown in fig. 4A, in a myocyte pretreated for at least 30 min with the PKC inhibitor staurosporine (100 nM), genistein had no effect on I_{Ca}, whereas the current was still stimulated by isoproterenol, ruling out a rundown of the channels. These experiments were repeated with 18 myocytes, and the results are indicated in Table 1. Similar suppression of the effect of genistein was also obtained using another PKC inhibitor, calphostin C (2 ± 13 vs. 128 ± 10% in calphostin C (n = 7) and control conditions (n = 7), respectively, P < 0.01). 

The effect of genistein was then studied in myocytes pretreated with PMA to stimulate PKC. In 77% of the cells studied, PMA had a biphasic effect on I_{Ca}, characterized by an initial increase in amplitude (75 ± 17%, n = 15, P < 0.05) (22) followed by a decrease at ~5 min (36 ± 5%, n = 15, P < 0.001) (Fig. 4B and Table 1). The fall in I_{Ca} observed after prolonged PMA application was distinct from that observed during classic rundown of L-type Ca^{2+} channels, with a steeper slope of current decline in PMA than in control conditions (2.8 ± 0.3 ms^{-1}, P < 0.05) (26). In addition, PMA had no significant inhibitory effect on I_{Ca} in myocytes pretreated with staurosporine (n = 5; Fig. 4C). Moreover, the inactive analog of PMA, 4α-PDD, had no effect on I_{Ca} (2.7 ± 0.4 pA/pF vs. 2.8 ± 0.4 pA/pF in control conditions and after 4α-PDD exposure, respectively, n = 8) and did not prevent that of PMA (47 ± 5% of I_{Ca} inhibition caused by PMA in 4α-PDD-treated cells, n = 8). At the steady state of I_{Ca} inhibition by PMA, application of genistein still caused a marked increase
in I_{Ca} (Fig. 4B), and comparison of the effect of genistein on I_{Ca} in control and PMA-treated myocytes showed a higher percentage of current enhancement in the latter (323 ± 25% vs. 120 ± 13%, n = 8, P < 0.05) (Table 1). Finally, pretreating myocytes with genistein reduced the inhibitory effect of PMA on I_{Ca} (percentage of I_{Ca} inhibition caused by PMA: 40 ± 5% in control (n = 15) vs. 20 ± 7% in genistein (n = 15), P < 0.05).

**DISCUSSION**

We found that various agents known to inhibit TK, such as genistein, lavendustin A, and tyrphostin 23, stimulated I_{Ca} in human atrial myocytes. The effects of these compounds on the I_{Ca} appear to result from their ability to inhibit TK, because 1) they increased the I_{Ca} at concentrations known to inhibit TK activity, and 2) these compounds, which are structurally unrelated, all had the same effect on I_{Ca}, suggesting a common mechanism of action (2, 14, 7).

In human atrial myocytes, TK inhibition is accompanied by an apparent change in the degree of phosphorylation of L-type Ca^{2+} channels, as indicated by the slow increase in current amplitude and the shift of the voltage-relationship toward negative potentials (10). Clearly, this effect of TK inhibitors on I_{Ca} cannot be attributed to the modulation of cAMP-dependent processes, especially because inhibition of cAMP-dependent protein kinase did not suppress the effect of genistein. However, our results do not eliminate the possibility that in human atrial myocytes, as in guinea pig ventricular myocytes, TK regulate the β-adrenergic responsiveness of Ca^{2+} channels (6).

Our finding that the effects of TK inhibitors on I_{Ca} were modulated by compounds known to alter PKC activity suggests a link between these two enzymes. It is unlikely that staurosporine or calphostin C inhibited the effect of genistein via an effect of genistein on PKC, because 1) the genistein concentration used was much lower than that required for PKC blockade (2), and 2) lavendustin A, which is devoid of any significant PKC-inhibiting action (7), had the same effect as genistein on the I_{Ca}. Similar cases of cooperative regulation of ion channels by PKC and TK have been observed in other cell types. For instance, in rat and human retinal epithelial cells, genistein stimulates L-type Ca^{2+} channels when PKC is prestimulated with PMA (21). Furthermore, the G protein-coupled m_{3}-muscarinic acetylcholine receptor inhibits Kv1.2 channels expressed in Xenopus oocytes and in cell lines through a phospholipase C (PLC)/PKC-dependent mechanism that controls direct tyrosine phosphorylation of K^{+} channels (9). These effects involved a cytosolic proline-rich tyrosine kinase (PYK2), which can be activated by phosphorylation in response to various stimuli such as intracellular Ca^{2+} and PKC activation (13, 20). Interestingly, in cat atrial myocytes it has been reported that a cytosolic (nonreceptor) TK may be responsible for the inhibition

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Values are means ± SE; n = no. of cells. I_{Ca}, L-type Ca^{2+} current; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate. Comparisons between groups were done by ANOVA followed by Scheffé's F test. Differences were considered statistically significant when P < 0.05. *P < 0.05; †P < 0.01, current density before vs. after genistein exposure. ¶P < 0.05, genistein exposure vs. basal condition.

