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Activation of cardiac chloride conductance by the tyrosine kinase inhibitor, genistein

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1 Genistein (GST), an inhibitor of protein tyrosine kinase (PTK), Na_3VO_4 (VO_4), an inhibitor of phosphotyrosine phosphatase (PTPase), and forskolin (FSK), an activator of the cyclic AMP-dependent, cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel, were applied to guinea-pig ventricular myocytes to probe for a possible role of tyrosine phosphorylation in the regulation of cardiac Cl^- channels.

2 Myocytes in the standard whole-cell configuration were pulsed to various potentials and Cl^- current (I_{Cl}) measured as the difference from control background current. GST (1-500 μM) activated a current that had similar biophysical properties (time- and voltage-independent; Cl^--dependent reversal potential and outward rectification) as I_{Cl} activated by 5 μM FSK. The EC_{50} for activation of Cl^- conductance (g_{Cl}) by GST was approximately 100 μM, and g_{Cl} activated by GST (500 μM) was as large as g_{Cl} activated by maximally-effective FSK (5 μM). Daidzein, a GST analogue with little effect on PTK, was at least one order less effective than GST.

3 GST responses were rapidly and reversibly inhibited by 0.1-1 mM VO_4 treatments that had little effect on FSK-activated I_{Cl}.

4 Niflumic acid (100-200 μM) reversibly depressed GST (100 μM)-activated g_{Cl} by 55%.

5 GST (50 μM) strongly incremented current in myocytes with cyclic AMP-dependent CFTR I_{Cl} already activated by maximally-effective FSK 5 μM.

6 Based on these results, and on evidence of a synergistic interaction between GST and FSK, we conclude that inhibition of tyrosine phosphorylation by GST causes an activation of cardiac CFTR that is not mediated by an elevation of cyclic AMP.

Keywords: Guinea-pig ventricular myocytes; Cl^- current; genistein; daidzein; forskolin; niflumic acid; protein tyrosine kinase; protein kinase A; orthovanadate; CFTR

Introduction

Genistein (GST) is an isoflavonoid that inhibits both soluble and membrane-bound protein tyrosine kinase (PTK) (Ogawara *et al.*, 1986; Akiyama *et al.*, 1987). The inhibitor has a number of properties that make it a useful tool for the investigation of tyrosine kinase involvement in cellular events. It is active on external application, reversible, and has little effect on adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase A (PKA) and protein kinase C (PKC) (Akiyama *et al.*, 1987; Akiyama & Ogawara, 1991; Hidaka & Kobayashi, 1992).

GST (30-100 μM) has been shown to inhibit a variety of membrane ion channels. These include voltage-gated K^+ channels in human T lymphocytes (Hess *et al.*, 1992) and rat vascular smooth muscle cells (Smirnov & Aaronson, 1995), as well as Ca^{2+} channels in human T lymphocytes (Hess *et al.*, 1992), swine carotid media tissues (Gould *et al.*, 1995), and isolated smooth muscle cells (Wijetunge *et al.*, 1992; Kusaka & Sperelakis, 1995). GST also inhibited the activation of swelling-activated Cl^- conductance in dog atrial myocytes (Sorota, 1995). However, this drug is not an indiscriminate channel inhibitor; it had no effect on K^+ current carried by a delayed rectifier K^+ channel expressed in oocytes, nor did it suppress Ca^{2+}-activated Cl^- current (I_{Cl}) in these cells (Huang *et al.*, 1993). In fact, GST and related compounds may have stimulatory actions on some ionic channels, including the PKA-regulated cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. The secretory activity of CFTR-expressing T84 cells was enhanced by 100 μM quercetin, a GST-related dietary flavanol (Nguyen *et al.*, 1991), and 50 μM GST transiently increased ^{125}I efflux in 3T3 cells transfected with CFTR (Illek *et al.*, 1995).

We report that GST activates I_{Cl} in guinea-pig ventricular myocytes, and that this action is antagonized by Na_3VO_4 (VO_4), an inhibitor of phosphotyrosine phosphatase (PTPase) (Swarup *et al.*, 1982; Gordon, 1991). Comparison of I_{Cl} activated by GST and I_{Cl} activated by forskolin (FSK) suggests that the channel activated by GST is the cyclic AMP-dependent CFTR channel.

Methods

Cell isolation

In accord with national and local regulations on animal experimentation, guinea-pigs (ca. 300 g) were killed by cervical dislocation, and excised hearts sequentially perfused (37°C) with oxygenated normal Tyrode solution, Ca^{2+}-free Tyrode solution ($CaCl_2$ omitted), Ca^{2+}-free Tyrode containing collagenase (0.05-0.1 mg/ml^{-1}; Yakult, Tokyo, Japan), and storage solution. The ventricles were cut into chunks, and cells dispersed by mechanical agitation and kept in storage solution at room temperature. All experiments were carried out within 12 h of cell isolation.

Electrophysiology

An aliquot of storage solution containing myocytes was transferred to the experimental chamber positioned on top of an inverted microscope stage (Nikon Diaphot, Tokyo, Japan). The chamber was perfused with normal Tyrode solu-

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tion heated to 35–36°C. Pipettes were pulled from thick-walled borosilicate glass capillaries (Jencons, Bedfordshire, U.K.) in the usual two-step process (Hamill *et al.*, 1981). They had an inside tip diameter of 2–4 μm and resistance 2–3 MΩ when filled with pipette solutions. The voltage-clamp amplifier was an EPC-7 (List Medical Electronic, Darmstadt, Germany), and a flowing 3 M KCl, Ag-AgCl reference electrode was used to minimize changes in liquid junction potential. Currents and voltages were recorded on a video cassette recorder through an A/D PCM-2-B adapter (Medical Systems Corp., Greenvale, NY, U.S.A.) prior to computer analysis with pCLAMP 6 software (Axon Instruments, Inc., Foster City, CA, U.S.A.). Currents were filtered at 2 kHz and digitized at 5 kHz.

Solutions

Myocytes were superfused with K^+-, Ca^{2+}-free Tyrode solution that contained (mM) NaCl 140, $MgCl_2$ 1.15, glucose 10, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) 10, $CdCl_2$ 0.2, and $BaCl_2$ 1 (pH 7.4 with NaOH). The standard pipette solution contained (mM) CsCl 30, Cs aspartate 110, MgATP 5, ethylene glycol-*bis*(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) 5, and HEPES 5 (pH 7.2 with CsOH). In some experiments, the Cl^- concentration of the pipette solution was reduced to 10 or 20 mM (replacement with aspartate), or elevated to 40, 50, 115 or 130 mM (replacement of aspartate).

The storage solution contained (mM): KCl 30, KOH 80, glutamic acid 50, KH_2PO_4 30, $MgSO_4$ 3, taurine 20, glucose 10, EGTA 0.5, and HEPES 10 (pH 7.4 with KOH).

Drugs

GST, daidzein, FSK, and niflumic acid were prepared as stock solutions (100 mM, 100 mM, 10 mM, and 100 mM) in dimethyl sulphoxide (DMSO), and stored at −20°C. Appropriate amounts of stock solutions were added to external solutions, and corresponding amounts of DMSO (≤0.05% in most cases; 0.5–1% when GST ≥ 200 μM, daidzein 500 μM, or FSK 100 μM were used) were also added to the control external solutions. Aqueous stock (100 mM) solutions of Na_3VO_4 were freshly prepared and pH was adjusted to ~10 (cf. Gordon, 1991). Appropriate amounts of stock solution were added to the superfusate just before use, and the pH readjusted to pH 7.4 with NaOH. All agents were purchased from Sigma (St. Louis, MO, U.S.A.) with the exception of daidzein (Calbiochem (La Jolla, CA, U.S.A.)) and Na_3VO_4 (Fisher Scientific (Nepean, ON, Canada)).

Statistics

Results are expressed as means ± s.e. mean. Comparisons were made by one-way analysis of variance (ANOVA) or Student's two-tailed paired *t* test. A difference was considered to be significant when $P < 0.05$.

