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Protein Phosphatase 2B-dependent Pathway of Insulin Action on Single Cl^- Channel Conductance in Renal Epithelium

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Abstract. The apical membrane of distal nephron epithelium (A6) has a Ca^{2+}-dependent outwardly rectifying Cl^- channel with single channel conductances of 3 pS for outward current and 1 pS for inward current under the basal condition. The single channel conductance for inward currents increased as cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) was elevated, while the single channel conductance for outward currents did not change at the range of $[Ca^{2+}]_c$ from 10 nM to 1 mM. Insulin (100 nM) increased the single channel conductance for the inward current by increasing the sensitivity to cytosolic Ca^{2+} by 400-fold, but did not affect the single channel conductance for the outward current. Further, insulin increased the open probability of the channel. These effects of insulin were completely blocked by cyclosporin-A, an inhibitor of protein phosphatase type 2B (PP2B) which dephosphorylates phospho-tyrosine in addition to phospho-serine/threonine, but not by okadaic acid, an inhibitor of protein phosphatase type 1 and 2A. Further, these effects of insulin were also completely blocked by W7, an antagonist of calmodulin which is required for activation of PP2B. Lavendustin A, an inhibitor of protein tyrosine kinase (PTK), mimicked these effects of insulin; this action of lavendustin A required 1 hr after its application, while within 30 min after its application lavendustin A had no significant effects on the single channel conductance. On the other hand, lavendustin A blocked the insulin action for a relatively short time period (i.e., within 30 min after their application). However, H89 (an inhibitor of protein kinase A) or H7 (an inhibitor of protein kinases A, C and G) did not mimic the insulin action. Application of PP2B or protein tyrosine phosphatase to the cytosolic surface of the inside-out patch membrane increased the single channel conductance and the open probability as did insulin in cell-attached patches. The insulin-induced increases in single channel conductance and open probability were reversibly decreased by application of PTK catalytic subunit in the presence of ATP through a decrease in the sensitivity to cytosolic Ca^{2+}, but not by protein kinase A. These observations suggest that as intracellular signalling of insulin action, PP2B-mediated dephosphorylation of phospho-tyrosine of the channel protein (or channel-associated protein) is a novel mechanism for regulation of single channel conductance, and that at least two different types of PTKs regulate the channel characteristics.

Key words: Protein tyrosine kinase — Cyclosporin A — H89 — Lavendustin A — Single channel conductance — Ca^{2+}

Introduction

It is well recognized that insulin has various effects on cell metabolism, glucose uptake and ion transport [11, 21, 29, 47]. The first step of insulin action is activation of protein tyrosine kinase (PTK) involved in the insulin receptor, which is one of the most important signal-transduction pathways in insulin action [10, 15, 23, 40]. On the other hand, the function of ion channels is modulated by insulin [9, 29, 32, 49]. However, it is still unclear how insulin acts on remote effectors like ion channels in the apical membrane after activating the PTK of the receptor located in the basolateral membrane of the cell. Ion channels have been reported to be regulated by cytosolic Ca^{2+}, nucleotide, phosphorylation and dephosphorylation [19, 20, 39, 44, 45, 48]. We performed single channel recording experiments to study the mechanism of insulin action on the single channel con-

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ductance of the Ca^{2+}-dependent Cl^- channel in the apical membrane of a renal cell line, A6 cell. Here, we report that insulin increased the single channel conductance of the Ca^{2+}-dependent Cl^- channel by increasing the sensitivity to cytosolic Ca^{2+} through dephosphorylation of phospho-tyrosine of the channel or channel-associated protein through activation of protein phosphatase type 2B (PP2B). The present study is the first report that the insulin action is mediated through PP2B.

Materials and Methods

Cell Culture

A6 cells were purchased from the American Type Culture Collection (Rockville, MD) in the 68th passages, and were maintained in plastic culture flasks at 26°C in an incubator with 4% CO_2 in air [35–37]. All the experiments were carried out on the 75th ~ 83rd passages. The culture medium was NCTC-109 medium modified for amphibian cells (100 mM NaCl, 20 mM $NaHCO_3$, pH 7.4), in which 10% fetal bovine serum (FBS), 1 μM aldosterone, 100 μg/ml streptomycin and 100 U/ml penicillin were added. For patch clamp experiments, A6 cells were subcultured for 10 ~ 14 days on a permeable support filter (Nunc Tissue Culture Inserts, Roskilde, Denmark) to form a polarized monolayer.

Record of Single Channel Currents

Standard patch clamp techniques were used [14, 29–31]. Patch pipettes were made from LG 16 glass (Dagan Corporation, Minneapolis, MN) and fired-polished to produce tip diameters of about 0.5 μm. The patch pipette was applied from the apical side. Then, we made a GΩ seal (>400 GΩ) on the apical membrane of cells. Single channel currents from cell-attached and excised inside-out patches were obtained at 22 ~ 23°C with an Axopatch 1D patch clamp amplifier (Axon Instruments, Foster City, CA). Current signals were recorded on a digital video recorder (HF860D, Sony, Tokyo, Japan) with pulse-code modulation (1-DR-390, Neuro Data Instruments Corporation, New York, NY), and then digitized and analyzed with a continuous-data acquisition program. A 100-Hz low-pass filter was used to demonstrate the single channel current. The single channel conductance shown in the present study means a chord conductance between 0 and +100 mV for outward currents or between 0 and −100 mV for inward currents. The open probability was measured at ±100 mV [29, 31]. The amplitudes of the single channel currents and conductances shown in the present study were not significantly due to the filtering effects, since they were not significantly altered by the filter at least in the range of 50–300 Hz.

