BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **235,** 74–78 (1997)
ARTICLE NO. RC976739

Genistein Directly Induces Cardiac CFTR Chloride Current by a Tyrosine Kinase-Independent and Protein Kinase A-Independent Pathway in Guinea Pig Ventricular Myocytes

Chern-En Chiang,[1] Shih-Ann Chen, Mau-Song Chang, Cheng-I Lin,* and Hsiang-Ning Luk*
*Division of Cardiology, Veterans General Hospital-Taipei and National Yang-Ming University School of Medicine; and *Department of Pharmacology, National Defense Medical Center, Taipei, Taiwan, Republic of China*

Received April 29, 1997

With one-suction electrode voltage-clamp technique, we demonstrated that genistein, a tyrosine kinase (TK) inhibitor, could directly activate cystic fibrosis transmembrane regulator (CFTR) chloride current in guinea pig ventricular myocytes. The activation showed concentration-dependent effect with the estimated IC_{50} of 39.7 μM. Tyrphostin 51, another TK inhibitor, had no effect, suggesting that genistein's effect might be unrelated to TK inhibition. After the chloride current had been activated by the maximally elevated intracellular cAMP content by saturating concentration of isoproterenol, forskolin and IBMX, genistein could further enhance the current. Pre-treatment with saturating concentration of a specific protein kinase A (PKA) inhibitor, H-89, or other protein kinase inhibitors H-8 and H-9 in the perfusate or intracellularly could not prevent the activation of the current by genistein, suggesting a PKA-independent activity. Furthermore, saturating concentration of calyculin A, a specific inhibitor of phosphotase 1 and 2A, in the perfusate or intracellularly could not block genistein's action. It is possible that genistein opens the channels directly or inhibits the dephosphorylation process of CFTR, which is not sensitive calyculin A. © 1997 Academic Press

Genistein is an isoflavonoid that has widely been used as a specific inhibitor of protein tyrosine kinase (1). Experimental evidences from recent investigation have shown that genistein not only inhibits protein tyrosine kinase but also other ATP-binding enzymes (2, 3). In addition, a variety of ionic channels in the cell membrane are susceptible to its blocking effects (4, 5).

In our previous study, we demonstrated that genistein directly inhibited transmembrane L-type calcium current in guinea pig ventricular myocytes (6). We also found that genistein could potentiate cardiac cystic fibrosis transmembrane regulator (CFTR) chloride current in the same experiment. Similar findings have been demonstrated in different experimental conditions that genistein can activate CFTR chloride current without any increase in intracellular content of 3′:5′-cyclic monophosphate (c-AMP) (7, 8).

In this study, we show that genistein, possibly by a tyrosine kinase-independent and protein kinase A-independent pathway, activates cardiac CFTR chloride current in guinea pig ventricular myocytes.

MATERIALS AND METHODS

Isolation of single ventricular myocytes. The investigation conformed with the Guiding Principles in the Care and Use of Animals as approved by the Council of the American Physiologic Society. Single ventricular myocytes were isolated from guinea pigs using enzymatic digestion method (9). Guinea pigs of either sex (300 to 500 g) were killed by cervical dislocation. The heart was immediately excised. After the aorta was cannulated, the heart was mounted onto a Langendorff apparatus. The coronary arteries were perfused retrogradely with oxygenated normal Tyrode solution at 37°C. Immediately after the blood was washed out, the perfusate was switched to the nominally calcium-free Tyrode solution for 5 min. Subsequently, collagenase (type A, Boehringer, Manheim, Germany; or type 2, Worthington, New Jersey, USA) 1 mg/ml and protease (type XIV, Sigma, St Louis, USA) 0.04 mg/ml in nominally calcium-free Tyrode solution were perfused for 2 to10 min. The heart was then shifted to perfusate with low-calcium (0.18 mM) enzyme-free Tyrode solution for 5 min. The left ventricle was dissected into small pieces, and gently agitated. The dispersed cells were stored in normal Tyrode solution for later use within 10 hours.

Voltage clamp and recording technique. The isolated single ventricular myocytes were placed in the recording chamber (2 ml in volume) which was mounted on an inverted microscope (IMT-2, Olympus, Japan), and perfused with Tyrode solution at a flow rate of 2 ml/min with different compositions as indicated. Whole-cell voltage clamp technique (10) was applied at room temperature. The suction pipette was made of borosilicate (2.00 mm o.d. and 1.25 mm i.d.,

[1] To whom correspondence should be addressed at Division of Cardiology, Veterans General Hospital-Taipei, 201, Sec 2, Shih-Pai Road, Taipei, Taiwan, R.O.C. Fax: 886-2-8735656. E-mail: cechiang@vghtpe.gov.tw.

