

# Insulin Stimulates Membrane Conductance in a Liver Cell Line

EVIDENCE FOR INSERTION OF ION CHANNELS THROUGH A PHOSPHOINOSITIDE 3-KINASE-DEPENDENT MECHANISM\*

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**Activation of insulin receptors stimulates a rapid increase in the ion permeability of liver cells. To evaluate whether this response involves insertion of ion channels, plasma membrane turnover was measured in a model liver cell line using the fluorescent membrane marker FM1-43. Under basal conditions, the rate of constitutive membrane turnover was  $\sim 2\% \text{min}^{-1}$ , and balanced exocytosis and endocytosis maintained the total cell membrane area constant. Exposure to insulin stimulated a transient increase in membrane turnover of up to 10-fold above constitutive rates. The response was concentration-dependent (0.001–10  $\mu\text{M}$ ). Insulin also caused a parallel increase in membrane conductance as measured by whole-cell patch clamp recording due to opening of  $\text{Cl}^-$ - and  $\text{K}^+$ -selective ion channels. The insulin-stimulated membrane turnover did not appear to involve the constitutive recycling compartments, suggesting that a distinct pool of vesicles may be involved. The effects of insulin on membrane turnover and membrane conductance were inhibited by blockers of phosphoinositide 3-kinase LY294002 and wortmannin or by disrupting microtubule assembly with nocodazole. Taken together, these findings indicate that insulin stimulates recruitment of new membranes through phosphoinositide 3-kinase-dependent mechanisms. Thus, regulated insertion of a separate population of ion channel-containing vesicles may represent one mechanism for mediating the changes in membrane conductance that are essential for the cellular response to insulin.**

Liver cells exhibit rapid regulation of solute transport across the plasma membrane in response to changing physiological demands. Changes in the concentration of solutes in the extracellular space are followed by increased uptake of glucose, amino acids, and bile acids as well as an increase in the activity of specific enzymes that regulate glucose metabolism and bile formation. While many of the individual transport proteins involved have been molecularly defined, little is known regarding the cellular strategies for regulating the number, type, and activity of transporters in the plasma membrane under defined physiological conditions.

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In other cells, insertion or retrieval of transport proteins through vesicular exocytosis or endocytosis regulates the protein composition of the plasma membrane (1). In adipocytes, for example, insulin-stimulated glucose uptake is mediated by insertion of glucose transporter GLUT4-containing vesicles, leading to an increase in total transport capacity (2). These effects appear to be mediated in part by phosphoinositide 3-kinase (PI 3-kinase).<sup>1</sup> This kinase phosphorylates phosphatidylinositols at the D3 position, and the resulting lipid-signaling molecules have been implicated in recruitment or activation of proteins essential for vesicular transport (3–6). In liver, insulin receptor binding leads to an increase in ion channel activity, and the resulting increases in membrane conductance appear to be an early and essential step necessary for subsequent changes in transport and metabolic activity. For example, insulin activates a nonselective cation conductance, resulting in  $\text{Na}^+$  and  $\text{Ca}^{2+}$  influx (7, 8) and an increase in liver cell volume (9), a signal proposed to mediate many of the insulin effects (10). However, the mechanisms that couple receptor binding to the increase in membrane conductance have not been defined.

In general, liver cells are thought to function as constitutive secretory cells where export of secretory proteins occurs at a constant rate. Under defined physiological conditions, the insertion of specific membrane transporters into the plasma membrane of hepatocytes has been proposed to be a mechanism that contributes to bile formation (11–13). Furthermore, there is evidence that membrane and secretory proteins are transported by separate vesicular carriers (14). Nevertheless, the relationship between membrane transport and membrane trafficking in liver cells is poorly understood. Because insulin stimulates bile formation (15, 16) and activates PI 3-kinase (17–19), the aim of the present work was to examine the rate of membrane turnover in a model liver cell line and to assess the effects of insulin on membrane trafficking and conductance. Our findings support the presence of active constitutive membrane trafficking that is regulated in part by PI 3-kinase. In addition, insulin receptor binding transiently stimulates membrane turnover that contributes to the insertion of  $\text{Cl}^-$ - and  $\text{K}^+$ -selective ion channels into the plasma membrane.

## EXPERIMENTAL PROCEDURES

**Cell Preparation and Solutions**—All studies were performed in HTC cells derived from rat hepatoma using methods described previously (20, 21). These cells have been widely used as a stable model of hepatocyte ion transport because they express insulin receptors, signaling pathways, and ion channels analogous to those found in primary hepatocytes (7, 20, 22, 23). In brief, cells were maintained at 37 °C in a 5%  $\text{CO}_2$  and 95% air atmosphere in minimal essential medium (Life Technologies, Inc.) containing 5% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. For all experiments, HTC cells were plated on coverslips, and the culture medium was replaced

<sup>1</sup> The abbreviation used is: PI 3-kinase, phosphoinositide PI 3-kinase.

with standard extracellular solution that contained 142 mM NaCl, 4 mM KCl, 1 mM  $\text{KH}_2\text{PO}_4$ , 2 mM  $\text{MgCl}_2$ , 2 mM  $\text{CaCl}_2$ , 10 mM D-glucose, 10 mM HEPES/NaOH (pH 7.25). The osmolarity of the external solution was 295–300 mosmol/kg.