Solutions

In experiments of cell attached patches, the bathing and pipette solutions contained (in mM): 120 NaCl, 3.5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES with pH of 7.4. In experiments of inside-out patches, the pipette contained the same solution as that used in cell-attached patches and the bathing (cytosolic) solution contained the same ionic composition as that used in cell-attached patches except for the free Ca concentration. To vary the free Ca concentration of the bathing (cytosolic) solution used in inside-out patches, the appropriate amounts of $CaCl_2$ were added into the pipette solution containing 10 mM EGTA. The free Ca concentration was adjusted using the known $CaCl_2$ and EGTA (10 mM as pure EGTA) concentrations, calculated with pK_d values of 10.86 for $EGTA^{4-}$ and 5.25 for $HEGTA^{3-}$. The solution of the patch pipette contained 100 μM amiloride to block amiloride-sensitive Na^+ channels that were frequently observed in the apical membrane of A6 cells [28].

Chemicals and Hormone

Insulin, protein kinase A (PKA) catalytic subunit, H7, aldosterone and amiloride were obtained from Sigma Chemical (St. Louis, MO); lavendustin A from CALBIOCHEM (San Diego, CA); PTP 34 kDa fragment from Boehringer Mannheim GmbH (Mannheim, Germany); PTK catalytic subunit (p60c-src, c-src kinase) from Upstate Biotechnology (Lake Placid, NY); okadaic acid from Research Biochemicals International (Natick, MA); cyclosporin-A (CYCL), H89 and W7 from CALBIOCHEM; NCTC-109 medium, FBS, streptomycin and penicillin from GIBCO (Grand Island, NY). PP2B and calmodulin were generous gifts from Drs. N. Yokoyama and J. H. Wang (Department of Biochemistry, University of Western Ontario Faculty of Medicine, London, Ontario, Canada).

Data Presentation

The data are represented as means ± SD. Where SD bars are not visible, they are smaller than the symbol. The Student's *t*-test or ANOVA was used for statistical analysis as appropriate and the P value < 0.05 was considered significant. When the P value was less than 0.05, it is marked by * or #.

Results

Insulin Action on Single Channel Currents and Current-Voltage Relationship

As we previously reported [26, 27], the apical membrane of A6 cells had an outwardly rectifying Cl^- channel. The permeability to Cl^- (P_{Cl}) of the channel was much larger than the permeability to Na^+ (P_{Na}) or K^+ (P_K): P_{Cl}/P_{Na} and P_{Cl}/P_K were larger than 17, respectively [27]. Figure 1 shows typical traces of single channel currents through this Cl^- channel obtained from a cell-attached patch formed on the apical membrane before and 20 min after application of 100 nM insulin applied to the basolateral solution. The interesting action of insulin on this channel was to increase the magnitude of the inward current through this Cl^- channel and the open probability (Fig. 1). The insulin-untreated channel had an outwardly rectified current-voltage relationship (open squares in Fig. 2; Control); the single channel conductances for outward and inward currents were 3 and 1 pS, respectively. On the other hand, the insulin-treated channel had a linear current-voltage relationship (closed circles in Fig. 2*A*; Insulin); the single channel conductances for both outward and inward currents were 3 pS. Figure 3 shows the time course of the single channel conductance for the inward current at 100 mV more negative than the resting membrane potential. The single

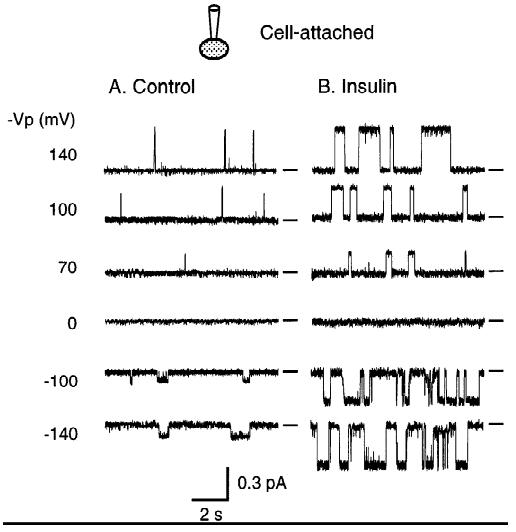

Fig. 1. Single channel current traces obtained from a cell-attached patch before (*A.* Control) and 20 min after (*B.* Insulin) 100 nM insulin application. −Vp means the replacement of the holding potential from the resting apical membrane potential. For example, −Vp = 100 mV means that the intracellular potential of the patch membrane, referred to as the intrapipette potential (extracellular potential), was 100 mV more positive than that of the resting membrane potential. The closed level of the single channel current is marked with a horizontal dash at the right of each trace. Downward deflection indicates inward currents across the patch membrane.

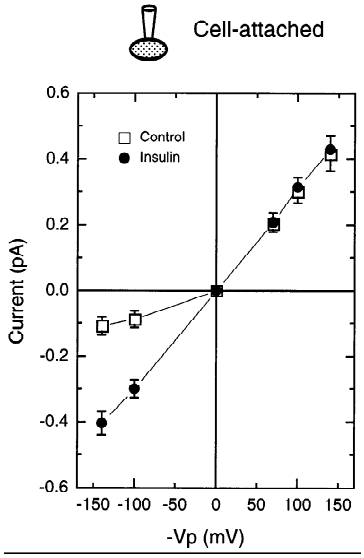

Fig. 2. Current-voltage relationship obtained from a cell-attached patch before (open squares; Control) and 20 min after (closed circles; Insulin) 100 nM application. −Vp = −100 mV, which means that the intracellular potential of the patch membrane, referred to as the intrapipette potential (extracellular potential), was 100 mV more negative than that of the resting membrane potential. $n = 5$.

channel conductance started to increase immediately after insulin application, reaching steady values within 20 min (Fig. 3).