Results

I_{Cl} in the guinea-pig ventricular myocytes was investigated under conditions expected to minimize K^+ currents (K^+-free, Ba^{2+}-containing superfusate; K^+-free, Cs^+ dialysate), Na^+

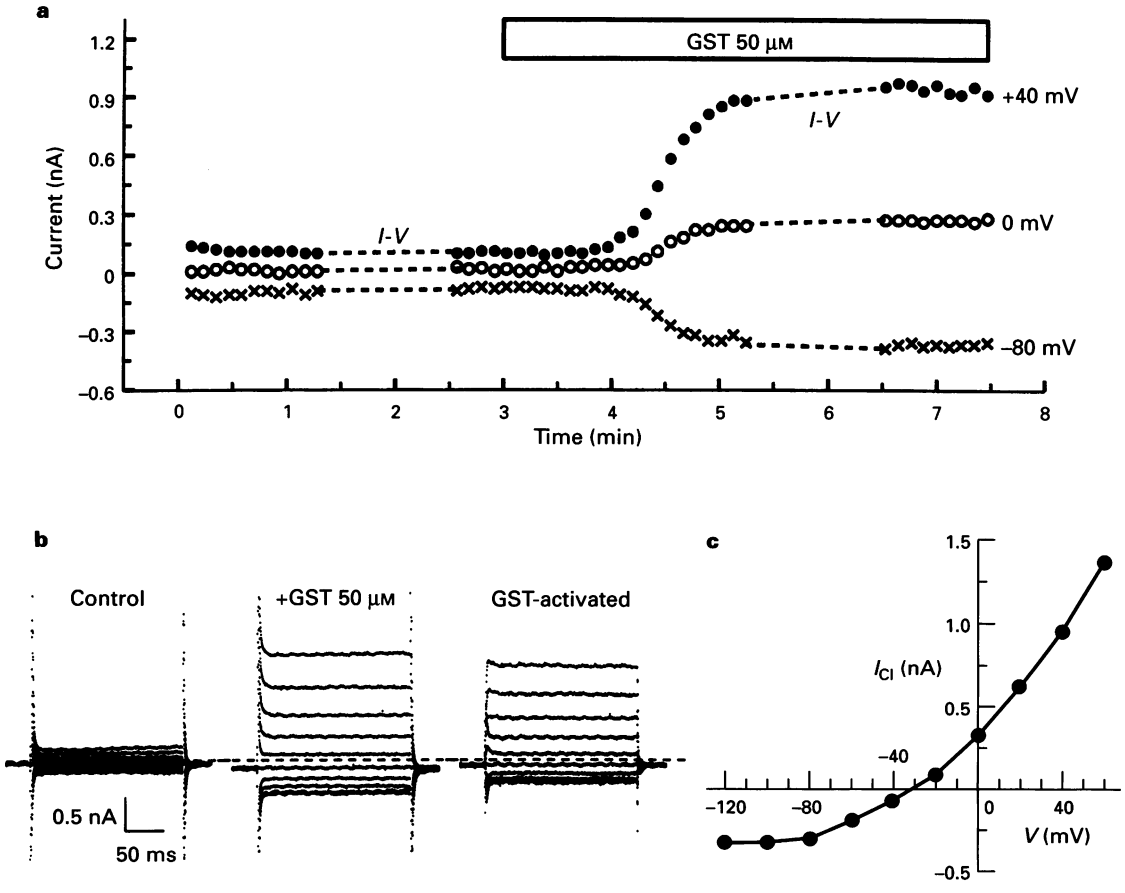

Figure 1 Activation of I_{Cl} by GST 50 μM. (a) Time course of GST-induced changes in membrane current monitored at −80, 0 and +40 mV. Time in this and other figures refers to post-patch-breakthrough. (b) Current records from the experiment in (a). The currents were elicited by 200 ms test pulses from prepulse −40 to voltages between +60 mV (top traces) and −120 mV (bottom traces) in 20 mV increments. Control (pre-GST) currents (left) were subtracted from GST-stimulated currents (middle) to obtain GST-activated currents (right). The dashed line indicates zero current level. (c) The *I–V* relationship of the GST-activated current.

current (voltage protocol), Ca^{2+} currents (Ca^{2+}-free, Cd^{2+}-containing superfusate), Na^+-K^+ pump current (K^+-free superfusate; Na^+-free dialysate), and Na^+-Ca^{2+} exchange current (low Ca^{2+} dialysate). In most experiments, the external Cl^- concentration (Cl^-_0) was 145 mM, and the dialysate concentration was 30 mM, to give a calculated Cl^- equilibrium potential (E_{Cl}) of −42 mV. Currents were elicited by 100–200 ms depolarizations or hyperpolarizations applied at 0.1–0.2 Hz from prepulse −40 or 0 mV (−80 mV holding potential), and current-voltage ($I-V$) relationships were generated from mean current amplitudes measured during the last 20 ms of pulses.

Activation of I_{Cl} by GST

Figure 1 shows a representative response to GST (50 μM). After a lag of ~1 min, current monitored at −80, 0 and +40 mV increased to a new steady state within 2 min (Figure 1a). Currents elicited by 200-ms test pulses to potentials between −120 and +60 mV were essentially time-independent both before and during exposure to GST (Figure 1b). The $I-V$ relationship of the current activated by GST (GST-stimulated minus control background current) exhibited strong outward rectification and reversed at −32 mV (Figure 1c). This reversal potential (E_{rev}) is close to that expected for a Cl^--dominated current when E_{Cl} is −42 mV because cardiac Cl^- channels are moderately permeable to the other anion (110 mM aspartate) supplied in the dialysate (cf. Tseng, 1992; Vandenberg *et al.*, 1994; Shuba *et al.*, 1996b).

The two features of GST-activated current identified above, time-independent waveform on test pulses, and outward-going rectification in myocytes dialysed with low Cl^- solution, are well-established properties of cardiac Cl^- currents activated by PKA or PKC (Bahinski *et al.*, 1989; Harvey & Hume, 1989; Walsh & Long, 1994; Zhang *et al.*, 1994; Shuba *et al.*, 1996a). We compared the responses elicited by GST (50 μM) with those elicited by a maximally-effective concentration of FSK (5 μM) (cf. Tareen *et al.*, 1991; Hwang *et al.*, 1993). Myocytes were treated with GST or FSK for 3–5 min, and the $I-V$ relationships of the activated currents were normalized by setting I_{Cl} activated at +40 mV equal to 1.0. The resultant mean curves in Figure 2a (GST) and b (FSK) indicate that