**Measurement of Membrane Turnover**—The rate of membrane turnover was assessed by real time imaging using a fluorescent probe FM1-43 (Molecular Probes, Inc., Eugene, OR). FM1-43 has two specific properties that permit its use in this capacity. First, it binds to membranes but does not cross lipid bilayers. Second, it is not fluorescent in solution, but when it binds to biological membranes its quantum yield increases about 350 times (24). Thus, the fluorescence intensity is directly proportional to the amount of membrane exposed to FM1-43. For these studies, FM1-43 was added to the external solution at a concentration of 4  $\mu\text{M}$ . Initially, FM1-43 partitions into the plasma membrane exposed to the external solution. Subsequently, when vesicles fuse with the plasma membrane, FM1-43 equilibrates with the new membrane, resulting in an increase in the apparent fluorescence. Consequently, the overall change in FM1-43 fluorescence provides in real time a measure of the sum of all exocytic events. Since the binding of FM1-43 to the membranes is reversible, the fluorescence intensity that remains after removal of dye from the external medium provides a quantitative measure of endocytosis. The loss of internalized FM1-43 fluorescence can be further exploited to study exocytosis as labeled vesicles fuse with the plasma membrane and FM1-43 diffuses away from the membrane and into the medium. These methods have been utilized previously to evaluate membrane recycling in neuronal cells (25–31) and constitutive secretory cells (32).

**Imaging and Analysis**—Coverslips with HTC cells were perfused in a chamber at a rate that allowed complete exchange of chamber volume in about 1–2 min. Cells were viewed with a Nikon  $\times 60$  water immersion objective (NA = 1.2). The fluorescence of FM1-43 was excited with band pass filters (peak at 480 nm) and collected with an emission filter (peak at 535 nm). The duration of fluorescent light exposure was 50 ms. Images were acquired once every 30 s with a 12-bit-cooled CCD IMAGO digital camera (9.9  $\times$  9.9- $\mu\text{m}$  chip size) controlled by TILLvisION version 3.3 software (both TILL Photonics). Using the  $\times 60$  objective, the pixel size was 0.165  $\mu\text{m}$ . Quantitative analyses of fluorescent images were performed on a Macintosh computer using NIH Image (National Institutes of Health, Bethesda, MD) and IgorPro3 (WaveMetrics) software. Total cellular FM1-43 fluorescence was measured from the region containing the cell. For background subtraction, fluorescence was measured in the same way from regions containing no cells. After background subtraction, the total fluorescence was normalized to the values obtained immediately after staining the plasma membrane with FM1-43.

To evaluate the potential effect of photobleaching on FM1-43 fluorescence, cells were incubated in FM1-43 for 15 min. During this period, via constitutive membrane turnover, the FM1-43 was internalized and selectively labeled only constitutive endocytic compartments (32). After removing the dye from external medium, the loss of internalized fluorescence was monitored to assess the rate of membrane recycling from endocytic compartments (see also Fig. 4). To assess the potential effect of photobleaching, the fluorescent images were acquired by varying the duration of light exposure. With an exposure of 50 ms, the destaining time constant was 7.5 min (Fig. 4). When the duration of fluorescence light exposure was 100 ms (5 cells) or 200 ms (7 cells), the destaining time constants were 7.3 and 7.4 min, respectively (not shown). The similarity of these measures suggests that the fluorescence originating from FM1-43-labeled membrane compartments is not bleached significantly with the exposure of 50 ms used for these studies.

**Current and Conductance Measurements**—Whole-cell currents were measured using the patch clamp technique (33). Cells were maintained in the standard external solution described above and were dialyzed with a standard pipette solution that contained 130 mM KCl, 10 mM NaCl, 1 mM EGTA, 0.5 mM  $\text{CaCl}_2$ , 2 mM  $\text{MgCl}_2$ , 10 mM HEPES/NaOH (pH 7.25,  $\sim 272$  mosmol/kg). In some experiments to decrease  $[\text{Cl}^-]$  in the intracellular medium the cells were dialyzed with a pipette solution containing 130 mM potassium glutamate, 20 mM NaCl, 1 mM EGTA, 0.5 mM  $\text{CaCl}_2$ , 2 mM  $\text{MgCl}_2$ , 10 mM HEPES/NaOH (pH 7.25,  $\sim 276$  mosmol/kg). After obtaining a stable whole-cell configuration, membrane capacitance and access resistance were compensated. Whole-cell currents in response to voltage pulses were filtered with an eight-pole Bessel filter at a 1-kHz cut-off frequency and sampled every 0.5 ms. To determine reversal potential ( $E_r$ ), a voltage ramp from  $-90$  to  $90$  mV (duration 1 s) was applied. For ion substitution experiments, the external solution was changed to high  $\text{K}^+$  or NMDG $^+$  solutions. The high  $\text{K}^+$  solution contained 150 mM KCl, 2 mM  $\text{CaCl}_2$ , 10 mM D-glucose, 10 mM HEPES/KOH (pH 7.25,  $\sim 297$  mosmol/kg). The high NMDG $^+$  solution contained

150 mM NMDG-Cl, 2 mM  $\text{CaCl}_2$ , 10 mM D-glucose, 10 mM HEPES/HCl (pH 7.25,  $\sim 299$  mosmol/kg). Reversal potentials were calculated taking into account the corrections for liquid junction potentials. With standard pipette solution and high NMDG $^+$ , the liquid junction potential was  $-2$  mV. With standard external solution and low  $[\text{Cl}^-]$  pipette solution, the liquid junction potential was 5 mV.

In some experiments, the whole-cell membrane conductance was measured rather than whole-cell membrane current. Membrane conductance was determined every 3 s by applying 4-ms voltage pulses ( $-20$  mV) from a holding potential of  $-40$  mV. The steady state current response was used to calculate the conductance as described in Ref. 34. Where indicated, insulin (0.01–10  $\mu\text{M}$ ) was added to the external medium about 1–2 min after obtaining whole-cell configuration.