Effects of Protein Phosphatase Inhibitors and Calmodulin Antagonist on Insulin Action

We tested whether an inhibitor of protein phosphatase type 2B (PP2B) blocks the insulin action on the single channel conductance and the open probability. Figure 4*A* shows single channel currents at 100 mV more negative than the resting membrane potential recorded from cell-attached patches formed on the apical membrane of cells under various conditions: *a*, without insulin or any other blockers (Fig. 4*A-a*); *b*, with insulin (Fig. 4*A-b*); *c*, with insulin after pretreatment of cyclosporin-A (1 μM, an inhibitor of PP2B; Fig. 4*A-c*); *d*, with insulin after pretreatment of W7 (100 μM, an inhibitor of calmodulin; Fig. 4*A-d*); *e*, with insulin after pretreatment of okadaic acid (1 μM, an inhibitor of protein phosphatase type 1 (PP1) and protein phosphatase type 2A (PP2A); Fig. 4*A-e*). Insulin increased the amplitude of the single channel current for the inward current (Fig. 4*A-a,b*). In the presence of cyclosporin-A, insulin failed to increase the amplitude of the single channel current for the inward current (Fig. 4*A-a,b,c*). The insulin-increased amplitude

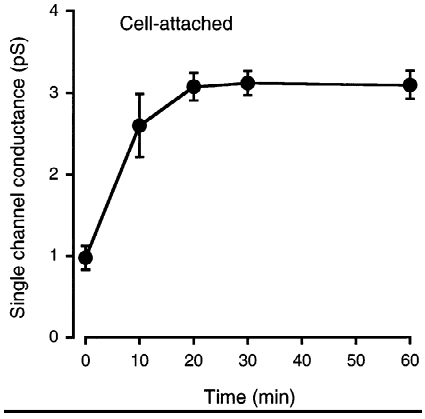

Fig. 3. Time courses of single channel conductance for inward current after insulin application in cell-attached patches at −Vp = −100 mV, which means that the intracellular potential of the patch membrane, referred to as the intrapipette potential (extracellular potential), was 100 mV more negative than that of the resting membrane potential. The single channel conductance started to increase immediately after insulin application, reaching steady values within 20 min. $n = 6$.

was also reversibly decreased to the basal level by cyclosporin-A 1 hr later after application of cyclosporin-A. Since PP2B is a Ca^{2+}-dependent enzyme that is activated by calmodulin (6,42), we studied whether an inhibitor of calmodulin, W7, blocks the insulin action. W7 (100 μM) abolished the insulin action (Fig. 4*A-a,b,d*) as did cyclosporin-A. However, okadaic acid (1 μM), an inhibitor of

