Activation of Background Membrane Conductance by the Tyrosine Kinase Inhibitor Tyrphostin A23 and Its Inactive Analog Tyrphostin A1 in Guinea Pig Ventricular Myocytes

Toshitsugu Ogura*, Sunao Imanishi and Toshishige Shibamoto
Second Department of Physiology, Kanazawa Medical University, 1-1, Daigaku, Uchinada-machi, Kahoku-gun, Ishikawa 920-0293, Japan
Received May 30, 2001 Accepted September 27, 2001

ABSTRACT—The effects of the tyrosine kinase (TK) inhibitor tyrphostin A23 and its inactive analog tyrphostin A1 on background membrane conductance were investigated in guinea pig ventricular myocytes. TK-inhibiting A23 reversibly increased membrane conductance under conditions designed to minimize Na⁺, Ca²⁺, K⁺, and Na⁺-K⁺ pump currents. Similar stimulatory action was obtained with TK-inactive A1. The tyrphostin-induced current was inhibited by omitting external Na⁺ or Ca²⁺, suppressed by chelating internal Ca²⁺, blocked by external Cd²⁺ and Ni²⁺, and insensitive to changes in internal Cl⁻ concentration. We conclude that tyrphostins have a direct, TK-independent action that increases membrane conductance probably by stimulating Na⁺-Ca²⁺ exchange.

Keywords: Tyrphostin, Tyrosine kinase, Na⁺-Ca²⁺ exchange

Tyrphostins, synthetic inhibitors of tyrosine kinase (TK) (1, 2), are widely used to assess the roles of tyrosine phosphorylation in cell function, including regulation of membrane ionic channels and transporters. A recent study on L-type Ca²⁺ current in heart cells has demonstrated that tyrphostins inhibit the current, whereas a TK-inactive analog does not, and the tyrosine phosphatase inhibitor antagonizes the tyrphostin-induced inhibition (3). These results led to the conclusion that tyrosine phosphorylation is involved in the regulation of cardiac Ca²⁺ channels, and a similar conclusion has been reached in regard to Na⁺-K⁺ pump (4), non-selective Ca²⁺-permeable cation channels (5) and volume-regulated Cl⁻ channels (6) in cardiovascular preparations. However, tyrphostins have a variety of TK-independent actions, including block of ATP-sensitive K⁺ channels (7), hyperpolarization-activated cation channels (8) and non-selective cation channels (9) in heart and smooth muscle cells. In the present study on guinea pig ventricular myocytes, we have investigated the effects of tyrphostin A23 and its negative analog tyrphostin A1 (1) on background membrane conductance elicited under conditions where Na⁺, Ca²⁺, K⁺ and Na⁺-K⁺ pump currents were suppressed.

Experiments were performed in accord with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society. Guinea pig ventricular myocytes were enzymatically isolated as described previously (3). The myocytes were superfused at 36°C with modified Tyrode’s solution that contained 140 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM BaCl₂, 10 mM glucose, 0.01 mM verapamil and 5 mM HEPES (pH 7.4 with NaOH) and dialyzed with pipette-filling solution that contained 30 mM CsCl, 106 mM caesium aspartate, 8 mM NaCl, 5 mM MgATP, 5 mM EGTA and 5 mM HEPES (pH 7.2 with CsOH, pCa 7 by adding 2 mM CaCl₂). These solutions were modified in some experiments as noted below. Whole-cell membrane currents were recorded using an EPC-8 amplifier (HEKA, Lambrecht, Germany) and PULSE software (HEKA) at a sampling rate of 10 kHz. The series resistance ranged between 3 and 7 MΩ and was compensated by 60–80%. The cell membrane was held at −40 mV, and 200-ms test steps to 0 mV were applied at 0.2 Hz, except for periodic sequences of pulses to other potentials to measure current-voltage (I-V) relationships. Tyrphostins, A23 and A1 (Calbiochem, La Jolla, CA, USA), and genistein (GST; Sigma, St. Louis, MO, USA) were prepared as 100 mM stock solutions in DMSO. Corresponding amounts of DMSO (≤0.1%) were also added to the control superfusates. Results are ex-
pressed as means ± S.E.M., and comparisons were made using Student’s t-test. A difference was considered significant when P<0.05.