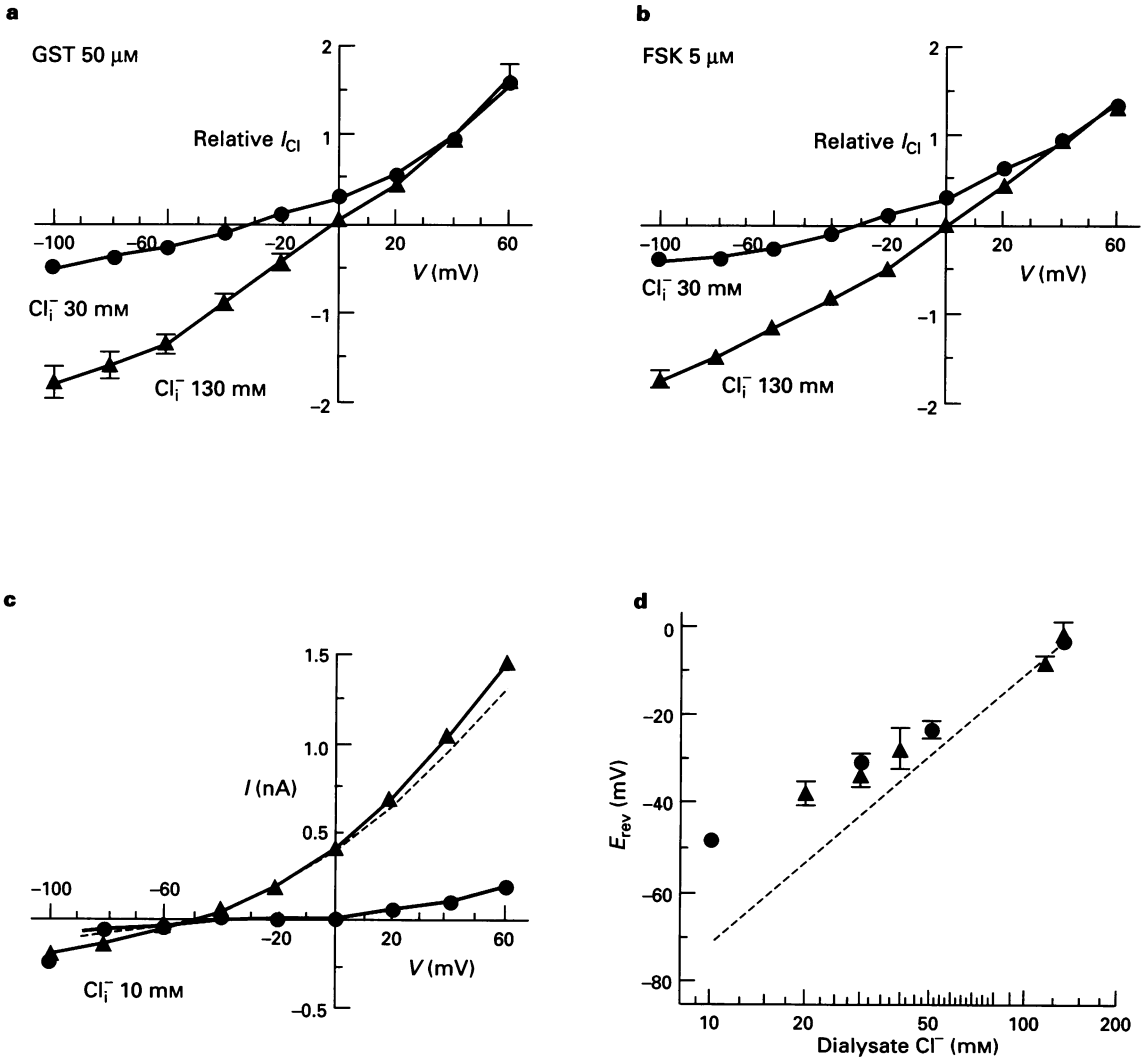

Figure 2 Properties of I_{Cl} activated by 50–100 μM GST or 5 μM FSK. (a) The $I-V$ relationships of currents activated by 3–5 min exposures to 50 μM GST. Currents at each potential were normalised relative to the amplitude of the current at +40 mV. The average results are from myocytes dialysed with solution that contained either 30 mM Cl^- (●, $n=13$) or 130 mM Cl^- (▲, $n=6$). (b) Normalised relationships from different myocytes dialysed with 30 mM Cl^- (●, $n=8$) or 130 mM Cl^- (▲, $n=7$) solutions and treated with FSK for 3–5 min. (c) $I-V$ relationships measured before (●) and 5 min after addition of 100 μM GST (▲) to a myocyte dialysed with 10 mM Cl^- solution. The dashed line is the difference current. (d) Dependence of the E_{rev} of activated current on dialysate Cl^- concentration: (●) 50–100 μM GST (10 mM Cl^-, $n=6$; 30 mM Cl^-, $n=13$; 50 mM Cl^-, $n=7$; 130 mM Cl^-, $n=6$); (▲) 5 μM FSK (20 mM Cl^-, $n=4$; 30 mM Cl^-, $n=8$; 40 mM Cl^-, $n=4$; 115 mM Cl^-, $n=6$; 130 mM Cl^-, $n=7$). The dashed line represents E_u calculated with the Nernst equation.

there was no significant difference in E_{rev} (-30 ± 1.7 mV ($n=13$), versus -33 ± 2.3 mV ($n=8$)). The degree of outward rectification (defined as chord conductance between E_{rev} and $E_{rev}+60$ mV, divided by chord conductance between E_{rev} and $E_{rev}-60$ mV) of the $I-V$ relationships was also similar (2.0 ± 0.1 versus 2.2 ± 0.2). In other myocytes that were dialysed with 130 mM Cl^- solution, the average degrees of outward rectification were much smaller (1.3 ± 0.12 (GST); 1.3 ± 0.03 (FSK)) than in the myocytes dialysed with 30 mM Cl^- solution (Figure 2a,b). This change in degree of outward rectification with high versus low Cl^- dialysate is similar to that previously reported by others for cyclic AMP-dependent I_{Cl} (e.g. Bahinski *et al.*, 1989; Overholt *et al.*, 1993).

Figure 2a,b indicates that the E_{rev}s of GST- and FSK-activated current in myocytes dialysed with 130 mM Cl^- solution were similar (-2 ± 1.6 mV and 0 ± 1.5 mM, $n=6,7$) and close to calculated E_{Cl} of -3 mV. The E_{rev} of GST-activated I_{Cl} was also measured in myocytes dialysed with 10 or 50 mM Cl^- solution. An example of $I-V$ relationships obtained from a myocyte dialysed with 10 mM Cl^- solution is shown in Figure 2c. The GST-activated current (dashed line) rectified strongly in the outward direction, and reversed at -52 mV. A summary plot of E_{rev} versus dialysate Cl^- concentration (Figure 2d) indicates that, with due account for the moderate permeability of aspartate, the reversal of GST-activated current (circles) is dictated by the Cl^- gradient in a manner similar to that of FSK-activated I_{Cl} (triangles).

Niflumic acid has been shown to inhibit a variety of anion transporters and Cl^- channels (e.g. Knauf & Mann, 1984; Gray & Ritchie, 1986; White & Aylwin, 1990; Hughes & Segawa, 1993; Janssen & Sims, 1993). We examined the effects of 100–200 μM niflumic acid on I_{Cl} activated by GST (100 μM), and an example of strong reversible inhibition of GST-activated I_{Cl} by 200 μM niflumic acid is shown in Figure 3a. The current monitored at 0 mV declined from 170 pA to 30 pA within 2 min of addition of the inhibitor, and recovered to 170 pA within 2 min of its removal. The chord Cl^- conductance (E_{rev} to 0 mV) (g_{Cl}) activated by GST was reduced from 5.6 ± 1.7 nS ($n=5$) to 2.6 ± 1.2 nS by 100 μM niflumic acid, and from 6.1 ± 1.5 nS ($n=4$) to 2.5 ± 1 nS by 200 μM inhibitor. Pooled niflumic acid g_{Cl} (2.6 ± 0.8 nS, $n=9$) was significantly smaller ($P<0.02$) than pooled control (5.8 ± 1.1 nS) (Figure 3b).

The magnitude of the I_{Cl} activated by GST was dependent on the concentration of the drug. Figure 4a illustrates that in myocytes that responded to GST (50 μM) with a relatively small activation of I_{Cl}, larger concentrations of the drug (e.g. 200 μM) were capable of a further activation of current. At the highest GST concentration tested (500 μM) (Figure 4c), the activation proceeded with a much shorter lag, and reached a plateau much more quickly, than with 50 μM GST.

Chord Cl^- conductance (E_{rev} to 0 mV) activated by the drug was measured in myocytes exposed to a single concentration of GST between 1 and 500 μM (Figure 5a). Concentrations <30 μM were relatively ineffective, whereas 500 μM activated a g_{Cl} of 9.4 ± 1.9 nS ($n=8$). The concentration-response curve in the figure is drawn according to the equation $g_{Cl}=g_{Cl(max)}\ /\ (1+(EC_{50}/[GST])^n)$ where maximal $g_{Cl}=9.5$ nS, $EC_{50}=103$ μM and slope factor $n=1.02$. An experimental detail that deserves mention here concerns the solubility of high concentrations of GST in the bathing solution. Avoidance of precipitation when solutions contained $\geqslant100$ μM GST required higher DMSO concentrations and a raising of pH (e.g. 1% DMSO and pH 7.8 for 500 μM GST) compared to solutions that contained $\leqslant50$ μM GST ($\leqslant0.05$% DMSO, pH 7.4). Although the addition of 1% DMSO during treatment with 50 μM GST had no effect on I_{Cl} at potentials between -100 and $+60$ mV ($n=6$), we cannot be certain that the combination of higher DMSO and pH did not influence responses to high concentrations of GST. However, we can be sure that 200 μM GST is significantly more potent than 50 μM in activating Cl^- conductance in given myocytes. Warmed 200 μM GST reservoir solution (0.2% DMSO, pH 7.5) that