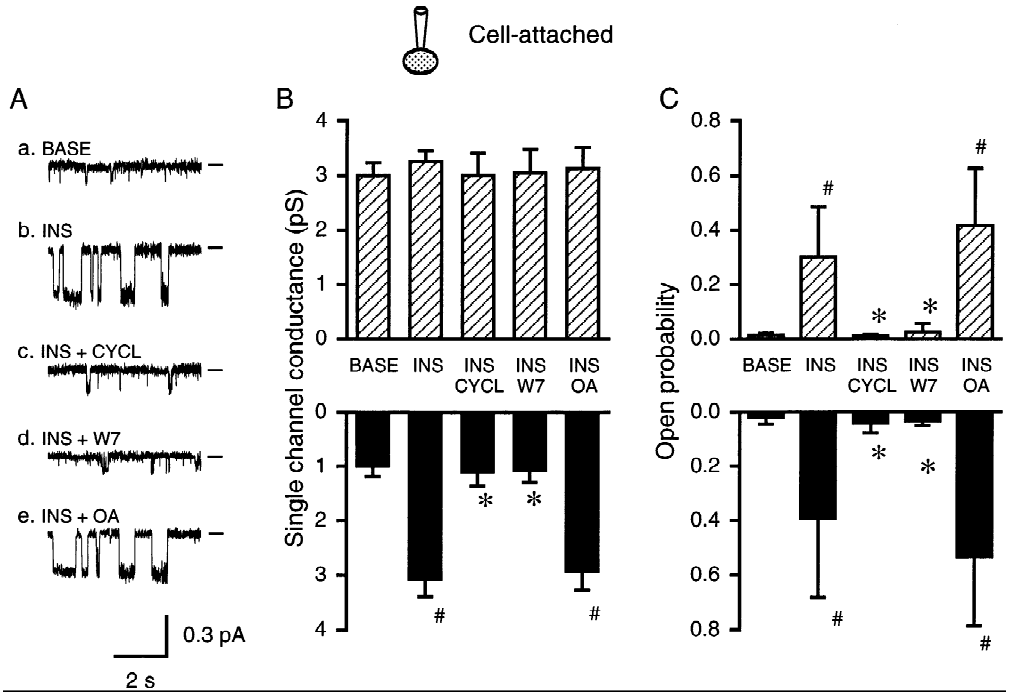

Fig. 4. Single 3 pS Cl^- channel activity. (*A*) Single channel currents were recorded in cell-attached patches at $-Vp = -100$ mV, which means that the intracellular potential of the patch membrane, referred to as the intrapipette potential (extracellular potential), was 100 mV more negative than that of the resting membrane potential. *a*, Base (without any treatment, BASE); *b*, 20 min after addition of 100 nM insulin (INS); *c*, 20 min after addition of 100 nM insulin (INS) in the presence of 1 μM cyclosporin-A (CYCL) which was applied 1 hr before addition of insulin; *d*, 20 min after addition of 100 nM insulin (INS) in the presence of 100 μM W7 which was applied 30 min before addition of insulin; *e*, 20 min after addition of 100 nM insulin (INS) in the presence of 1 μM okadaic acid (OA) which was applied 1 hr before addition of insulin. (*B*) Statistical results of single channel conductances obtained from cell-attached patches formed on cells under the condition shown in Fig. 4*A*. The upper and lower panels show the single channel conductances for outward and inward currents at $-Vp = 100$ and -100 mV, respectively. Insulin significantly increased the single channel conductance for inward current (#, $P < 0.0001$, compared with the base), while cyclosporin-A or W7 completely blocked the insulin action (*, $P < 0.0001$; compared with insulin only). Okadaic acid had no significant effects on the insulin action; in the presence of OA, insulin increased the single channel conductance for the inward current (#, $P < 0.0001$). However, the single channel conductance was not significantly affected by insulin or other agents. $n = 6$ except okadaic acid; $n = 3$ for okadaic acid. (*C*) Statistical results of open probability obtained from cell-attached patches formed on cells under the condition shown in Fig. 4*A*. The upper and lower panels show the open probability for outward and inward currents at $-Vp = 100$ and -100 mV, respectively. Insulin significantly increased the open probability (#, $P < 0.025$, compared with the base), while cyclosporin-A or W7 completely blocked the insulin action (*, $P < 0.05$; compared with insulin only). Insulin increased the open probability even in the presence of OA (#, $P < 0.025$; compared with base); okadaic acid had no significant effects on the insulin action (compared with insulin only). $n = 6$.

protein phosphatase type 1 (PP1) and protein phosphatase type 2A (PP2A), did not significantly affect the insulin action (Fig. 4*A-a,b,e*). These observations about the single channel conductance for inward currents are summarized in Fig. 4*B* (solid bars). On the other hand, neither insulin nor these inhibitors had significant effects on the single channel conductance for the outward current (slashed bars in Fig. 4*B*). Further, insulin increased the open probability of the channel and the effect of insulin on the open probability was blocked by cyclosporin-A or W7, but not by okadaic acid (Fig. 4*C*). These observations suggest that the insulin action on the single channel conductance and the open probability is mediated through PP2B-dependent pathways.

Effects of Protein Kinase Inhibitors on Insulin Action

As shown above, our observations indicate that insulin may increase the single channel conductance and the open probability by dephosphorylating the channel or channel-associated protein through PP2B-dependent pathways. So, we tested what kind of protein kinase phosphorylates the protein which regulates the single channel conductance and the open probability. In cell-attached patches, lavendustin A (10 μM for 1 hr treatment), an inhibitor of protein tyrosine kinase (PTK), mimicked the insulin action; i.e., lavendustin A significantly increased the single channel conductance for in-

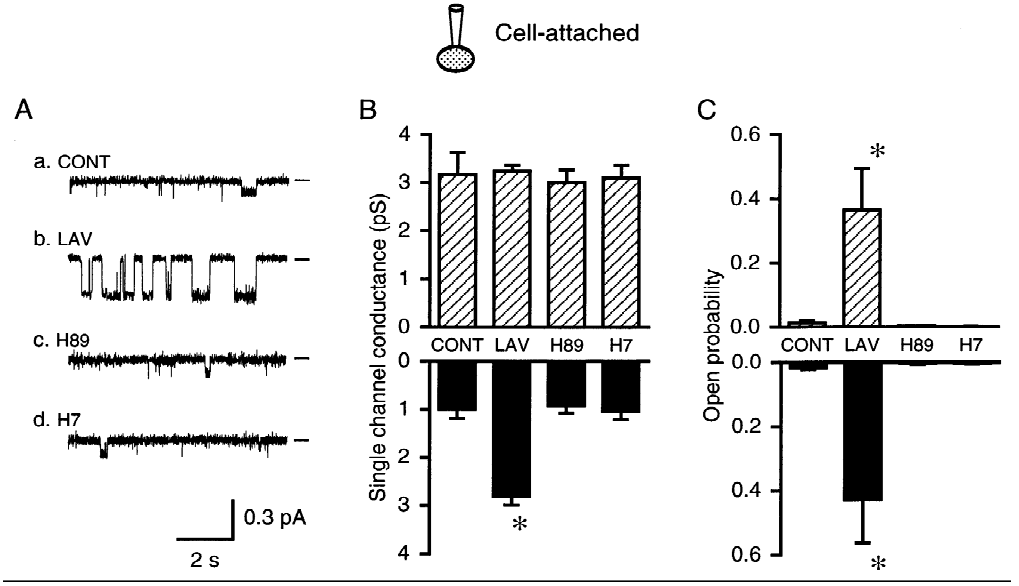

Fig. 5. The effect of inhibitors of protein kinases on the single channel conductance. (*A*) Actual traces of single channel currents obtained from cell-attached patches at −Vp = −100 mV. *a*, control (CONT); *b*, lavendustin A (LAV, 10 μM) action was measured 1 hr after its application; *c*, H89 (0.5 μM) action was measured 1 hr after its application; *d*, H7 (50 μM) action was measured 4 hr after its application. (*B*) Statistical results of single channel conductances obtained from cell-attached patches formed on cells. The upper and lower panels show the single channel conductances for outward and inward currents at −Vp = 100 and −100 mV, respectively. Lavendustin A significantly increased the single channel conductance for inward currents (*, $P < 0.0001$), while Lavendustin A had no significant effects on the single channel conductance for outward currents. H89 and H7 had no significant effects on the single channel conductance for outward or inward currents. $n = 4$. (*C*) Statistical results of open probability obtained from cell-attached patches formed on cells. The upper and lower panels show the open probability for outward and inward currents at −Vp = 100 and −100 mV, respectively. Lavendustin A significantly increased the open probability (*, $P < 0.0001$). H89 or H7 did not increase the open probability. $n = 6$.

ward current (Fig. 5*A-a,b* and solid bars in Fig. 5*B*), but did not affect the single channel conductance for the outward current (slashed bars in Fig. 5*B*). On the other hand, H89 (an inhibitor of cAMP-dependent protein kinase (PKA), 0.5 μM for 1 hr treatment) or H7 (an inhibitor of PKA, protein kinase C (PKC) and cGMP-dependent protein kinase (PKG), 50 μM for 4 hr treatment) had no significant effects on the single channel conductance in cell-attached patches (Fig. 5*A-c,d,B*), indicating that these compounds failed to mimic the insulin action. Our previous (43) and preliminary observations indicate that H89 and H7 blocked the cAMP action on the Cl^- channel in A6 cells. This suggests that the failure of H89 or H7 to mimic the insulin action would not be due to the lack of the inhibitory action on PKA or other kinases in the cell. Further, lavendustin A increased the open probability, but H89 or H7 had no stimulatory effects on the open probability (Fig. 5*C*). These observations indicate that tyrosine in the channel or channel-associated protein is phosphorylated by PTK and is dephosphorylated by PP2B.