0006-291X/97 $25.00

Jencons, Leighton Buzzard, UK) and pulled in a Flamming/Brown micropipette puller (model P-87, Sutter Instrument Company, Novato, USA). The tip of electrode was fabricated with a fire-polisher (MF-83 microforge, Narishige, Tokyo, Japan). The pipette tip resistance was 2 to 5 MΩ when filled with the pipette solution. The current was recorded using a patch clamp amplifier (Axopatch 1-D, Axon Instruments, Inc., Foster City, CA, USA). The cells were first perfused with modified Tyrode solution to record L-type Ca^{2+} current. The holding potential was −40 mV to inactivate sodium current. The membrane potential was depolarized to +10 mV for a duration of 300 ms, and then repolarized to −40 mV. A large L-type Ca^{2+} current was usually observed. The perfusate was then shifted to modified Tyrode solution to record CFTR chloride current, which contained Cd^{2+} ion (1 mM) and verapamil (20 μM) to block L-type Ca^{2+} current, and no Ca^{2+} ions were added. In some experiments, ramp test from −100 mV to +100 mV for a duration of 10 sec (20 mV/sec) was performed. Data were sampled at 2 kHz and filtered at 1 kHz (−3dB cutoff frequency) with an eight-pole Bessel-type filter (VBF/8.03, Kemo, Beckenham Kent, UK) and analyzed using pCLAMP software (6.03, Axon Instruments, Inc., Foster City, CA, USA). The data are expressed as mean ± standard error of the mean. The data before and after intervention were compared by paired Student's *t*-test. A p value $< .05$ was considered statistically significant.

Solutions and drugs. The normal Tyrode solution for isolation of single ventricular myocytes contained (in mM): NaCl 137, KCl 4, $MgCl_2$ 0.5, $CaCl_2$ 1.8, HEPES 10, glucose 5.5 (pH = 7.4, titrated with NaOH). External calcium was omitted in nominally calcium-free Tyrode solution. Low calcium-Tyrode solution contained 0.18 mM calcium. The modified Tyrode solution to record L-type Ca^{2+} current contained (in mM): NaCl 137, CsCl 4, $MgCl_2$ 0.5, $CaCl_2$ 3.6, HEPES 11.8, glucose 5.5. The modified Tyrode solution to record CFTR chloride current contained (in mM): NaCl 137, CsCl 4, $MgCl_2$ 0.5, $CdCl_2$ 1, HEPES 11.8, glucose 5.5 and verapamil 20 μM. All the perfusates were titrated with NaOH to pH=7.4. The pipette solution used for recording either the L-type Ca^{2+} current or the CFTR chloride current contained (in mM): CsCl 120, TEA-Cl 20, MgATP 5, EGTA 20, HEPES 10 (pH = 7.2, titrated with CsOH). Possible contamination from Na/Ca exchange current was ruled out by omitting intracellular Na^+ and Ca^{2+} and chelating Ca^{2+} with 20 mM EGTA in the pipette solution. Na/K pump current was inhibited by either ouabain or omitting Na^+ and K^+ on both sides of cell membrane. Tetrodotoxin-sensitive Na^+ current was inactivated by holding the membrane potential at −40 mV or replacing the NaCl in the perfusate with equimolar Tris-Cl in some experiments. Tetrodotoxin was also added in some experiments.

Forskolin, IBMX, genistein, daidzein, H-8, H-9, H-89 (Research Biochemicals International, Natick, MA, USA), tyrphostin 51 (Sigma, St. Louis, Missouri, USA), and calyculin A (Gibco BRL Life Technologies, Inc., Grand Island, NY, USA) were prepared in DMSO. Possible vehicle effect was excluded when concentration of DMSO was less than 0.1%.