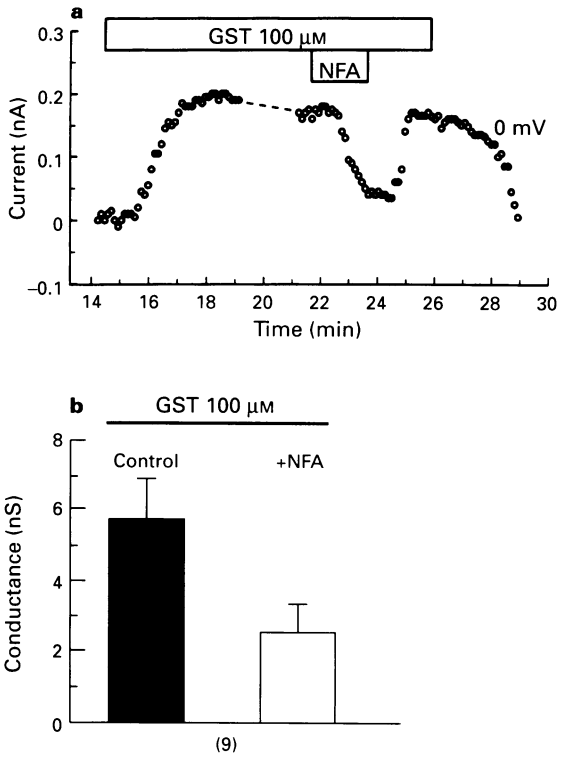

Figure 3 Inhibition of GST-activated current by niflumic acid. (a) Reversible inhibition by 200 μM niflumic acid (NFA) of current monitored at 0 mV. (b) Summary of results obtained with 3-min application of 100 or 200 μM niflumic acid. Chord Cl^- conductance (E_{rev} to 0 mV) activated by 100 μM GST was significantly reduced by the inhibitor ($P<0.02$). Number of myocytes in parentheses.

was essentially free of precipitate augmented I_{Cl} in myocytes pretreated with 50 μM GST (Figure 4a). In experiments on four myocytes pretreated with 50 μM GST, 200 μM GST increased g_{Cl} by 3.7 ± 0.5 nS. An additional concern with very high concentrations of GST is that the drug may inhibit protein kinases other than PTK (Akiyama *et al.*, 1987; Hidaka & Kobayashi, 1992). Any spillover inhibition of PKA or PKC by high GST in the myocytes investigated here would probably have led to an underestimation of $g_{Cl(max)}$ if the latter reflected activation of CFTR (see Discussion). Despite this possible underestimate, g_{Cl} activated by 500 μM GST (9.4 ± 1.9 nS, $n=8$) was as large as g_{Cl} activated by 5 μM FSK (8.3 ± 1.0 nS, $n=21$) (Figure 5b).

Experiments with daidzein and orthovanadate

To provide direction on whether GST was acting via an inhibition of PTK, we examined the effects of daidzein, a GST analogue that has minimal effects on PTK (Lavens *et al.*, 1992), as well as the effects of VO_4, an inhibitor of PTPase (Swarup *et al.*, 1982; Gordon, 1991; Hunter, 1995).

Daidzein was a relatively ineffective activator of g_{Cl}. Figure 6a shows one of the larger responses to 500 μM daidzein in a myocyte dialysed with 30 mM Cl^- solution. An 8-min application of the analogue provoked small reversible (<50 pA) increases in current at -80 and $+40$ mV. Compared to 50 μM GST (activation of 3.5 ± 0.6 nS, $n=33$), 50 μM daidzein was essentially inactive (0.4 ± 0.2 nS, $n=8$) (Figure 6b). At ten fold higher concentrations of the drugs, the conductances were 9.4 ± 1.9 nS ($n=8$) for GST, and 1.3 ± 0.5 ($n=7$) for daidzein.

External application of millimolar VO_4 suppresses a variety of GST effects thought to be related to inhibition of tyrosine phosphorylation in non-cardiac cells (Swarup *et al.*, 1982; Illek *et al.*, 1995). Figure 7a–c shows that 1 mM VO_4 inhibited I_{Cl} activated by GST (50 μM). The inhibition was rapid (<1 min),

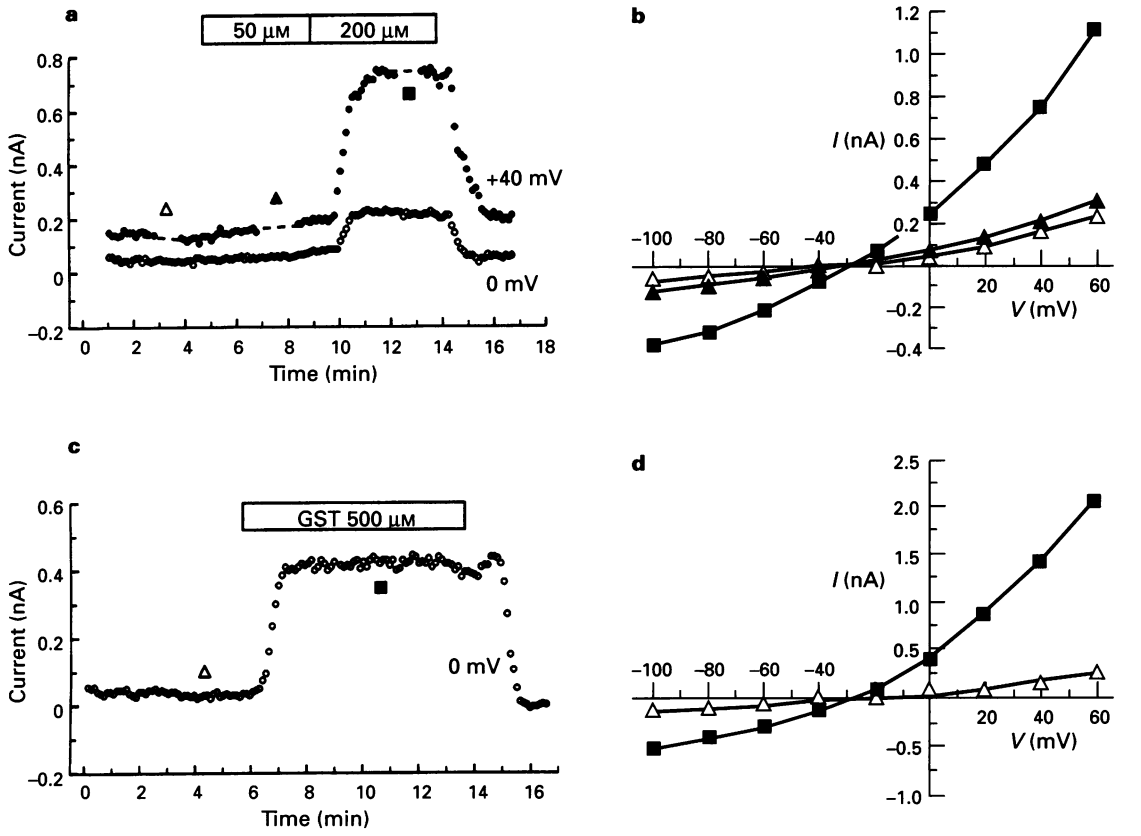

Figure 4 Activation of I_{Cl} by moderate and high concentrations of GST. (a) Modest response to 50 μM GST was followed by a large response to a 200 μM concentration. (b) $I-V$ relationships from this experiment. The symbols are keyed to the time plot in (a). (c) Large rapid activation of I_{Cl} by 500 μM GST. (d) $I-V$ relationships from the experiment in (c).

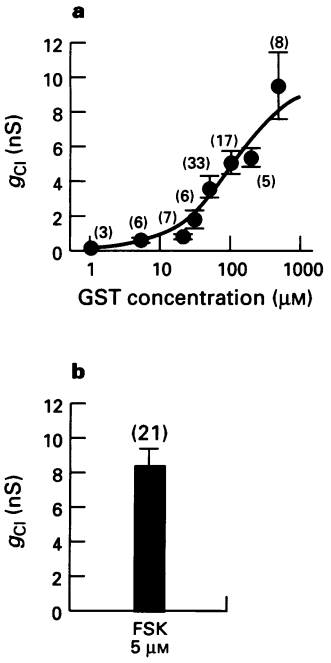

Figure 5 Dependence of Cl^- channel activation on the concentration of GST. (a) GST concentration-g_{Cl} (E_{rev} to 0 mV) relation. The data are from myocytes treated for 3–5 min with 1, 5, 20, 30, 50, 100, 200 or 500 μM GST. The number of myocytes is given in parentheses. The curve describing the data is $g_{Cl} = g_{Cl(max)}/(1+(EC_{50}/[GST])^n)$, where $g_{Cl(max)} = 9.5$ nS, $EC_{50} = 103$ μM, and $n = 1.02$. (b) Magnitude of the Cl^- conductance (E_{rev} to 0 mV) activated by 5 μM FSK in other myocytes.