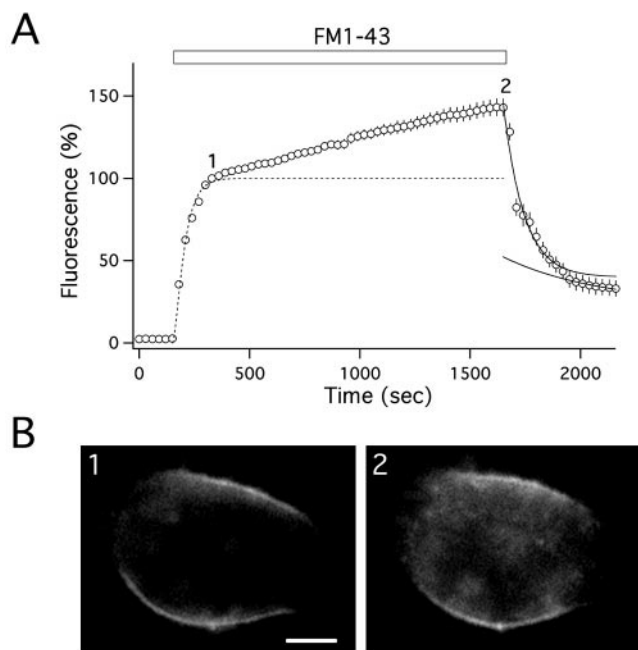
**Cell Treatments and Reagents**—For pharmacological studies, cells were incubated in culture medium at 37  $^\circ\text{C}$  with different drugs. To assess the role of PI 3-kinase, cells were treated with blockers of PI 3-kinase, LY294002 (10  $\mu\text{M}$ ) or wortmannin (100 nM), for 15 min. In other experiments, the role of microtubules was assessed by incubating the cells with the microtubule-depolymerizing compound nocodazole (30  $\mu\text{M}$ ) for 1 h. LY294002 was purchased from Calbiochem, and all other drugs were purchased from Sigma. All experiments were performed at 24  $^\circ\text{C}$ . Data are expressed as mean  $\pm$  S.E., unless otherwise stated. Results were compared using Student's *t* test on paired or unpaired data.

## RESULTS

**Evidence for Constitutive Membrane Turnover in Liver Cells**—To evaluate whether the plasma membrane of HTC cells is stable in composition or undergoes changes with time, cellular fluorescence was measured in the presence of a constant concentration of FM1-43. The time course of changes in total cell fluorescence is shown in Fig. 1A. After exposure to FM1-43, fluorescence increases in two phases. A rapid initial rise corresponding to diffusion of the dye into the external medium is fitted with a single exponential (*dashed line*). Initially, FM1-43 stains only the plasma membrane (Fig. 1B, *left panel*). Subsequently, there is a slower rate of continuous accumulation of FM1-43 fluorescence at a rate of  $2.0 \pm 0.1\% \text{min}^{-1}$  (16 cells). After 22 min, total fluorescence increased by  $43 \pm 2\%$  (8 cells), and the pattern of fluorescence changed to include both the plasma membrane and the cell interior (Fig. 1B, *right panel*). Thus, new exocytic membranes were added to the plasma membrane, resulting in an increase in total cellular fluorescence.

To test whether the new membranes increased total plasma membrane area, the time course of fluorescence loss was measured following removal of FM1-43 from the medium. Notably, the internalized fluorescence that remained after washing dye out of the plasma membrane was  $40 \pm 1\%$ , a value not different from the fluorescence increase that occurred in the presence of the dye ( $p > 0.15$ ). The similarity of these two measures indicates that the total plasma membrane area was not increased. These results suggest that HTC cells have a constitutive mechanism for continuous exocytosis and compensatory endocytosis that actively replaces membrane at a rate of  $\sim 2\% \text{min}^{-1}$  but maintains the plasma membrane area constant.

**Regulation of Membrane Turnover by Insulin**—In hepatocytes, the formation of bile is thought to be mediated in part by the insertion of specific transport proteins into the plasma membrane through vesicular exocytosis (11–13). Since activation of insulin receptors is a potent stimulus for bile formation (15, 16, 35), the effect of insulin on membrane turnover rates was assessed in an analogous manner as shown in Fig. 2A. In the presence of FM1-43, exposure to insulin stimulated a transient increase in fluorescence above the constitutive rate, and the effects were detectable within  $87 \pm 3$  s (51 cells). Subsequently, the rate of increase returned toward the constitutive values observed in the absence of insulin. To quantify the effects, the change in FM1-43 fluorescence ( $\Delta F$ ) was measured as the difference between the linear fit describing the consti-