Effects of Lavendustin A on the Insulin Action

The insulin receptor is thought to be coupled to PTK, suggesting that a PTK inhibitor could block the insulin action. As described above, lavendustin A, an inhibitor of PTK, mimicked the insulin action on the single channel conductance and the open probability. This observation seems to be inconsistent with the coupling of the insulin signalling to the PTK. Therefore, we studied the relationship between the insulin and lavendustin A action. Lavendustin A itself increased the single channel conductance 1 hr after its application, however lavendustin A had no significant effects on the single channel conductance within 30 min after its application (open bars in Fig. 6). The insulin action was significantly observed within 10 min after its application (Fig. 6). The insulin action was significantly diminished in the presence of lavendustin A during the period of 30 min after application of insulin, while 60 min after insulin application in the presence of lavendustin A the single channel conductance was increased (solid and slashed bars in Fig. 6). This increase in the single channel conductance was also observed in the presence of lavendustin A alone for 60 min without insulin (open bars in Fig. 6). Therefore, lavendustin A inhibited the insulin action, although the co-application of insulin and lavendustin A for a relatively long period (60 min) induced an increase in the single channel conductance due to the action of lavendustin A itself but not due to the insulin action.

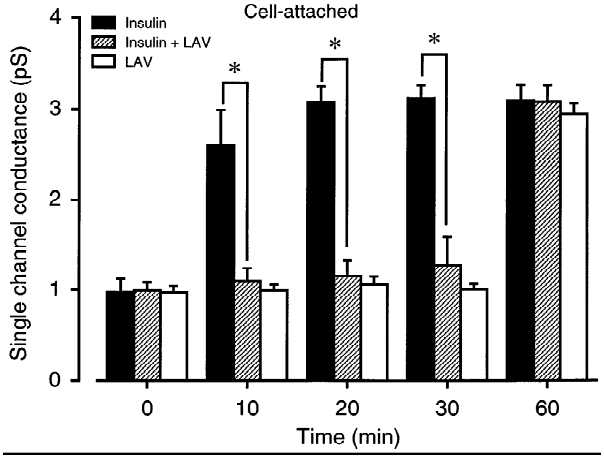

Fig. 6. Time course of single channel conductance for inward currents after application of insulin, lavendustin A or insulin with lavendustin A in cell-attached patches at $-Vp = -100$ mV, which means that the intracellular potential of the patch membrane, referred to as the intrapipette potential (extracellular potential), was 100 mV more negative than that of the resting membrane potential. Lavendustin A had no significant effects on the single channel conductance within 30 min after its application, while lavendustin A increased the single channel conductance at 60 min after its application. Insulin was applied in the presence of lavendustin A (lavendustin A was applied 10 min before addition of insulin). The insulin action on the single channel conductance was completely blocked by the presence of lavendustin A within 30 min after application of insulin (*, $P < 0.001$); there was no significant differences between lavendustin A and Insulin + lavendustin A. At 60 min after application of insulin in the presence of lavendustin A, the single channel conductance was increased to a level similar to that in the absence of lavendustin A. $n = 6$.

Effects of Protein Phosphatases on Insulin Action

To further confirm the role of dephosphorylation of tyrosine in the insulin action, we performed experiments by directly applying PP2B to cytosolic surface of inside-out patches containing the Cl^- channel in a bathing (cytosolic) solution with 1 μM Ca^{2+}. PP2B (15.6 μg/ml) with calmodulin (an activator of PP2B, 30 μg/ml) increased the single channel conductance for the inward current and the open probability without any significant effects on the single channel current for the outward current as did insulin in cell-attached patches (Fig. 7*A-a,b,B* and *C*). Calmodulin itself applied to the cytosolic surface in inside-out patches had no significant effects on the single channel conductance or the open probability (*data not shown*). Further, to confirm dephosphorylation of phospho-tyrosine by PP2B, we also studied the effect of protein tyrosine phosphatase (PTP) on the single channel conductance. PTP (25 mU/ml) applied to the cytosolic surface of the inside-out patches containing the Cl^- channel in a bathing (cytosolic) solution with 1 μM Ca^{2+} increased the single channel conductance for the inward current and the open probability without any significant effects on the single channel conductance for the outward current as did insulin in cell-attached patches (Fig. 7*A-a,c,B* and *C*) as did PP2B. Taken together, these observations suggest that both PP2B and PTP would increase the single channel conductance for inward current and the open probability by dephosphorylating phosphotyrosine of the channel or channel-associated protein.