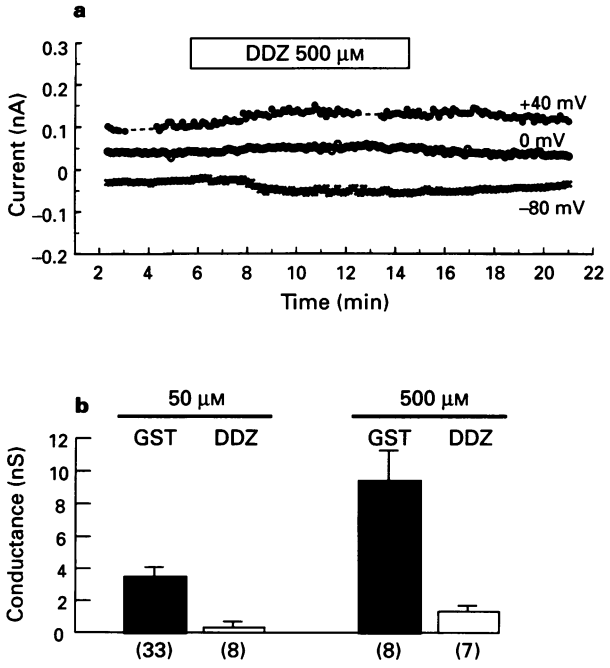

Figure 6 Ineffective activation of Cl^- conductance by the GST-analogue daidzein. (a) Effect of 500 μM daidzein (DDZ) on current monitored at −80, 0 and +40 mV (b) Chord Cl^- conductance (E_{rev} to 0 mV) measured from myocytes treated with 50 or 500 μM GST or daidzein. The number of myocytes is given in parentheses.

pronounced, and reversible (Figure 7a). We did not determine the full concentration-response relationship for VO_4, but established that the concentration required for half-maximal inhibition was <0.1 mM, i.e., g_{Cl} activated by 50 μM GST was reduced from 2.9 ± 0.8 to 0.7 ± 0.3 nS ($n=4$) after 3 min 0.1 mM VO_4, and from 4.1 ± 0.8 to 0.8 ± 0.5 nS ($n=7$) after 1 mM VO_4 (Figure 7d,e).

VO_4 did not inhibit I_{Cl} when the current was activated by FSK. Figure 8a,b shows that 2 min pretreatment with 1 mM VO_4 had little effect on background membrane conductance and did not prevent a large activation of I_{Cl} by 3 μM FSK. Neither subsequent washout of VO_4 in the continued presence of FSK, nor a second exposure to 1 mM VO_4, had any effect on the current activated by FSK. Addition of 1 mM VO_4 to six

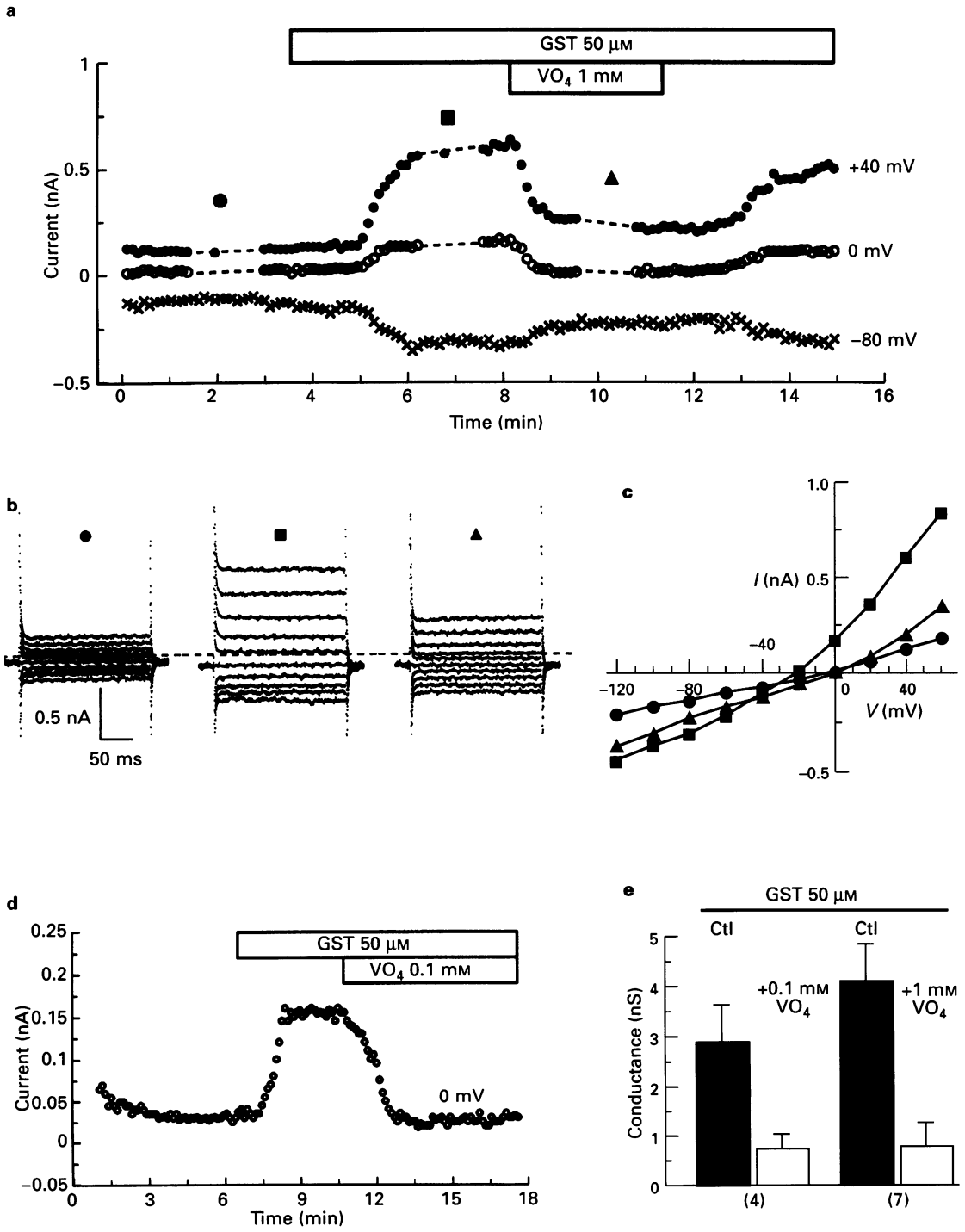

Figure 7 Reversible inhibition by orthovanadate of I_{Cl} activated by 50 μM GST. (a) Time course of activation by 50 μM GST and reversible inhibition by 1 mM VO_4. (b) Current records (+60 mV top, −120 mV bottom, 20 mV increments) obtained at the times indicated by the symbols referenced to (a). (c) $I-V$ relationships from the same experiment. The inhibition by VO_4 was probably larger than indicated, i.e., note the small leak (difference between control (●) and VO_4 (▲) centred at 0 mV) that developed during the experiment. (d) Example of the inhibition by 0.1 mM VO_4 of GST-activated current. (e) Summary of inhibition by 0.1 mM VO_4 ($n=4$) and 1 mM VO_4 ($n=7$). Myocytes were treated with 50 μM GST for 3–5 min for measurement of activated g_{Cl} (E_{rev} to 0 mV) and then with VO_4 for 2–3 min.

other myocytes treated with 3–5 μM FSK also had little effect; FSK-activated g_{Cl} was an insignificant 7.6±4.5% ($n=7$) smaller after 2 min VO_4.