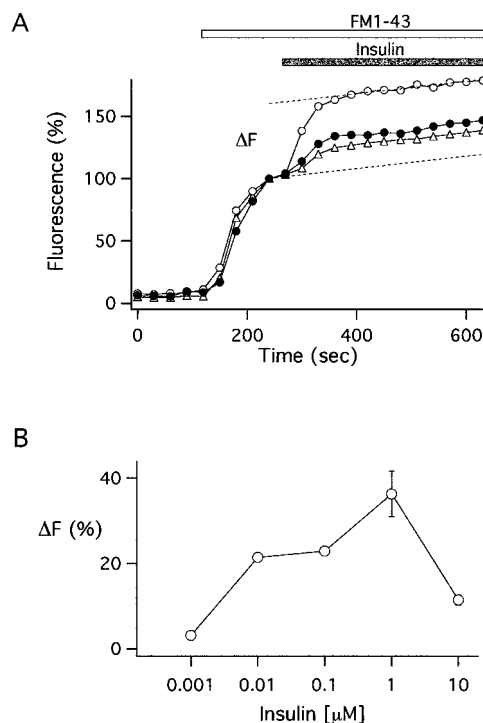


**FIG. 1. Constitutive membrane turnover in HTC cells.** *A*, HTC cells were exposed to FM1-43 ( $4 \mu\text{M}$ , bar), and total cellular fluorescence was measured over time. Circles represent the mean  $\pm$  S.D. of eight cells. Initially, diffusion of the dye into external medium resulted in a rapid increase in fluorescence, which could be fitted with a single exponential rise (dashed line). Subsequently, there was a continuous accumulation of fluorescence at a slower rate, representing constitutive membrane turnover. Following the removal of FM1-43 from the bath, the rate of fluorescence loss was characterized with two components (solid lines). The steady state of the faster exponential decay for each cell was taken as the fluorescence that remained after dye wash out from the plasma membrane, and a slower decay ( $\tau = 7.3$  min) represented membrane turnover from endocytic compartments (see also Fig. 4). *B*, fluorescence images of a representative cell from *A* measured after introduction of FM1-43 when only the plasma membrane was fluorescent (1) and after 22 min in FM1-43 when fluorescence transfer to endocytic compartments was apparent (2). Note that the overall cell fluorescence increased. Scale bar,  $5 \mu\text{m}$ .

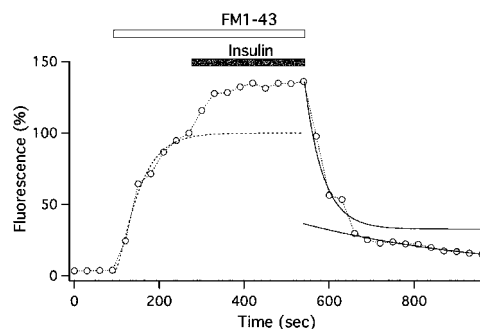
tutive rise in the fluorescence in the presence *versus* absence of insulin (Fig. 2*A*, dashed lines). The effects were concentration-dependent, with maximal responses at concentrations near  $1 \mu\text{M}$  (Fig. 2*B*). Interestingly, at higher insulin concentrations of  $10 \mu\text{M}$ , the effect was smaller. These observations suggest that insulin stimulates a transient increase in the rate of exocytosis in a concentration-dependent manner.

To test whether the insulin-stimulated insertion of new membranes increased plasma membrane area, the time of FM1-43 exposure was varied to assess the degree of dye internalization. In Fig. 3 the decay of the cellular fluorescence was fitted with two exponentials as described for Fig. 1*A*. The internalized fluorescence that remained after washing the dye from the plasma membrane was  $34 \pm 1\%$  (11 cells), and the value was not different from the increase in fluorescence of  $35 \pm 1\%$  measured just before removal of FM1-43. These results indicate that the transient addition of new membranes by insulin is accompanied by a compensatory retrieval of membrane that maintains the plasma membrane area constant.

Over time, exposure to FM1-43 resulted in the detection of fluorescence in intracellular compartments, consistent with accumulation of FM1-43 in the membranes of endocytic compartments responsible for recycling of plasma membrane receptors (32). After removal of FM1-43 from the external medium, the loss of fluorescence was used to monitor membrane recycling related to exocytosis of the vesicles that originated from endo-

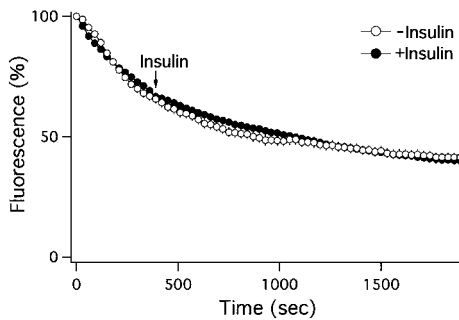


**FIG. 2. Insulin stimulates transient membrane turnover.** *A*, cells were exposed to FM1-43 and then stimulated with insulin ( $0.01 \mu\text{M}$  (triangles),  $0.1 \mu\text{M}$  (filled circles), and  $1 \mu\text{M}$  (open circles)) indicated by bars. Insulin increased the fluorescence within 2 min by  $\Delta F$ . *B*, the effects of insulin on  $\Delta F$  are shown, and each point represents the mean  $\pm$  S.D. of 6–17 cells. Error bars of some points were small and are not visible in the graph.



**FIG. 3. Insulin-evoked membrane turnover involves exocytosis and endocytosis.** After a 3-min exposure to insulin ( $1 \mu\text{M}$ ), cellular fluorescence increased by  $25 \pm 1\%$  (11 cells). Following removal of external FM1-43, the decrease in fluorescence could be characterized by two components as also shown in Fig. 1*A*. Fast decay corresponds to the dye removal from plasma membrane. A slower time constant of 7.6 min is likely to reflect the dye loss from endocytic recycling compartments (see also Fig. 4). Note that the internalized fluorescence that remained after washing the plasma membrane is similar to the total fluorescence increase, indicating balanced rates of exocytosis and endocytosis.

cytic compartments. For these studies, HTC cells were incubated with FM1-43, and after washing the dye out from the external solution and the plasma membrane, the internalized fluorescence was measured in the absence *versus* presence of insulin (Fig. 4). If the  $\Delta F$  induced by insulin involved a transient exocytosis of the vesicles derived from endocytic recycling compartments, then insulin would be expected to enhance the rate of fluorescence loss. However, insulin did not increase the rate of loss of fluorescence, suggesting that the transient exocytosis stimulated by insulin does not involve rapid recycling from endocytic compartments. These findings further suggest that recruitment of new membranes by insulin may involve a



**FIG. 4. Insulin does not stimulate membrane turnover from constitutive recycling compartments.** Internal membranes of HTC cells were labeled by preincubation with FM1-43 for 15 min. After the removal of FM1-43 from the external medium, the rate of decline in fluorescence was assessed. The fluorescence decay (mean  $\pm$  S.D.) from nine cells is shown in the absence of insulin (open circles) and from 11 cells (closed circles) that were stimulated with  $1 \mu\text{M}$  insulin at the arrow. The time constants of decay for control and insulin-stimulated cells were 7.5 and 9.5 min, respectively. Note that insulin did not increase the rate of destaining (exocytosis) of FM1-43 from endocytic compartments.