RESULTS

Genistein Alone Activates Chloride Current

As shown in Figure 1 (a, b), genistein (50 μM) itself directly activated an inward current at −40mV. The induced current was very similar to that induced by isoproterenol (0.8 μM) and forskolin (10 μM) in the same cell (Fig. 1, c, d). Both induced currents had the reversible potentials approximately the same as the equilibrium potential for chloride ions (E_{Cl}= −1 mV). When the extracellular chloride concentration was changed by replacing it with aspartate, the reversible potential also followed the calculated equilibrium potential for the new chloride gradient. The induced current was presumed to be CFTR chloride current (see Discussion for more details). In the 80 cells tested, we could observe this phenomenon in 64 cells (80%). The mean peak current induced by 50 μM genistein was 1.55 ± 0.95 pA/pF ($p<0.001$). Figure 2 shows the concentration-dependent effect of genistein. The calculated IC_{50} was 39.7 μM.

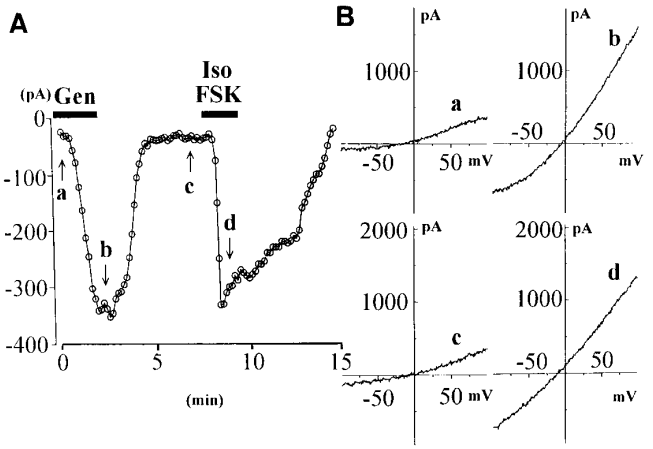

FIG. 1. Direct activation of CFTR chloride current by genistein. A. The time course of the inward shift of the holding current when the holding potential was −40 mV. B. The current-voltage curve obtained by ramp test at different time in panel A as indicated. The reversal potentials for both currents approximated the equilibrium potential of chloride ion (−1 mV). Gen: genistein (50 μM); Iso: isoproterenol (0.8 μM); FSK: forskolin (10 μM).

Genistein Activates the CFTR Chloride Current by a Tyrosine Kinase-Independent Pathway

Although the inactive analogue, daidzein (50 μM), could not induce CFTR chloride current (Fig. 3A), another tyrosine kinase inhibitor, tyrphostin 51 (50 μM), could not induce the current either (Fig. 3B), suggesting that the induction of CFTR chloride current by genistein might be due to other mechanisms not related to tyrosine kinase inhibition.

Genistein Induces CFTR Chloride Current at Least Partly by a Protein Kinase A-Independent Pathway

In the presence of maximally elevated intracellular content of cAMP by saturating doses of isoproterenol (0.8 μM), forskolin (50 μM), and IBMX (10 μM), genistein still could augment the current significantly with a mean increase of 47.9 ± 10.1% (n=5, $p<0.001$)(Fig. 4A). On the other hand, when the myocytes were pretreated with a specific protein kinase A inhibitor, H-89 (10 μM, 200 times the IC_{50} for inhibition of protein kinase A) (11), to completely inhibit protein kinase A, genistein could still induce it to a similar extent as the control value ($p > .05$) (Fig. 4B). Furthermore, H-89

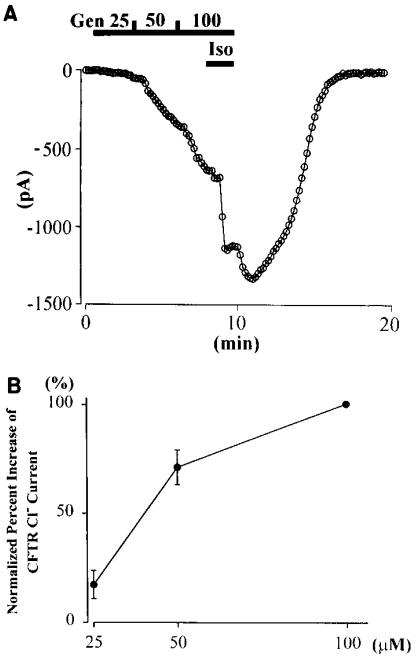

FIG. 2. A. The concentration-dependent activation of CFTR chloride current by genistein. The induced current by genistein could be further enhanced by isoproterenol (n=12). B. The dose-response curve of genistein's effect (n=12). We found that 100 μM genistein was the maximal dose while no further increase of the current could be observed beyond this concentration. Gen: genistein (μM); Iso: isoproterenol (0.8 μM).