Effects of Protein Kinases on Insulin Action

We further tested whether the insulin-increased single channel conductance is reversibly decreased by application of PTK. We first made cell-attached patches on the apical membrane without any treatment. After getting high seal resistances (>400 GΩ), we applied insulin to the basolateral solution, while keeping the high seal resistance in cell-attached patches. Then, after observing the insulin-induced increase in the amplitude of the single channel current and the open probability, we made inside-out patches. In a bathing (cytosolic) solution containing 1 μM Ca^{2+}, the amplitude of the single channel current was 0.3 pA at the membrane potential of −100 mV (Fig. 8*A-a;* Insulin) and its single channel conductance for the inward current was 3 pS (solid bars in Fig. 8*B;* Insulin), which was much larger than that obtained from insulin-untreated cells (Fig. 7*A-a* and solid bar in Fig. 7*B;* CONT). We applied PTK catalytic subunit (12.5 U/ml) with ATP (2 mM) to the cytosolic surface of the patch membrane obtained from insulin-treated cells. The amplitude and conductance of the inward single channel current were decreased (Fig. 8*A-a, b* and solid bar in Fig. 8*B* (Insulin + PTK)) instantaneously after the PTK application. However, the single channel conductance for the outward current was not affected by PTK (slashed bars in Fig. 8*B*). On the other hand, PKA catalytic subunit (10 μg/ml) with ATP (2 mM) to the cytosolic surface of the patch membrane obtained from insulin-treated cells did not reversibly decrease them (Fig. 8*A-a, c* and *B*). The open probability was also reversibly decreased by PTK, but not by PKA (Fig. 8*C*).

Sensitivity of Single Channel Conductance to Cytosolic Ca^{2+} and Effects of Insulin and PTK on the Sensitivity to Cytosolic Ca^{2+}

We also studied the sensitivity of the single channel conductance to cytosolic Ca^{2+}. The sensitivity to cytosolic Ca^{2+} in Control in Fig. 9 was obtained from inside-out patches formed on the apical membrane of cells without insulin pretreatment. In the cases of cells with the insulin pretreatment, we used the same protocol as done in experiments shown in Fig. 8 applying insulin before making inside-out patches. Insulin increased the sensitivity of the channel to cytosolic Ca^{2+} by 400-fold (compare circles and triangles in Fig. 9). PTK catalytic

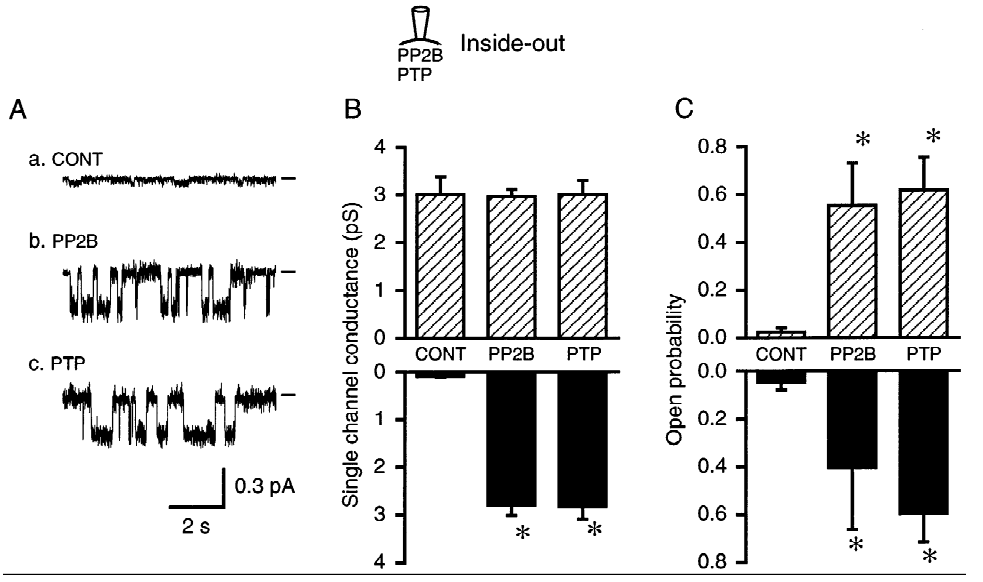

Fig. 7. Effects of protein phosphatase type 2B (PP2B) and protein tyrosine phosphatase (PTP) on the single channel conductance. (*A*) Actual traces of single channel currents obtained from inside-out patches obtained from cells without insulin treatment at −100 mV. The bathing (cytosolic) solution contained 1 μM Ca^{2+}. *a*, control (CONT); *b*, PP2B (15.6 μg/ml) action was measured 10 min after its application from the cytosolic surface in the presence of calmodulin (30 μg/ml); *c*, PTP (25 mU/ml) action was measured 10 min after its application from the cytosolic surface. (*B*) Statistical results of single channel conductances obtained from inside-out patches under the condition shown in Fig. 5*A*. The upper and lower panels show the single channel conductances for outward and inward currents at 100 and −100 mV, respectively. PP2B and PTP significantly increased the single channel conductance for inward currents (*, $P < 0.0005$), while they had no significant effects on the single channel conductance for outward currents. $n = 4$. (*C*) Statistical results of open probability obtained from cell-attached patches. The upper and lower panels show the open probability for outward and inward currents at 100 and −100 mV, respectively. PP2B and PTP significantly increased the open probability (*, $P < 0.05$). $n = 5$.

subunit reversibly, but not completely, decreased the insulin-increased sensitivity of the single channel conductance to cytosolic Ca^{2+} (Fig. 9). The Table summarizes the maximum values of the single channel conductance for the inward current and EC_{50} of cytosolic Ca^{2+} on the single channel conductance in inside-out patches obtained from control (insulin-untreated) cells and insulin-treated cells with and without application of PTK catalytic subunit. On the other hand, the single channel conductance for the outward current (about 3 pS) did not change in the range of $[Ca^{2+}]_c$ from 10 nM to 1 mM, irrespectively of the condition (basal, insulin or insulin +PTK) (*data not shown*).

Discussion

Protein Phosphatases

Various studies indicate that insulin activates some type of protein phosphatases; e.g., insulin has been reported to activate PP1 [1–3, 5, 46], PP2A [3] and pyruvate phosphatase [24]. However, no reports indicate the insulin action on PP2B. Further, although in vitro PP2B has been shown to dephosphorylate phospho-tyrosine [4, 8, 38], no information is available on the role of PP2B in dephosphorylation of phospho-tyrosine in vivo (cell level). The present study is the first report indicating that insulin acts through PP2B-dependent pathways through dephosphorylation of phospho-tyrosine of protein in vivo (cell level).