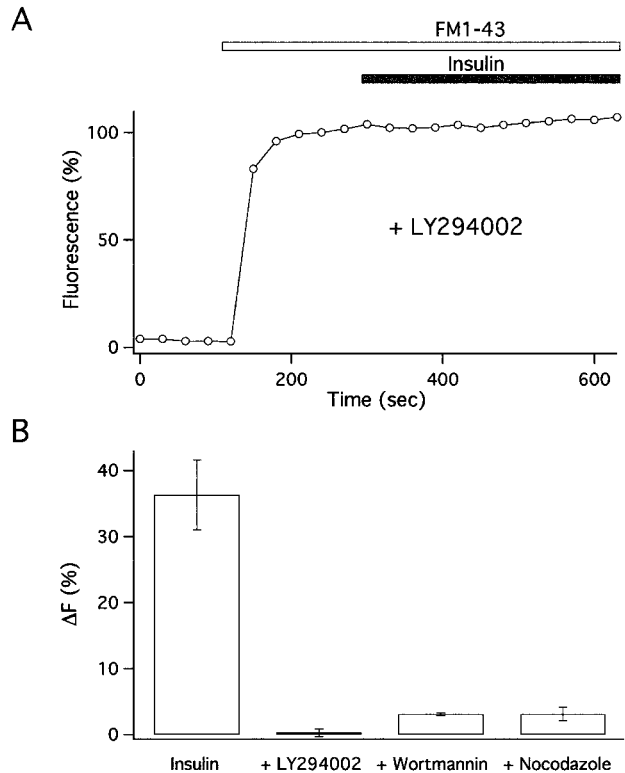
distinct vesicular population.

**Regulation of Membrane Turnover by PI 3-Kinase and Microtubules**—Activation of PI 3-kinase is one of the major signaling pathways triggered by insulin receptor binding (36). Since the lipid products of PI 3-kinase appear to be essential for vesicular transport in many cells (3, 6), we examined the role of PI 3-kinase inhibitors on insulin-stimulated changes in fluorescence. In Fig. 5A, cells were preincubated with LY294002, which abolished the  $\Delta F$  associated with  $1 \mu\text{M}$  insulin exposure ( $0 \pm 1\%$ , 8 cells; compare with Fig. 2A). The same results were obtained with  $10 \mu\text{M}$  insulin ( $0 \pm 1\%$ , 8 cells). Similarly, wortmannin, another blocker of PI 3-kinase, was also effective in inhibiting the response to insulin (Fig. 5B).

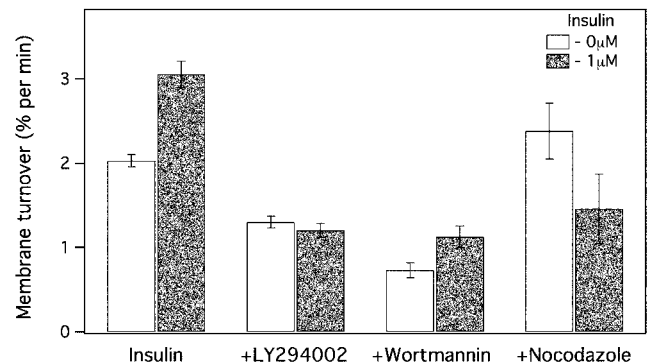
Interestingly, these PI 3-kinase inhibitors also decreased the rate of constitutive membrane turnover detected in the absence or presence of  $1 \mu\text{M}$  insulin (Fig. 6). The slow rate of accumulation of FM1-43 fluorescence measured in the presence of insulin of  $3\% \text{min}^{-1}$  was greater than the constitutive rate of  $2\% \text{min}^{-1}$  observed in the absence of insulin. However, inhibition of PI 3-kinase with concentrations of LY294002 or wortmannin sufficient to completely inhibit the  $\Delta F$  response to insulin only partially inhibited constitutive membrane turnover.

Vesicular exocytosis in liver also has been linked to changes in microtubule stability (37). Consequently, additional studies were performed in cells preincubated with the microtubule-depolymerizing compound nocodazole ( $30 \mu\text{M}$ ). Nocodazole effectively inhibited the fluorescence response to insulin ( $p < 0.005$ ; Fig. 5B) but had no effect on constitutive membrane turnover rates ( $p > 0.15$ ; Fig. 6). Collectively, these findings indicate that the insulin-evoked increases in membrane turnover are mediated by PI 3-kinase and require intact microtubules. In contrast, constitutive membrane turnover may be regulated by different mechanisms, since it is only partially inhibited by PI 3-kinase blockers and is not affected by disruption of microtubules.