Membrane currents were recorded under conditions expected to minimize Na⁺ and T-type Ca²⁺ currents (by –40 mV holding potential), L-type Ca²⁺ current (by verapamil), K⁺ currents (by K⁺-free Ba²⁺-containing superfusate and Cs⁺-rich dialysate), and Na⁺-K⁺ pump current (by K⁺-free superfusate). The results shown in Fig. 1A indicate that exposure to 100 μM tyrphostin A23 caused a rapid and reversible increase in the membrane slope conductance between –40 and 0 mV (G_{40/0}). A23-induced currents obtained by subtracting pre-A23 records from A23 records were essentially time-independent and exhibited a near linear I-V relationship. G_{40/0} activated by 100 μM A23 was 3.4 ± 0.2 nS (n = 9, P<0.0001), whereas 1 and 10 μM A23 had no significant effects (0.1 ± 0.2 and 0.2 ± 0.3 nS changes, respectively; n = 4 each). Exposure of four other myocytes to 100 μM tyrphostin A1, an analog with minimal effects on TK (1), caused a significant increase in G_{40/0} (1.6 ± 0.3 nS, P<0.05) (Fig. 1: B and C). On the other hand, 100 μM A23 did not significantly affect G_{40/0} when the superfusate was supplemented with 1 mM Cd²⁺ (0.1 ± 0.3 nS change, n = 4) or 5 mM Ni²⁺ (0.2 ± 0.2 nS change, n = 5) instead of verapamil.

The findings are possibly explained as tyrphostin-

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**Fig. 1.** Stimulatory actions of tyrphostins A23 and A1 on membrane conductance. A, Effects of A23. Slope conductance was measured between –40 and 0 mV (G_{40/0}). Current-voltage (I-V) relationship was measured from A23-induced currents obtained by subtracting control records from A23 records (b – a). Dashed line indicates zero current. B, Comparative effects of A23 and A1. C, Dose-dependence of A23 and A1 effects. *P<0.05 and **P<0.0001 vs pre-drug, †P<0.005 vs A23; n = 4 – 9 myocytes.
induced increase in membrane conductance through activating CFTR Cl\(^-\) current (see Refs. 3 and 10). However, this was not the case because the reversal potential (\(E_{\text{rev}}\)) of 100 \(\mu\)M A23-induced currents (-13 ± 2 mV, \(n = 5\)) was more positive than calculated Cl\(^-\) equilibrium potential (\(E_C\)) of -33 mV (external Cl\(^-\) = 146 mM, internal Cl\(^-\) = 42 mM), and elevation of dialysate Cl\(^-\) concentration from 44 to 148 mM (replacement of aspartate) did not significantly shift the \(E_{\text{rev}}\) (-17 ± 3 mV vs \(E_C = 0\) mV, \(n = 4\)). Furthermore, the Cl\(^-\) channel blocker anthracene-9-carboxylic acid (1 mM) had no significant effect on \(G_{40/0}\) activated by 100 \(\mu\)M A23 (-0.4 ± 0.3 nS change, \(n = 3\)).

To test whether the non-Cl\(^-\) conductance activated by A23 was generated by Na\(^+\) flux, membrane currents were measured from A23-treated myocytes before and after removal of external Na\(^+\). The myocytes were superfused with 100 \(\mu\)M A23-containing solution and dialyzed with Na\(^+\)-free pCa 7 solution, and then the superfusate was switched to Na\(^+\)-free solution (replacement of tetramethylammonium) in the continued presence of A23. Figure 2A depicts that 100 \(\mu\)M A23 increased \(G_{40/0}\) from 3.2 to 6.8 nS, and the subsequent removal of external Na\(^+\) reduced it to 3.7 nS. In three myocytes, Na\(^+\)-free treatment for 4 min significantly reduced the 100 \(\mu\)M A23-activated \(G_{40/0}\) by