Potentiation by GST of I_{Cl} activated by maximally-effective concentrations of FSK

GST had a pronounced stimulatory action on I_{Cl} preactivated by micromolar FSK. An example of one of the strongest activations by GST under these circumstances is shown in Figure 9. The myocyte was treated with FSK (1 μM) for 12 min, and this caused activation of a moderate-sized well-maintained current (0.7 nA total current at +40 mV) (last 5 min shown in Figure 9a). Addition of GST (50 μM) in the continued presence of 1 μM FSK boosted the current at +40 mV by an extremely large 2 nA within 2 min. Subsequent removal of FSK reduced the current by 1.8 nA within 4 min, despite the continued presence of GST. The current records (Figure 9b) and outwardly-rectifying $I-V$ relationships (Figure 9c) from this experiment indicate that all of the foregoing changes were centred around an E_{rev} of −32 mV. In terms of g_{Cl} (E_{rev} to 0 mV), FSK activated a conductance of 2.5 nS, GST increased it to 27.2 nS, and removal of FSK in the continued presence of GST reduced it to 4.7 nS.

To obtain unequivocal evidence that GST can augment I_{Cl} maximally activated by FSK, we performed two sets of experiments. In the first, myocytes were treated with 5 μM FSK for 3–5 min, and then with 100 μM FSK for a further 3–5 min. The experimental example in Figure 10a indicates that increasing FSK concentration to 100 μM did not activate a larger I_{Cl} than that achieved with 5 μM. In five myocytes, application of 100 μM FSK after pretreatment with 5 μM FSK had no significant effect on g_{Cl} (Figure 10b). In the second set of experiments, 50 μM GST was added after preactivation of I_{Cl} by 5 μM FSK. The representative time plot in Figure 10c shows that the PTK inhibitor incremented the FSK-activated current at 0 mV by approximately 45%. In nine myocytes, g_{Cl} increased from 9.5±2.3 nS to 12.7±2.8 nS after addition of 50 μM GST for 2–3 min ($P < 0.001$) (Figure 10d).

Discussion

GST activated a time-independent membrane current in guinea pig ventricular myocytes. This current was identified as a Cl^- current on the basis of its reversal near calculated E_{Cl}, and outward-going rectification dependent on dialysate Cl^-. The activation of the current by GST reached a steady state within several minutes, and deactivation of the current occurred over a similar time period upon removal of the drug. In all of these respects, the response elicited by GST was similar to that elicited by FSK. However, an important difference was detected

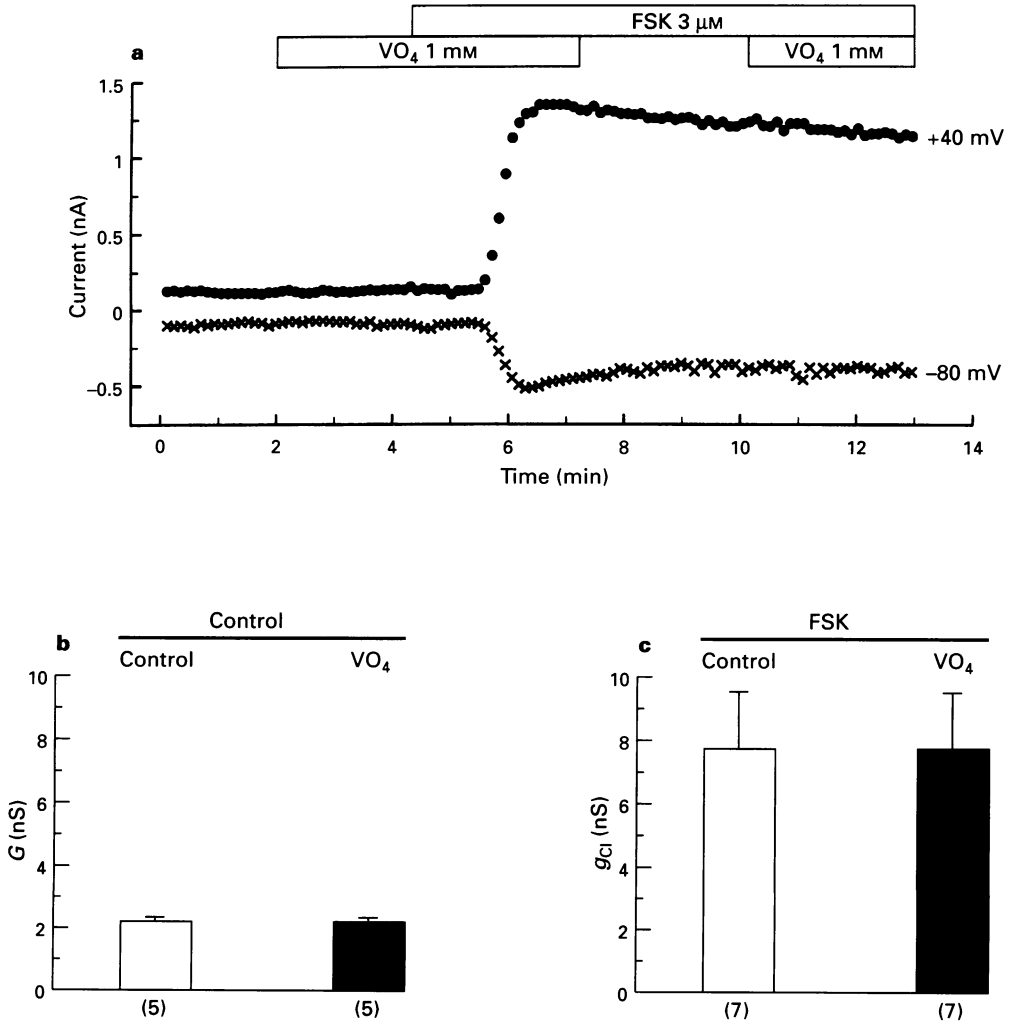

Figure 8 Lack of effect of 1 mM VO_4 on I_{Cl} activated by micromolar FSK. (a) VO_4 neither prevented nor inhibited activation of I_{Cl} by 3 μM FSK. (b) VO_4 (2–4 min exposure) had no effect on background chord (−80 to +40 mV) conductance under control conditions. (c) VO_4 had little effect on FSK-activated g_{Cl} (E_{rev} to 0 mV). VO_4 was added for 2 min after 3–5 min pretreatment with 3–5 μM FSK. Number of myocytes in parentheses.