**Insulin Activates Membrane  $\text{Cl}^-$  and  $\text{K}^+$  Conductances**—Previous studies of HTC cells indicate that insulin-induced changes in ion channel activity represent an early and essential component of the response to receptor binding (7, 8). To evaluate the role of ion channels in the cellular response to insulin, whole-cell currents were measured in the absence or presence of insulin ( $10 \mu\text{M}$ ) using the patch clamp technique. Representative recordings are shown in Fig. 7. After 2–3 min of hormone exposure, in 18 out of 21 cells insulin activated cur-



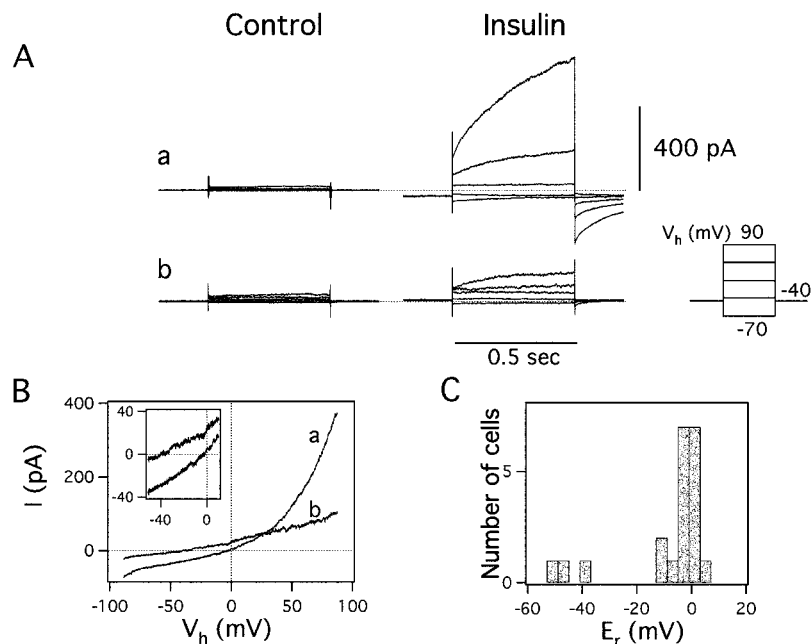
**FIG. 5. Insulin-evoked membrane turnover is regulated by PI 3-kinase and microtubules.** A, in this representative recording, the response to insulin was measured after inhibition of PI 3-kinase by LY294002. In the presence of LY294002, the  $sF$  response to insulin was eliminated. Compare with Fig. 2. B, average  $sF$  responses to  $1 \mu\text{M}$  insulin are shown under control conditions and after preincubation with PI 3-kinase inhibitors ( $10 \mu\text{M}$  LY294002 or  $100 \text{ nM}$  wortmannin for 15 min) or microtubule-depolymerizing agent ( $30 \mu\text{M}$  nocodazole for 1 h). The number of cells was 5–17. Each treatment caused a significant decrease in  $\Delta F$  ( $p < 0.001$ ) as compared with control values.



**FIG. 6. Constitutive membrane turnover is regulated by insulin, PI 3-kinase, and microtubules.** The slow component of fluorescence increase was measured by fitting the regions of continuous fluorescence increase with a straight line as shown in Fig. 2A. The slopes were taken as membrane turnover rates. Under control conditions, membrane turnover was  $\sim 2.0\% \text{min}^{-1}$  (16 cells). Lower concentrations of insulin ( $0.001$ – $1 \mu\text{M}$ ) caused an increase in turnover rates by  $\sim 25\%$ , while the higher concentration of insulin ( $10 \mu\text{M}$ ) caused a decrease in turnover rates by  $\sim 10\%$  ( $p < 0.05$ ; data not shown). The PI 3-kinase inhibitors LY294002 ( $10 \mu\text{M}$ ) and wortmannin ( $100 \text{ nM}$ ) partially decreased the membrane turnover rates in the absence or presence of  $1 \mu\text{M}$  insulin. Note that disruption of microtubules with nocodazole had no effect on constitutive turnover rates but partially inhibited the response to insulin.

rents that displayed outward rectification at positive potentials (Fig. 7A, panel a) and had a reversal potential near 0 mV (Fig. 7B). In 3 of 21 cells, insulin-activated currents had little or no rectification (Fig. 7A, panel b) and a reversal potential near

**FIG. 7. Insulin increases activity of ion channels in HTC cells.** *A*, whole-cell currents were measured in the absence and presence of  $10 \mu\text{M}$  insulin in the standard external solution. Current responses from two representative cells to voltage pulses of 500 ms in duration (*a* and *b*) are shown. The *dashed line* indicates the zero line of the whole-cell current. Holding potential was  $-40$  mV. Pulse protocol is shown on the *right*. *B*, whole-cell currents are shown *versus* holding potential for cells *a* and *b* from *A* during voltage ramp from  $-90$  to  $90$  mV (1-s duration). Reversal potentials ( $E_r$ ) for cells *a* and *b* were  $-3$  mV and  $-41$  mV, respectively. The *inset* is an expanded graph around the reversal potentials. *C*, histogram of reversal potentials of the whole-cell currents evoked by insulin obtained from 21 cells in standard pipette and external solutions. Note that 18 out of 21 cells have  $E_r$  close to  $0$  mV. *pA*, picoamperes.



$-40$  mV (Fig. 7B). Fig. 7C shows the reversal potentials of insulin-evoked whole-cell currents from different cells. Since most of the cells had reversal potentials between  $\text{K}^+$  and  $\text{Cl}^-$  reversal potentials ( $E_{\text{K}} = -82$  mV, and  $E_{\text{Cl}} = -2$  mV), additional ion substitution experiments were performed. By moving the  $E_{\text{Cl}}$  to  $-46$  mV using the pipette solution that contained low  $[\text{Cl}^-]$  ( $25$  mM), the reversal potential of insulin-evoked whole-cell currents decreased from  $-8.2 \pm 3.5$  mV (21 cells) to  $-20.5 \pm 3.0$  mV (9 cells). In another set of experiments, when the standard external solution was replaced with a high  $\text{K}^+$  solution ( $E_{\text{K}} = 4$  mV), keeping  $E_{\text{Cl}}$  constant, the reversal potential of insulin-evoked currents increased to  $5.2 \pm 0.6$  mV (5 cells). These results suggest that both  $\text{Cl}^-$ - and  $\text{K}^+$ -selective ion channels are activated by insulin receptor binding in HTC cells.