(10 μM) in the pipette solution could not prevent the activation of the current by genistein. Similar findings were observed when other protein kinase inhibitors, such as H-8 (20 μM, n=5) or H-9 (60 μM, n=6), were administered in the perfusate or in the pipette solution. The above findings suggest that genistein might activate CFTR chloride current, at least in part, by a protein kinase A-independent pathway.

The Effect of Genistein Is Not Due to Inhibition of Calyculin A-Sensitive Dephosphorylation Process

To test whether genistein inhibits the dephosphorylation process of the phosphorylated CFTR chloride channels, the effect of calyculin A, a specific inhibitor of phosphotase 1 and 2A, was examined. Saturating concentration of calyculin A, 20 nM (12), could not impair the direct activation of the chloride current by genistein (Fig. 5A), nor could it prevent the potentiation effect of genistein (Fig. 5B). Similar phenomena were observed when calyculin A was administered in the pipette solution.

DISCUSSION

We demonstrated that genistein, once esteemed as a specific blocker of protein tyrosine kinase, could activate CFTR chloride current in guinea pig ventricular myocytes by a tyrosine kinase-independent pathway. We also found that the increase of CFTR chloride current by genistein might involve, at least in part, a protein kinase A-independent pathway.

We believe that the induced current is CFTR chloride current itself by the following reasoning (13). It should not be a Ca^{2+}-activated chloride current because there are 20 mM EGTA in the pipette solution that the intracellular Ca^{2+} concentration should readily be buffered to extremely low level (14). Besides, there are adequate amount of Cd^{2+} and verapamil in the perfusate making Ca^{2+} induced-Ca^{2+} release an unlikely phenomenon. The isotonic condition on either side of the cell membrane suggests that it is not a swelling-induced chloride current (15). Furthermore, genistein has never been shown to have the capability of activating protein kinase C (PKC), so the induced current should not be PKC-activated chloride current (1).

Genistein has been widely used as a specific protein tyrosine kinase inhibitor for more than a decade (1). However, increasing evidences have shown that genistein can also inhibit a variety of enzymes, such as S6 kinase (2), histidine kinase (3), phospholipase C (16), and topoisomerase II (17). In addition, muscarinic receptor (18), purinergic receptor (19) and other intracellular biochemical process (20) all are potential targets for genistein. Recently, the ionic channels in the cell

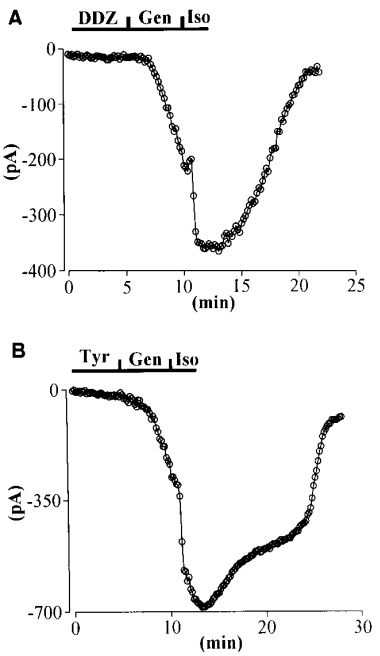

FIG. 3. The effect of daidzein and tyrphostin 51. A. Daidzein, an inactive analogue of genistein, could not activate CFTR chloride current (n=15). B. Tyrphostin 51, another potent inhibitor of tyrosine kinase, could not activate CFTR chloride current either (n=6). DDZ: daidzein (50 μM); Gen: genistein (50 μM); Iso: isoproterenol (0.8 μM); Tyr: tyrphostin (50 μM).

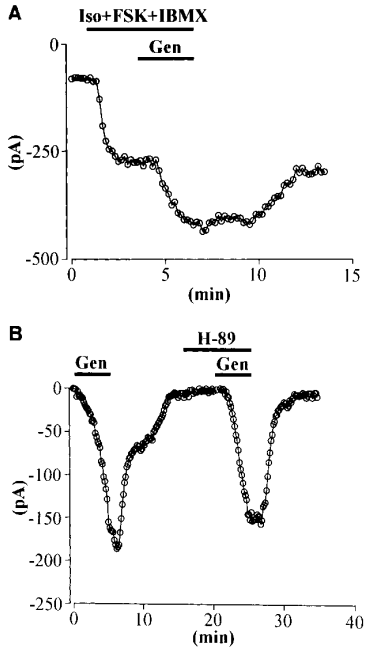

FIG. 4. The role of protein kinase A-cascade in the effect of genistein. A. When the intracellular content of cAMP was maximally elevated by saturating concentration of isoproterenol, forskolin and IBMX, genistein could further enhanced the current (n=5). B. When the cells were pretreated with saturating concentration of H-89 (10 μM), a specific inhibitor of protein kinase A, genistein still activated the chloride current, almost to the same extent as in the control condition (n=5). Gen:genistein (100 μM).

membrane have been shown to be susceptible to genistein's effect (4-6).