Fig. 4. A: time course of changes in I_{Ca} (test pulses to 0 mV) on exposure to genistein and genistein plus isoproterenol in myocytes pretreated with 100 nM staurosporine, where x-axis indicates time from beginning of current recording. Cell capacitance, 55 pF. B and C: time course of changes in I_{Ca} in response to phorbol 12-myristate 13-acetate (PMA) application and genistein application in presence of PMA in control (B; cell capacitance, 67 pF) and staurosporine-treated myocytes (C; cell capacitance, 124 pF), where x-axis indicates time from beginning of current recording. Gen, genistein.
of Ca\(^{2+}\) channels and, in turn, for the increase in \(I_{\text{Ca}}\) caused by genistein (24). Our observation that TK inhibitors stimulate \(I_{\text{Ca}}\) in whole cell patch-damped human atrial myocytes may appear to conflict with the presence of a cytosolic TK, which should be dialyzed by the patch pipette; however, it is possible that cytosolic TK is located in the proximity of ion channels in fuzzy subsarcolemmal spaces poorly accessible to dialysis (1).

In cat atrial myocytes, inhibition of PKC does not suppress the effect of genistein on the \(I_{\text{Ca}}\), suggesting that distinct soluble TK isoforms regulate \(I_{\text{Ca}}\) in cat and human atrial myocytes or that the nature of the stimuli (increase in intracellular Ca\(^{2+}\) concentration) that activate soluble TK varies with the species or pathophysiological conditions. In addition to soluble TK stimulated by PKC, other mechanisms might link the two enzyme activities. For instance, tyrosine phosphorylation of Goq11 protein facilitates coupling between Goq11 protein and glutamate receptor 1\(\alpha\) expressed in Chinese hamster ovary, leading to activation of a PLC-dependent signaling pathway (23). However, genistein still increased \(I_{\text{Ca}}\) in myocytes pretreated with PMA, which argues against the possibility that TK modulates PKC through a permissive effect on G protein coupling to a phospholipase, for instance. Finally, the observation that PMA still inhibited the current in myocytes pretreated with genistein, although less efficiently compared with the control, indicates that only part of the effect of PKC on L-type Ca\(^{2+}\) channels depends on TK activation. The relatively low density of \(I_{\text{Ca}}\) in staurosporine-treated myocytes appears to oppose the possibility of a tonic regulation of \(I_{\text{Ca}}\) by PKC via their coupling with TK. However, it is possible that staurosporine has some cellular toxicity, including disruption of the cytoskeleton (15) and resulting in a decreased density of \(I_{\text{Ca}}\) that was not caused by a rundown of the channel, as indicated by the persistence of the stimulatory effect of isoproterenol on \(I_{\text{Ca}}\) in staurosporine-treated myocytes.

The inhibitory effect of genistein on \(I_{\text{Ca}}\) observed in a smaller percentage of cells that, in some cases, preceded the increase in current may be caused by activation of receptor-bound TK distinct from the cytosolic TK, as reported in cat atrial myocytes (24). It is also possible that the inhibitory effect of genistein is in part TK-independent and caused by a direct effect of the compound on Ca\(^{2+}\) channels. This is suggested by the observation that the inhibition of \(I_{\text{Ca}}\) by genistein was a rapid process that did not occur with the other TK inhibitors tested and that also was observed at a concentration at which genistein is a weak TK inhibitor (Fig. 1D). In guinea pig ventricular myocytes, both genistein and its inactive analog daidzen inhibit \(I_{\text{Ca}}\), indicating that this effect is related not to suppression of TK activities but to direct effects of these drugs on L-type Ca\(^{2+}\) channels (6, 25). Our results, which are similar to those obtained in cat atrial myocytes but different from those obtained in guinea pig ventricular myocytes, point to the tissue and species specificity of L-type Ca\(^{2+}\) channel regulation by TK. Distinct regulatory mechanisms of \(I_{\text{Ca}}\) in human atrial myocytes are already known with regard to Ca\(^{2+}\) channel coupling to 5-HT\(_{1}\) receptors (17) and the effects of PDE inhibitors (19, 11); furthermore, the specificities of \(I_{\text{Ca}}\) regulation in human atrial myocytes may also be influenced by pathophysiological conditions (12).

Our observation of tonic \(I_{\text{Ca}}\) regulation by TK in human atrial myocytes raises important questions as to the significance of this regulatory process. Because L-type Ca\(^{2+}\) channels play a central role in excitation-contraction coupling of atrial myocytes (5), regulation of their activity by TK may have profound implications for the electrical and mechanical activity of these cells. In addition, it is conceivable that cellular Ca\(^{2+}\) influx regulation by TK contributes to modulation of tonic phenomena such as gene expression and cardiac phenotypic plasticity.

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Address for reprint requests and other correspondence: S. Hatem, INSERM U460, Faculté de Médecine Xavier Bichat, 16 rue Henri Huchard, 75018 Paris, France (E-mail: hatem@bichat.inserm.fr).

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