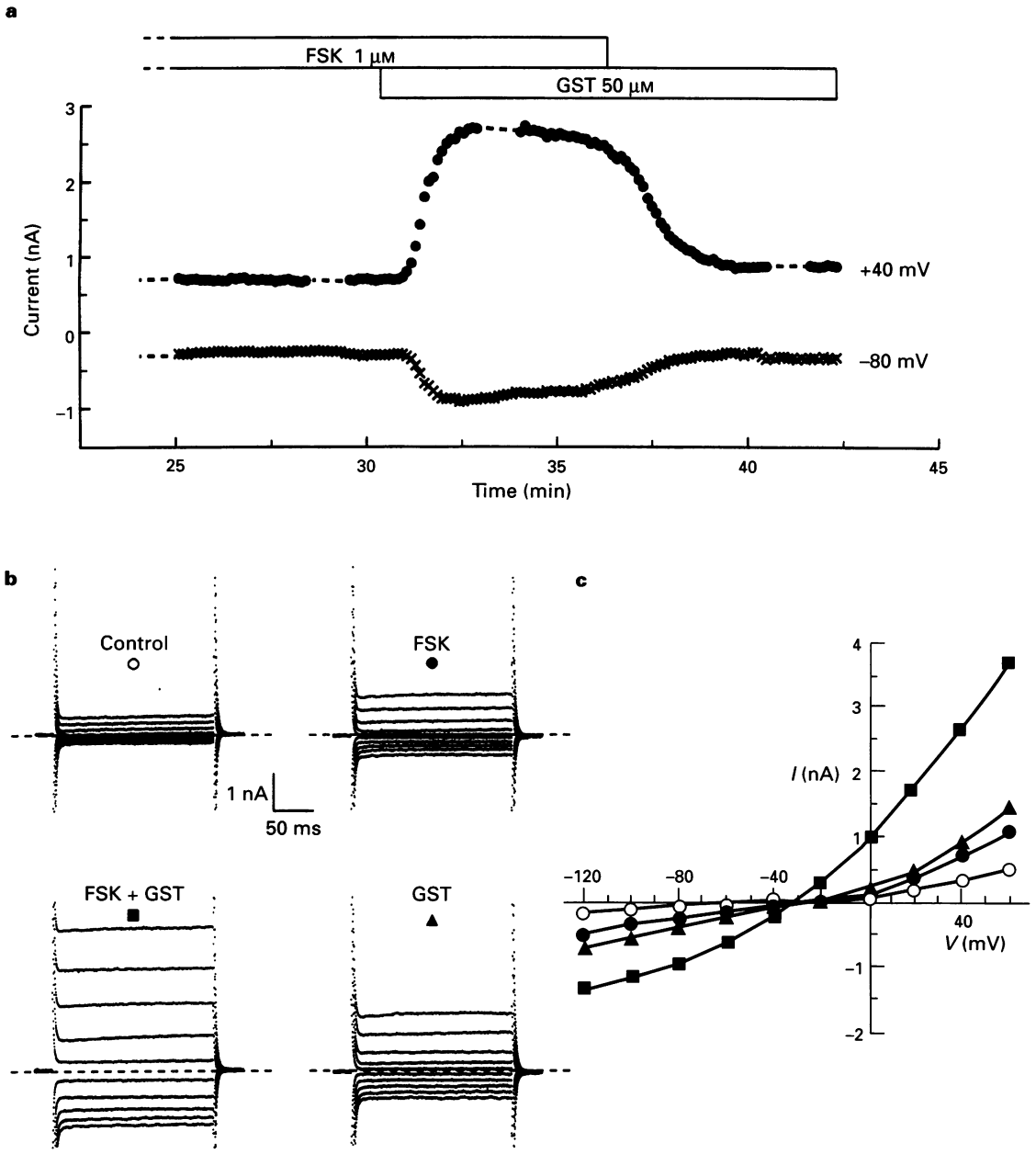

Figure 9 Synergistic activation of I_{Cl} by 50 μM GST and 1 μM FSK. (a) Time course of activation induced by GST, and deactivation caused by FSK withdrawal, in a myocyte stably activated by 12 min pretreatment with FSK (first 7 min not shown). (b) Current records (+60 mV top, −120 mV bottom, 20 mV increments) before FSK (control), just prior to addition of GST (FSK), after 3 min GST (FSK + GST), and 5 min after removal of FSK (GST). (c) $I-V$ relationships keyed to the symbols in (b).

by the use of 0.1 – 1 mM VO_4: GST-induced I_{Cl} was reversibly inhibited by this PTPase inhibitor, whereas FSK-induced I_{Cl} was unaffected. A further indication that the activation of current by GST is distinct from the activation by FSK was that GST strongly augmented I_{Cl} in myocytes with I_{Cl} already activated by a maximally-effective concentration of FSK.

These results raise a number of important issues. The primary ones are whether the activation of cardiac I_{Cl} by GST involves an inhibition of tyrosine phosphorylation, and whether this results in an interaction with the cyclic AMP pathway that culminates in enhanced opening of cyclic AMP-dependent CFTR Cl^- channels.

Involvement of tyrosine phosphorylation

GST inhibits both soluble and membrane-bound PTK (Ogawara *et al.*, 1986), but is far less active against other known kinases, including PKA and PKC (Akiyama *et al.*, 1987; Akiyama & Ogawara, 1991; Hidaka & Kobayashi, 1992). In view of this profile, cellular responses evoked by application of external GST are commonly attributed to an inhibition of tyrosine phosphorylation (e.g. Hidaka & Kobayashi, 1992; Lavens *et al.*, 1992; Huang *et al.*, 1993; Gould *et al.*, 1995; Illek *et al.*, 1995). However, GST may also have effects unrelated to inhibition of PTK. For example, Smirnov & Aaronson (1995) have concluded that the inhibition of K^+ current in vascular smooth muscle cells by GST may be due to a direct blocking action of the drug on K^+ channels.

It seems unlikely that the activation of I_{Cl} by GST in guinea-pig ventricular myocytes was due to a non-specific effect of the drug. The primary reasons for this view are that (i) daidzein, a structural analogue that has little inhibitory action on PTK (Lavens *et al.*, 1992), failed to activate large I_{Cl}, and (ii) VO_4, an inhibitor of PTPase (Swarup *et al.*, 1982; Gordon, 1991;

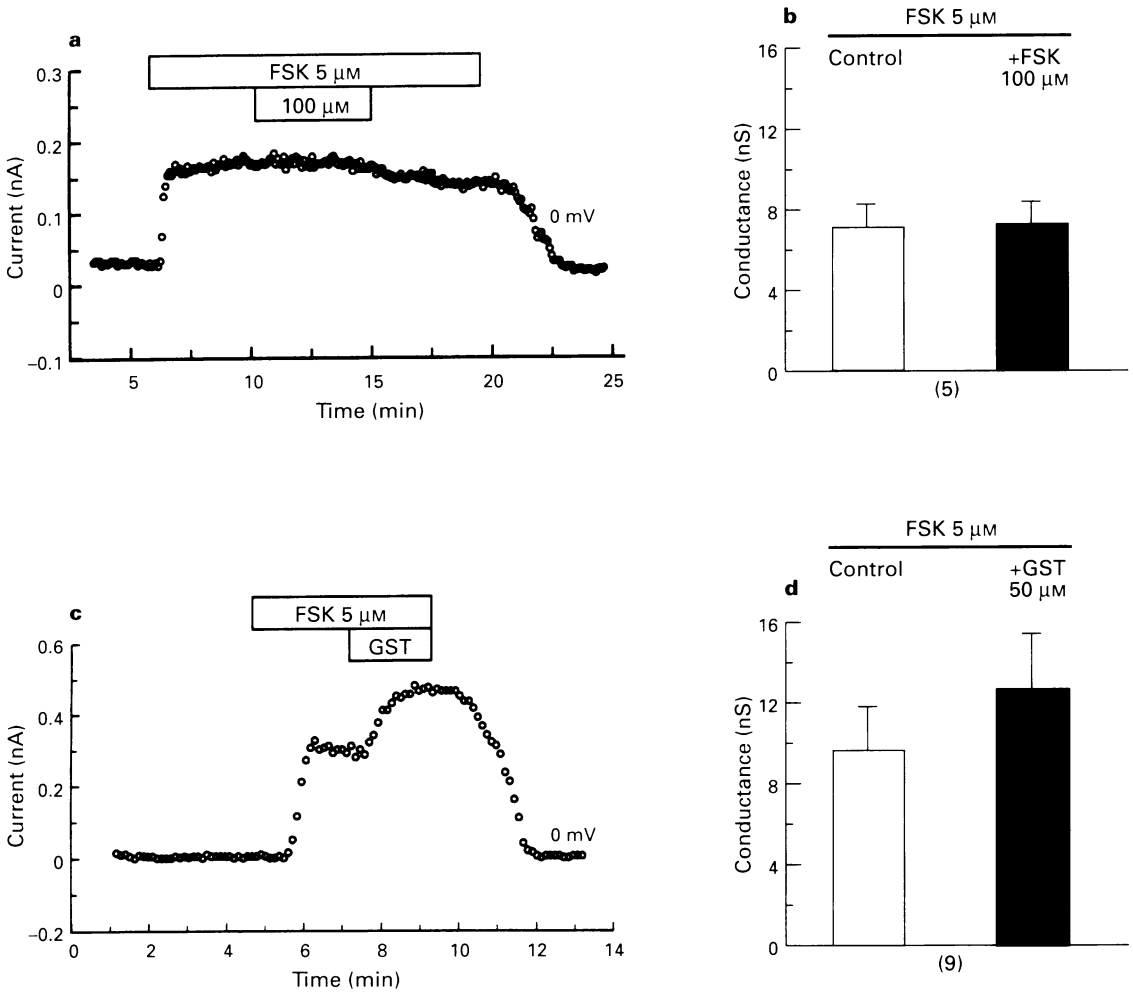

Figure 10 I_{Cl} activated by a maximally-effective 5 μM concentration of FSK is further activated by 50 μM GST. (a,b) Lack of effect of 100 μM FSK on I_{Cl} (0 mV) and g_{Cl} (E_{rev} to 0 mV) activated by 5 μM FSK. (c) Typical example of additional activation of I_{Cl} by 50 μM GST in a myocyte pretreated with 5 μM FSK. (d) Graph indicating the degree of additional activation of g_{Cl} (E_{rev} to 0 mV) by GST ($P < 0.001$). The myocytes were pretreated with 5 μM FSK for 2–3 min just before (control) and 2–3 min after addition of GST. Number of myocytes in parentheses.