Lehrich et al. (7) demonstrated that genistein could increase chloride secretion in shark rectal gland and provided the first evidence for an inhibitory effect of tyrosine phosphorylation in the regulation of chloride transport through CFTR-like channels. In the meantime, Illek et al. (8) and Sears et al. (21) had similar findings that genistein could activate CFTR chloride current by a cAMP-independent pathway in different cell lines and concluded that tyrosine kinase-dependent pathway might be involved in the regulation of CFTR chloride channels in their experimental systems. However, the ensuing studies showed that the effect of genistein was probably due to its inhibitory action on protein phosphotase (22, 23) or on the dephosphorylation process involved in the regulation of CFTR chloride channels (12), which had nothing to do with tyrosine kinase inhibition.

We first demonstrated that in guinea pig ventricular myocytes genistein can potentiate CFTR chloride current (6). Shuba et al. (13) later provided evidence that genistein but not daidzein could activate cardiac CFTR chloride current in guinea pig ventricular myocytes and concluded that inhibition of tyrosine phosphorylation by genistein caused an activation of cardiac CFTR chloride current. However, they did not test the effect of other tyrosine kinase inhibitors. In the present study, we showed that another potent tyrosine kinase inhibitor, tyrphostin 51 (24), was unable to activate CFTR chloride current, suggesting that genistein's effect probably is not related to tyrosine kinase inhibition. Since genistein has been demonstrated to have multiple biologic activities, it is not surprising that it can activate CFTR chloride current by other mechanisms.

In the present study, when we maximally increased the intracellular cAMP content by saturating concentration of isoproterenol, forskolin, and IBMX, genistein could further increase the current. Surprisingly, when we applied H-89 (11), a highly specific inhibitor of protein kinase A, in the perfusate or intracellularly with a dose of 200 times its IC_{50} of inhibition to completely inhibit protein kinase A, genistein again was able to augment the current. Similar findings were observed when other protein kinase inhibitors such as H-8 or H-9 were administered (25). In view of the growing evidences showing genistein would neither increase intracellular cAMP content (22, 23) nor activate protein kinase A (1), combining with the findings in this study, we propose that genistein can activate CFTR chloride current in mammalian cardiac myocytes by, at least in part, a protein kinase A-independent pathway.

Yang et al. (12) have shown that in Hi-5 insect cells genistein might inhibit calyculin A-insensitive dephosphorylation of CFTR chloride channels. We have similar findings here that pre-treatment with saturating concentration of calyculin A could not prevent the activation of CFTR by genistein. It is possible that genistein opens the channels directly or inhibits the dephosphorylation process of CFTR, which is not sensitive to calyculin A. Further study to examine the effect

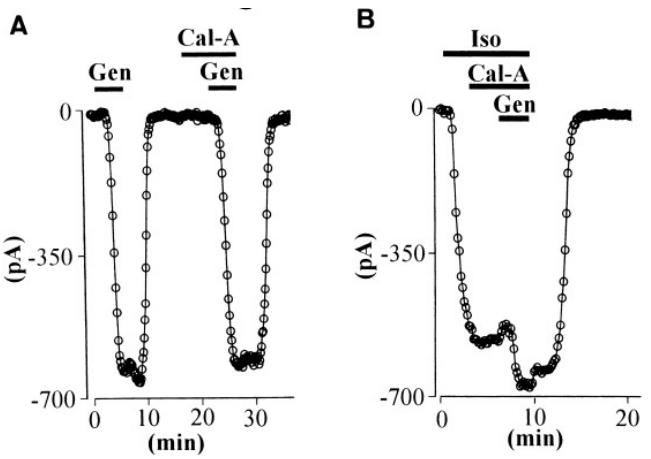

FIG. 5. The role of calyculin A sensitive dephosphorylation process in the effect of genistein. A. In the presence of maximal dose of calyculin A, genistein could induce similar extent of chloride current as compared with the control state (n=6). B. Maximal dose of calyculin A could not block the potentiation effect of genistein (n=6).

of genistein on the single channel recording by the giant patch technique will answer the question.