In HTC cells, insulin also activates small nonselective cation channels (7). To determine the contribution of these channels to the insulin-evoked current response, the standard external solution was replaced with a solution that contained no  $\text{Na}^+$  or  $\text{K}^+$ , but instead only  $\text{NMDG}^+$  as an impermeant monovalent cation. Under these conditions, the reversal potential of nonselective cation channels is expected to move toward negative membrane potentials. Interestingly, after activation of the whole-cell currents by insulin in standard external solution, subsequent replacement with high  $\text{NMDG}^+$  solution did not affect the current response (not shown). In addition, the reversal potential changed to  $1.6 \pm 3.9$  mV (5 cells), indicating that the overall contribution of nonselective cation channels to the insulin-evoked current response in HTC cells is small. Collectively, these results suggest that the activation of  $\text{Cl}^-$ - and  $\text{K}^+$ -selective ion channels is mainly responsible for the increase in membrane conductance resulting from activation of insulin receptors in HTC cells.

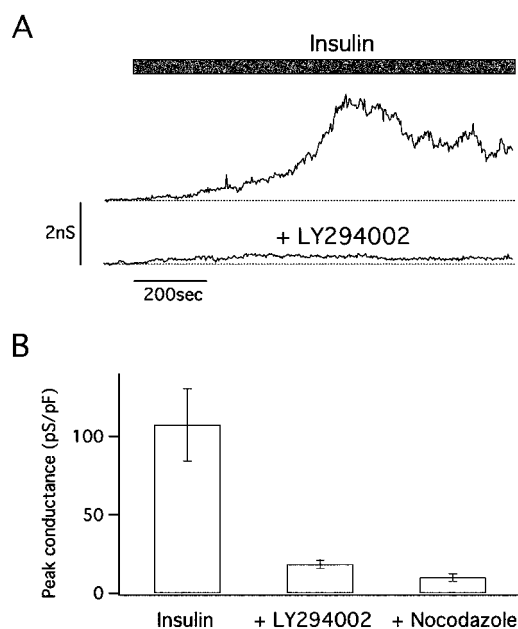
**Regulation of Insulin-evoked Membrane Conductance by PI 3-Kinase and Microtubules**—Previous studies indicate that the early response of HTC cells to insulin involves an increase in whole-cell current activity (7, 8). If these changes in whole-cell current are related to vesicular insertion of ion channels, then agents that inhibit insulin-induced changes in  $\Delta F$  would be anticipated to inhibit the whole-cell current response as well. Consequently, additional patch clamp studies were performed to assess the relationship between insulin-induced changes in

whole-cell current after inhibition of PI 3-kinase activity or disruption of microtubules. Since the reversal potential of insulin-evoked whole-cell currents varied (Fig. 7C), indicating involvement of more than one channel type, the whole-cell membrane conductance was measured in these studies, because it does not depend on reversal potential. In Fig. 8A, exposure to  $10 \mu\text{M}$  insulin resulted in a sustained increase in conductance in control cells ( $n = 13$ ). Lower insulin concentrations ( $0.01$ – $1 \mu\text{M}$ ) activated smaller conductance responses (data not shown). Preincubation with LY294002 to inhibit PI 3-kinase (6 cells) or nocodazole to depolymerize microtubules (5 cells) inhibited the insulin-stimulated changes in conductance (Fig. 8). Since these agents are not known to directly alter ion channel responses, these findings suggest that the insulin-stimulated changes in plasma membrane turnover and membrane conductance are related. Thus, one potential physiological role of the increase in membrane turnover by insulin may be to insert a new population of vesicles containing  $\text{Cl}^-$  and  $\text{K}^+$  selective ion channels into the plasma membrane.

#### DISCUSSION

The principal findings of this study in a model liver cell line are that (a) there is a constitutive membrane turnover that includes both exocytosis and endocytosis, (b) insulin receptor binding transiently stimulates membrane turnover through a PI 3-kinase- and microtubule-dependent mechanisms, and (c) inhibition of insulin-evoked membrane turnover blocks insulin-stimulated increases in membrane ion conductance. These findings suggest that a population of insulin-responsive vesicles contains ion channels and that regulated insertion of these channel-containing vesicles may represent one mechanism mediating the changes in membrane conductance essential for the cellular response to insulin.

We utilized FM1-43 fluorescence as a probe to evaluate the kinetics of membrane turnover in individual HTC cells. Under basal conditions, FM1-43 fluorescence continuously increased at a rate of  $2\% \text{min}^{-1}$ . This constitutive membrane turnover was associated with an equivalent accumulation of fluorescence into intracellular compartments, indicating that exocytic recruitment of new membranes is balanced by an equivalent rate of endocytic retrieval, which maintains the plasma membrane area constant. The rates of turnover correspond to endo-



**FIG. 8. Activity of insulin-responsive ion channels in HTC cells depends on PI 3-kinase and microtubules.** *A*, whole-cell membrane conductance was measured using the whole-cell patch clamp technique. Stimulation with  $1 \mu\text{M}$  insulin caused a sustained increase in conductance (*top trace*). The response was inhibited by preincubation with the PI 3-kinase inhibitor LY294002 ( $10 \mu\text{M}$ ) for 15 min (*bottom trace*). Holding potential was  $-40 \text{ mV}$ . *B*, the peak conductance evoked by insulin was significantly smaller in the cells that were treated with LY294002 or nocodazole ( $p < 0.03$  for both). For comparison, the peak conductance was normalized to the total membrane capacitance for each individual cell. *pS*, picosiemens; *pF*, picofarads.

cytosis of  $\sim 1.2$  times the plasma membrane every hour, a value similar to that defined using fluid phase endocytosis in hepatocytes (38) and other cells (39, 40).