![Fig. 2. Divergent responses of A23- and GST-activated currents to modified external solutions. Slope conductance was measured between -40 and 0 mV (\(G_{40/0}\)). Current-voltage (I-V) relationships were measured from Na\(^+\)-sensitive (A) and A23-induced (B) currents. A, Inhibition of A23-induced current by removal of external Na\(^+\). B, Activation of inwardly-directed currents by A23 in the absence of external Ca\(^{2+}\). Ca, Activation of \(G_{40/0}\) by GST in the presence of 5 mM Ni\(^{2+}\). Cb, Lack of inhibitory effect of Na\(^+\) removal on GST-activated \(G_{40/0}\).]
The dialysate containing 8 mM Na specifically point to the target of the drug. We used the Ca1.2 nS, n/G3d significantly increased G2+/Gb1 of A23-induced current (Fig. 2C): i) GST significantly increased G40.0 in myocytes superfused with Ca2+-free solution and dialyzed with pCa 7 solution. The A23-induced currents were inwardly-directed without exhibiting apparent Erev. Similar results were obtained from two other myocytes. Another set of experiments were performed on four myocytes superfused with Ca2+-free solution and dialyzed with pCa 10.5 solution (no addition of CaCl2). A 5-min exposure of these myocytes to 100 μM A23 caused no significant changes in membrane conductance (0.2 ± 0.4 nS change in G40.0).

GST is a TK inhibitor that is structurally different from tyrphostins (11), and its actions are frequently compared with those of tyrphostins (3, 5–8, 12). Representative results from myocytes treated with 100 μM GST indicate the divergent actions of these drugs (Fig. 2C): i) GST significantly increased G40.0 in myocytes treated with 5 mM Ni2+ (3.6 ± 1.0 nS, n = 3), ii) the activation was not significantly different from that in non-Ni2+-treated myocytes (3.8 ± 1.2 nS, n = 4), and iii) removal of extracellular Na+ reduced G40.0 by only 4–16% in the presence of GST (n = 3).

The present study has demonstrated that both TK-inhibiting A23 and its negative analog A1 cause a rapid and reversible increase in membrane conductance under conditions expected to minimize Na+, Ca2+, K+ and Na+-K+ pump currents. Regarding the site of action of A23, it is clear that the drug has no effect on Cl- channels, as reported earlier that tyrphostin A51 does not activate Cl- current in guinea pig ventricular myocytes (13). In contrast, the A23-induced current was i) inhibited by omitting external Na+ or Ca2+, ii) suppressed by tightly buffering the internal Ca2+ concentration, and iii) blocked by Cd2+ and Ni2+, but not by verapamil (see Ref. 14). Taken together, we assume that the tyrphostin-induced current is generated by Na+-Ca2+ exchange, although each of the above observations does not specifically point to the target of the drug. We used the superfusate containing 140 mM Na+ and 1 mM Ca2+ and the dialysate containing 8 mM Na+ and 100 mM free-Ca2+. Under these conditions, Erev of Na+-Ca2+ exchange calculated from Na+ equilibrium potential (EeqNa+) = +76 mV and Ca2+ equilibrium potential (EeqCa2+) = +112 mV by Erev = 3EeqNa+ - 2EeqCa2 is −16 mV; this potential is very close to the measured Erev of A23-induced current (−13 mV).

Tyrphostin A1 is a much weaker inhibitor of TK than other tyrphostins (IC50 >1250 μM for epidermal growth factor receptor (EGFR) kinase: Ref. 1), and therefore often used to differentiate TK-mediated effects of tyrphostins from other non-specific effects. In the present study, the activation of membrane conductance by A1 mimicked the stimulatory action of A23 (IC50 = 35 μM for EGFR kinase: Ref. 1). This result raises the possibility that the concentration of A1 used in this study was sufficient to cause a significant inhibition of TK. However, the interpretation seems unlikely because the effect of the TK inhibitor GST (IC50 = 2.6 μM for EGFR kinase: Ref. 11) on G40.0 was distinct from that of A23 with respect to the responses to Ni2+ and Na+-free treatments. We deem that the observed effects of tyrphostins are non-specific ones that are independent of TK inhibition. In this regard, tyrphostins are relatively low molecular weight compounds that compete with TK substrates and ATP (1, 2) and can directly act on other cellular targets (7, 9, 12, 15). Thus, it is possible to speculate that the stimulatory action of tyrphostins is due to opportunistic binding of tyrphostin molecules to the regulatory region of the exchanger protein and subsequent modulation of the exchanger activity. Further investigation using a specific TK inhibitor or “isolated” preparations (e.g., sarcolemmal vesicles, exchanger-expressing systems) will be needed to characterize the role of TK in the regulation of cardiac electrical activities.

Acknowledgments

This work was supported by Grant for Promoted Research from Kanazawa Medical University (500-15) to T.O. and Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (#11670717) to S.I. T.O. is grateful to Prof. T.F. McDonald at Dalhousie University (Halifax, Canada) for invaluable support.

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