Hunter, 1995), rapidly and reversibly inhibited GST-induced activation of I_{Cl}. This effect of VO_4 was not of an indiscriminate Cl^--channel-blocking nature because the vanadate compound had no significant inhibitory effect on I_{Cl} activated by FSK. An important point here is that Gadsby and colleagues (e.g. Baukrowitz *et al.*, 1994; Gadsby *et al.*, 1995) have previously shown that VO_4 can enhance PKA-mediated Cl^- conductance by locking activated channels in the open position. In those studies, 1–5 mM VO_4 was applied to the cytoplasmic face of membrane patches excised from guinea-pig ventricular myocytes. In the present case, 1 mM VO_4 was applied externally, and it had little effect on the FSK-activated I_{Cl}. One explanation is that the channel-locking effect requires millimolar internal VO_4, a cytoplasmic concentration unlikely to have been reached during the short external applications of 0.1–1 mM VO_4 used in the present study.

The EC_{50} for activation of g_{Cl} by GST was 103 μM (Figure 5a). This value is about five times higher than the concentration determined by Akiyama *et al.* (1987) for half-maximal inhibition of PTK phosphorylation. This discrepancy may be due to limited membrane permeability because (i) Akiyama *et al.* (1987) also reported that the IC_{50} for inhibition of epidermal growth factor receptor kinase was 111 μM when assessed in intact A431 cells, and (ii) the EC_{50} for inhibition of L-type Ca^{2+} current in smooth muscle cells is near 50 μM (Wijetunge *et al.*, 1992; Kusaka & Sperelakis, 1995). A further possible explanation for the high EC_{50} in the present study is that the pertinent PTK may be relatively insensitive to GST (cf. Geissler *et al.*, 1990)

Involvement of cyclic AMP

Although I_{Cl} in guinea-pig ventricular myocytes dialysed with EGTA solution under isosmotic conditions can be activated by stimulating non-cyclic AMP intracellular cascades (Matsuura & Ehara, 1992; Kaneda *et al.*, 1994; Walsh & Long, 1994; Shuba *et al.*, 1996a), the magnitude of the activation is far smaller than that observed with stimulation of the cyclic AMP pathway (e.g. Shuba *et al.*, 1996a) or than that reported here for GST. The potent effect of GST therefore raises the question of whether the activation of I_{Cl} by GST is due to an elevation of cyclic AMP which, in theory, could be caused by a stimulation of cyclic AMP formation or a retardation of its breakdown. In this regard, biochemical measurements have shown that 50 μM GST has no effect on basal (or FSK-stimulated) cyclic AMP levels in either rabbit parietal cells (Tsunoda *et al.*, 1993) or 3T3 cells (Illek *et al.*, 1995), and Akiyama & Ogawara (1991) reported that 370 μM GST had no effect on phosphodiesterase activity under *in vitro* conditions. While these findings suggest that GST did not activate I_{Cl} by elevating cyclic AMP in the myocytes investigated here, there is a need for more direct evidence on this point.

A strong argument excluding GST-mediated elevation of cyclic AMP can be made in regard to the extra stimulation of I_{Cl} induced by GST in myocytes pretreated with micromolar FSK. First, an additional stimulation of cyclic AMP formation by GST is an unlikely explanation because a presumably massive activation of adenylate cyclase by 100 μM FSK (cf. Rodger & Shahid, 1984) had no effect on I_{Cl} in myocytes that were pretreated with 5 μM FSK. In other words, cyclic AMP formation relevant to the magnitude of Cl^- channel activation was maximally achieved by application of 5 μM FSK to these myocytes. For the same reason, there would appear to be nothing further to be gained, as far as I_{Cl} activation is concerned, by an elevation of cyclic AMP concentration due to suppression of cyclic AMP breakdown. Furthermore, returning to the stimulation by GST alone, it is highly unlikely that inhibition of cyclic AMP hydrolysis under basal conditions could result in a rapid (<2 min) elevation in cyclic AMP concentration approaching that achieved by 1–2 min treatment with 5 μM FSK. In that regard, the effects of the phosphodiesterase-inhibiting 3-isobutyl-1-methylxanthine (IBMX) on cardiomyocyte I_{Cl} have been investigated in a number of previous studies. These have shown that prolonged treatment with 5–20 μM IBMX results in little if any activation of I_{Cl} (Tareen *et al.*, 1991; Nakashima & Ono, 1994; Ono & Noma, 1994).

Involvement of the cyclic AMP-dependent CFTR Cl^- channel in the I_{Cl} activated by GST

Illek *et al.* (1995) have previously investigated the effects of 40–50 μM GST on 3T3 cells transfected with CFTR. They observed that the drug transiently increased $^{125}I^-$ efflux, and stimulated multi-channel cell-attached patch currents attributed to Cl^- movement. Since (i) daidzein did not mimic GST responses, (ii) the GST-induced increase in I^- efflux was inhibited by VO_4 and (iii) the activation of membrane-patch current by GST was prevented by pretreatment with 1 mM VO_4, Illek *et al.* (1995) concluded that activation by GST was due to an inhibition of PTK. They also found that a prestimulation of I^- efflux with 1–10 μM FSK completely occluded further stimulation of efflux by 50 μM GST. Based on these results, they concluded that the channel affected by GST was the cyclic AMP-dependent CFTR.

In many respects, our results with GST alone, and with VO_4, are similar to those of Illek *et al.* (1995): (i) pretreatment with 1 mM VO_4 prevented activation of I_{Cl} by 50 μM GST, (ii) 0.1 mM VO_4 inhibited GST-activated g_{Cl} by about 75%, in close agreement with the near 70% inhibition of I^- efflux by 0.1 mM VO_4 in the Illek *et al.* (1995) study, and (iii) 50 μM GST (a concentration that they reported to be maximally-effective) activated ~50% of the Cl^- conductance activated by micromolar FSK, the same relative percentage of I^- efflux activated in their study. On the other hand, our results with the combination of GST and micromolar FSK are quite different from those of Illek *et al.* (1995). GST clearly augmented I_{Cl} in myocytes with CFTR I_{Cl} already activated by a maximally-effective concentration of FSK. Had FSK pretreatment occluded GST activation (as in the Illek *et al.*, 1995, study), it would have been an argument in favour of involvement of cardiac CFTR Cl^- channels in the GST response. However, the lack of occlusion does not rule CFTR out because saturating concentrations of FSK do not necessarily activate maximal CFTR current in guinea-pig ventricular myocytes (Hwang *et al.*, 1993; Gadsby *et al.*, 1995). Furthermore, the Cl^- currents activated by GST in the myocytes had a number of biophysical properties (time and voltage independence, anion selectivity, outward rectification) that were the same as those of CFTR currents activated by FSK. However, the most compelling evidence that GST activated the same myocyte Cl^- channel as FSK is presented in Figure 9. The results in this figure demonstrate that the current activated by the combination of drugs can be larger than the sum of the currents activated by each drug alone.

Regulation of Cl^- channel activity by tyrosine phosphorylation

We attribute the activation of I_{Cl} by GST to an inhibition of tyrosine phosphorylation, and reversal of GST action by VO_4 to a counteracting inhibition of PTPase-mediated dephosphorylation. Rapid activation of I_{Cl} upon application of GST, and rapid deactivation upon removal of GST, implies continuous phosphorylation and dephosphorylation influences on cardiac Cl^- channels by tyrosine kinase(s) and phosphatase(s). Depressed tyrosine phosphorylation of CFTR protein or of a CFTR regulatory factor may increase channel opening probability by promoting PKA-mediated phosphorylation. Variation in the expression of CFTR in regions of the guinea-pig ventricle (James *et al.*, 1995), as well as variation in local PTK, PTPase and PKA activity under basal conditions, may help explain (i) the variable activation of I_{Cl} by 50 μM GST (compare Figures 1 and 4a), (ii) the well-known variable response of guinea-pig ventricular I_{Cl} to elevations of cyclic AMP (e.g. Figure 10 of Matsuoka *et al.*, 1990), and (iii) the occasional dramatic potentiation of I_{Cl} by GST when channel activation by micromolar FSK is relatively weak (e.g. Figure 9).

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