Protein Tyrosine Kinase (PTK)

PTK reversibly decreased the insulin-increased single channel conductance, but PTK could not completely decrease the single channel conductance to a level without insulin treatment. The reason of this PTK-induced incomplete recovery of the increased single channel conductance is still unclear. Even though a PTK inhibitor mimicked the insulin action, it is still unknown whether under the in vitro condition where we made inside-out patches PTK catalytic subunits can completely phosphorylate tyrosine residue of protein regulating single channel conductance. On the other hand, some other phosphorylation of protein tyrosine caused by PTK catalytic subunits applied to the cytosolic surface of the inside-out patch membrane, which would not occur in vivo (intact cell level), may interfere the decreasing action of PTK on the single channel conductance in vivo

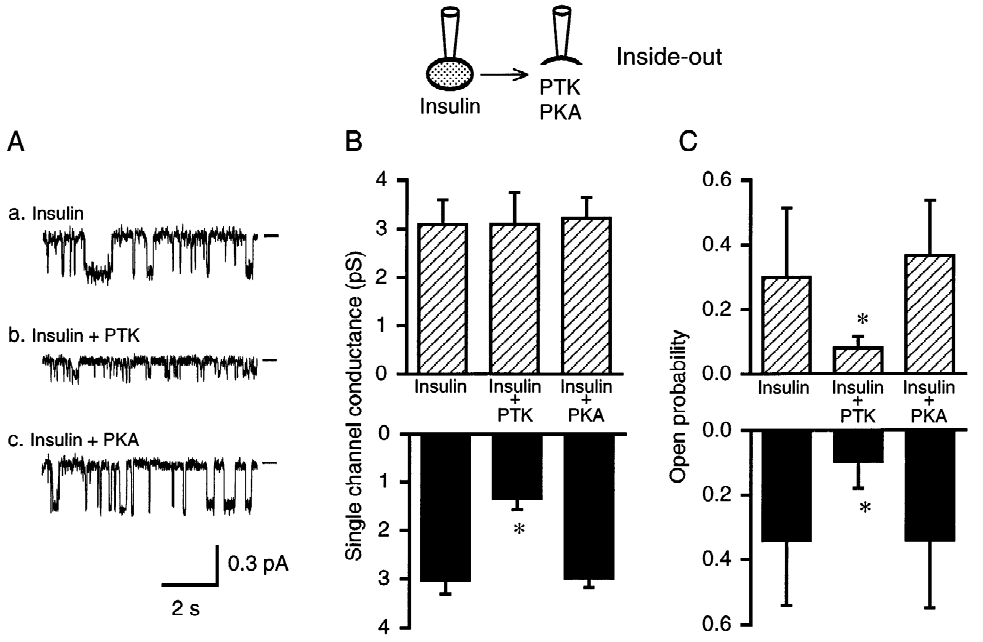

Fig. 8. Effects of protein tyrosine kinase (PTK) and cAMP-dependent protein kinase (PKA) on the single channel conductance of the insulin-activated channel in inside-out patches. We first made cell-attached patches on the apical membrane without insulin treatment. After getting high seal resistances (>400 GΩ), we applied 100 nM insulin to the basolateral solution, while keeping the high seal resistance in cell-attached patches. Then, after observing the insulin-induced increase in the amplitude of the single channel current, we made inside-out patches. The bathing (cytosolic) solution contained 1 μM Ca^{2+}. (*A*) Actual traces of single channel currents in inside-out patches at −100 mV obtained from cells pretreated with 100 nM insulin for 30 min. *a*, Insulin only; *b*, PTK (p60c-src, 12.5 U/ml) action was measured 10 min after application of PTK in the presence of ATP (2 mM) from the cytosolic surface in an inside-out patch obtained from insulin-treated cell; *c*, PKA (10 μg/ml) action was measured 10 min after application of PKA in the presence of ATP (2 mM) from the cytosolic surface in an inside-out patch obtained from insulin-treated cell. (*B*) Statistical results of single channel conductances obtained from inside-out patches under the condition shown in Fig. 8*A*. The upper and lower panels show the single channel conductances for outward and inward currents at 100 and −100 mV, respectively. PTK significantly decreased the single channel conductance for inward currents (*, $P < 0.0005$), but not for outward currents. PKA had no significant effects on the single channel conductance for the outward or inward current. $n = 5$. (*C*) Statistical results of open probability obtained from cell-attached patches under the condition shown in Fig. 8*A*. The upper and lower panels show the open probability for outward and inward currents at 100 and −100 mV, respectively. PTK significantly diminished the insulin action on the open probability (*, $P < 0.05$), however PKA had no significant effects on the open probability. $n = 7$.

(intact cell level). To confirm the phosphorylation site, further studies such as cloning of this channel may be required.

Different Sensitivity of Single Channel Conductance to Cytosolic Ca^{2+} at Cell-Attached and Inside-out Configuration

Our preliminary observation indicated that $[Ca^{2+}]_c$ of A6 cells was 40–100 nM and insulin had no significant effects on $[Ca^{2+}]_c$ of A6 cells. In cell-attached patches, in control (without insulin treatment) the single channel conductance was about 1 pS, while the single channel conductance in inside-out patches at the normal level of $[Ca^{2+}]_c$ (40–100 nM) was much smaller than 1 pS. In insulin-treated cells, the single channel conductance was 3 pS in cell-attached patches and 1 pS in inside-out patches at 100 nM $[Ca^{2+}]_c$. This difference between the single channel conductances in cell-attached and inside-out patches may result from lack of some cytosolic factor increasing the sensitivity to cytosolic Ca^{2+}. Our preliminary observation indicated that the single channel conductance for inward currents in the presence of ionomycin (1 μM) in cell-attached patches was about 3 pS almost similar to that in inside-out patches in a solution containing 1 mM Ca^{2+}. The $[Ca^{2+}]_c$ even in the presence of 1 μM ionomycin would not be larger than 10 μM (our preliminary results). This preliminary observation supports that some cytosolic factor would increase the sensitivity to cytosolic Ca^{2+}. Further, some reports indicate that cytosolic factors have been reported to modulate channel conductances [25], supporting our speculation.