ACKNOWLEDGMENTS

The authors are grateful to the National Science Council (NSC-85-2331-B-016-111), National Health Research Institute (DOH-85-HR-311), National Institute of Chinese Medicine Taipei, Taiwan, R.O.C., and United World Chinese Commercial Bank Culture and Charity Foundation for their grant support.

REFERENCES

1. Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S-I., Itoh, N., Shibuya, M., and Fukami, Y. (1987) *J. Biol. Chem.* **262,** 5592–5595.
2. Linassier, C., Pierre, M., Le Pecq, J-B., and Pierre, J. (1990) *Biochem. Pharmacol.* **39,** 187–193.
3. Huang, J., Naasr, M., Kim, Y., and Matthews, H. R. (1992) *J. Biol. Chem.* **267,** 15511–15515.
4. Wijetunge, S., Aalkjaer, C., Schachter, M., and Hughes, A. D. (1992) Tyrosine kinase inhibitors block calcium channel currents in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **189,** 1620–1623.
5. Smirnov, S. V., and Aaronson, P. I. (1995) *Circ. Res.* **76,** 310–316.
6. Chiang, C-E., Chen, S-A., Chang, M-S., Lin C-I., and Luk, H-N. (1996) *Biochem. Biophys. Res. Commun.* **223,** 598–603.
7. Lehrich, R. W., and Forrest, J. N. (1995) *Am. J. Physiol.* **269,** F594–F600.
8. Illek, B., Fischer, H., Santos, G. F., Widdicombe, J. H., Machen, T. E., and Reenstra, W. W. (1995) *Am. J. Physiol.* **268,** C886–C893.
9. Mitra, R., and Morad, M. (1985) *Am. J. Physiol.* **249,** H1056–H1060.
10. Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) *Pfugers Arch.* **391,** 85–100.
11. Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T., and Hidaka, H. (1990) *J. Biol. Chem.* **265,** 5267–5272.
12. Yang, I. C. H., Cheng, T-H., Wang. F., Price, E. M., and Hwang, T-C. (1997) *Am. J. Physiol.* **272,** C142–C155.
13. Shuba, L. M., Asai, T., Pelzer, S., and McDonald, T. F. (1996) *Br. J. Pharmacol.* **119,** 335–345.
14. Zygmunt, A. C., and Gibbons, W. R. (1991) *Circ. Res.* **68,** 424–437.
15. Tseng, G. N. (1992) *Am. J. Physiol.* **262,** C1056–C1068.
16. Higashi, K., and Ogawara, H. (1992) *Chem. Pharm. Bull.* **40,** 157–160.
17. Markovits, J., Linassier, C., Fosse, P., Couprie, J., Pierre, J., Jacquemin-Sablon, A., Saucier, J-M., Le Pecq, J-B., and Larsen, A. K. (1989) *Cancer Research* **49,** 5111–5117.
18. Otero, A. S., and Sweitzer, N. M. (1993) *J. Pharmacol. Exp. Ther.* **44,** 595–604.
19. Okajima, F., Akbar, M., Majid, M. A., Sho, K., Tomura, H., and Kondo, Y. (1994) *Biochem. Biophys. Res. Commun.* **203,** 1488–1495.
20. Young, S. W., Poole, R. C., Hudson, A. T., Halestrap, A. P., Denton, R. M., and Tavare, J. M. (1993) *FEBS.* **316,** 278–282.
21. Sears, C. L., Firoozmand, F., Mellanger, A., Chambers, F. G., Eromar, I. G., Bot, A. G. M., Scholte, B., De Jonge, H. R., and Donowitz, M. (1995) *Am. J. Physiol.* **269,** G874–G882.
22. Illek, B., Fischer, H., and Machen, T. E. (1996) *Am. J. Physiol.* **270,** C265–C275.
23. Reenstra, W. W., Yurko-Mauro, K., Dam, A., Raman, S., and Shorten, S. (1996) *Am. J. Physiol.* **271,** C650–657.
24. Levitzki, A. (1990) *Biochem. Pharmacol.* **40,** 913–918.
25. Casnellie, J. E. (1991) *Adv. Pharmacol.* **22,** 167–205.