Exposure to insulin transiently increased the rate of membrane turnover to values up to 10-fold above constitutive rates. This represents the first direct evidence for such an effect in a liver cell model. Several observations indicate that the insulin response involves recruitment of a separate population of exocytic vesicles and not a simple transient increase in the rate of constitutive membrane recycling. First, the rate of turnover from constitutive endocytic recycling compartments to the plasma membrane is not increased by insulin (Fig. 4). Second, there appear to be differences in the regulation of these vesicle populations. While the insulin response is completely inhibited by wortmannin, LY294002, or nocodazole, constitutive recycling is only partially inhibited by wortmannin or LY294002 and is not sensitive to nocodazole. Similarly, microtubules are necessary in liver for regulated exocytic insertion of canalicular multispecific organic anion transporters (13), for insertion of  $\text{Cl}^-/\text{HCO}_3^-$  exchange proteins (12), and for transport of polymeric IgA receptor to the plasma membrane (41).

The most direct interpretation of these observations is that insulin receptor binding mobilizes a separate population of vesicles that undergo fusion with the plasma membrane, resulting in a transient increase in FM1-43 fluorescence. This formulation is reminiscent of findings in adipocytes, where insulin-stimulated glucose uptake is mediated by the insertion of GLUT4-containing vesicles, leading to an increase in total glucose transport capacity (2). To identify candidate proteins that might be found in these insulin-responsive vesicles, the effects of inhibitors of membrane turnover on membrane ion conductance were assessed. Emphasis was placed on the conductance response because it takes place over a time course of minutes, similar to the changes in membrane turnover, and

constitutive membrane recycling is not associated with any change in membrane conductance over time (data not shown). Furthermore, the increase in conductance is essential for the liver cell response to insulin. The observation that inhibition of insulin-stimulated membrane turnover by two biochemically distinct mechanisms decreases the conductive response supports the hypothesis that ion channels are contained in the insulin-responsive vesicles.

Previous studies of HTC cells and primary hepatocytes indicate that the primary (but not the only) conductance response to insulin involves an increase in the activity of nonselective cation channels, which mediate influx of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  ions under physiological conditions (7, 8). In the present study, we identified at least two channel types that may be contained in insulin-responsive vesicles. A major contribution comes from the outwardly rectifying channels permeable to  $\text{Cl}^-$  ions. Also, it appears that there is a small contribution from  $\text{K}^+$  channels. Although the reversal potential of insulin-evoked whole-cell currents did not decrease when NMDG $^+$  replaced monovalent cations in the external solution, the presence of nonselective cation channels in insulin-responsive vesicles cannot be ruled out, since the amplitude of whole-cell currents measured in this work is about 10 times larger than macroscopic currents produced only by nonselective cation channels (7). Therefore, nonselective cation currents may have been masked by large  $\text{Cl}^-$  and  $\text{K}^+$ -selective currents. At present, the molecular identification of none of these insulin-responsive channels is established. This represents an important goal necessary for localization of specific channel proteins in insulin-responsive vesicles.

In intact livers, insulin stimulates bile formation by mechanisms that are poorly understood (15). The formation of bile in isolated perfused livers is stimulated by changes in cell volume through mechanisms that involve microtubule-dependent exocytosis as well as activation of  $\text{Cl}^-$  and  $\text{K}^+$  ion channels (42, 43). Thus, it is possible that the increase in membrane permeability to  $\text{Cl}^-$  and  $\text{K}^+$  ions through insulin-evoked insertion of  $\text{Cl}^-$  and  $\text{K}^+$  ion channels into the plasma membrane may represent a mechanism by which insulin stimulates bile formation in liver.

Assuming that these findings are relevant to liver cells *in vivo*, several limitations or uncertainties of these results merit emphasis. First, the transient change in membrane turnover induced by insulin appears to be completely dependent on the activity of PI 3-kinase. Consequently, rapid membrane recruitment would require very strong activity of PI 3-kinase during the first 2 min of insulin action. However, the time course of PI 3-kinase activity found in hepatocytes (17), Chinese hamster ovary cells (44), and adipocytes (45) follows a different profile, with more gradual accumulation of lipid mediators over time. This discrepancy may be related to localization of PI 3-kinase to specific cytosolic domains or to involvement of additional signaling mechanisms. Second, since the molecular identity of the insulin-responsive ion channels is not known, a direct effect of PI 3-kinase inhibitors or nocodazole on these channels that are already present in the plasma membrane cannot be completely excluded. This seems unlikely, since these inhibitors have no direct effect on ion channel activity in excised patches (46, 47).

There are emerging paradigms for receptor-induced trafficking of ion channels in other cell types, including insertion of cation-permeable channels into the plasma membrane of B lymphocytes by insulin-like growth factor-I (48) and insertion of potassium channels into the plasma membrane of myocytes from diabetic animals by insulin (49). Finally, since the maximal effect of insulin on membrane turnover was observed at lower concentrations than the maximal effects on membrane

conductance, it seems likely that ion channels are not the only protein components of insulin-responsive vesicles. It should be emphasized that insulin in intact liver has multiple effects, including stimulation of bile formation (15) and other membrane transport processes (10).

From a physiological perspective, regulated insertion of channel-containing vesicles may represent one mechanism for mediating the changes in membrane conductance essential for the cellular response to insulin. Consequently, identification of the signaling pathways and transport proteins involved is likely to provide novel strategies for modulating the metabolic and transport effects of insulin on liver cells. Moreover, it is attractive to speculate that other vesicular populations may differ in protein composition, providing a mechanism for selective regulation of ion transport to meet rapidly changing physiological demands.

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