Calmodulin

Antagonists of calmodulin abolish the stimulatory action of insulin on NaCl transport [17] and glucose uptake [13,

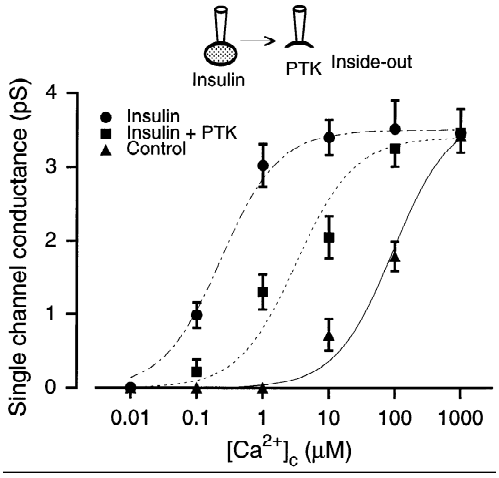

Fig. 9. The dependency of the single channel conductance on $[Ca^{2+}]_c$ in inside-out patches. The single channel conductances were measured at −100 mV. *Control:* we made inside-out patches obtained from cell without insulin treatment. *Insulin:* we first made cell-attached patches on the apical membrane without any treatment; after getting high seal resistances (>400 GΩ), we applied insulin (100 nμM) to the basolateral solution, while keeping the high seal resistance in cell-attached patches; after observing the insulin-induced increase in the amplitude of the single channel current, we made inside-out patches. *Insulin + PTK:* we made cell-attached patches on the apical membrane without any treatment; after getting high seal resistances (>400 GΩ), we applied insulin (100 nM) to the basolateral solution, while keeping the high seal resistance in cell-attached patches; after observing the insulin-induced increase in the amplitude of the single channel current, we made inside-out patches; we applied PTK catalytic subunit (p60c-src, 12.5 U/ml) with ATP (2 mM) to the cytosolic surface of the inside-out patches.

Table. The maximum values of the single channel conductance and EC_{50} of $[Ca^{2+}]_c$

Condition	Maximum value of the single channel conductance (pS)	EC_{50} (μM)
Control	3.7 ± 0.3	92 ± 25
Insulin	3.5 ± 0.7	0.23 ± 0.03*
Insulin/PTK	3.4 ± 0.2	3.2 ± 1.3*#

* $P < 0.001$ (compared with control); # $P < 0.005$ (compared with insulin); $n = 4$.

16]. On the other hand, insulin has also been reported to induce phosphorylation of calmodulin [7, 18, 41]. These observations suggest that the insulin may stimulate ion transport and glucose uptake by stimulating phosphorylation of calmodulin. However, even though it is suggested that some action of insulin is mediated through phosphorylation of calmodulin, it was still unknown how insulin stimulates ion or glucose transport through calmodulin-dependent pathways. Recent studies indicate that calmodulin activates PP2B, a Ca^{2+}-dependent enzyme [6, 42]. These observations suggest that insulin may act on the channel by activating PP2B via calmodulin-dependent pathways. Our present study clearly indicates that insulin acts on the Cl^- channel through PP2B-dependent pathways in a calmodulin-dependent manner.

Signaling Pathways of Insulin

PTK is well recognized to be one of the most important key enzymes in the signal transduction of the insulin action, which is involved in the insulin receptor [12, 22, 33, 40]. Autophosphorylation of the receptor's tyrosine results from the insulin-induced activation of receptor PTK [22, 33]. As described above, in A6 cells the insulin action was blocked by lavendustin A, a PTK inhibitor, suggesting that the insulin receptor is coupled to a PTK (PTK* in Fig. 10). On the other hand, lavendustin A also mimicked the insulin action with a different time course from the blocking action on the insulin effect. This observation suggests that another type of PTK ($PTK^{\#}$ in Fig. 10) is involved in the channel regulation. In epithelial tissues, the activated PTK involved in the insulin receptor located in the basolateral membrane does not directly affect effector molecules located in the apical membrane, but rather has a high affinity for potential cytoplasmic substrates and the ability to phosphorylate them [22]. The interaction of the insulin receptor with cytosolic proteins (e.g., insulin-receptor substrate-1 [12, 22, 34]) may enable post-receptor signalling to affect remote effectors such as ion channels located in the apical membrane apart from the basolateral membrane where the insulin receptor is located. The observations of the present study indicate that insulin would activate cytoplasmic PP2B in a calmodulin-dependent manner, and that the increase in PP2B activity causes the dephosphorylation of phospho-tyrosine in the Ca^{2+}-dependent Cl^- channel protein or channel-associated protein. This dephosphorylation of the channel or channel-associated protein augments its sensitivity to cytosolic Ca^{2+}, altering its single channel conductance. Dephosphorylation-induced modulation of the sensitivity to cytosolic Ca^{2+} may be the mechanism that regulates ion transports through the Ca^{2+}-dependent Cl^- channel. The identification of intermediate molecules in the post-receptor pathway may provide more details about insulin signalling for the regulation of ion channels and transport through PP2B-dependent pathways.

Conclusion

Taken together, our observations shown in the present study suggest that insulin increases the single channel conductance by increasing the sensitivity to cytosolic

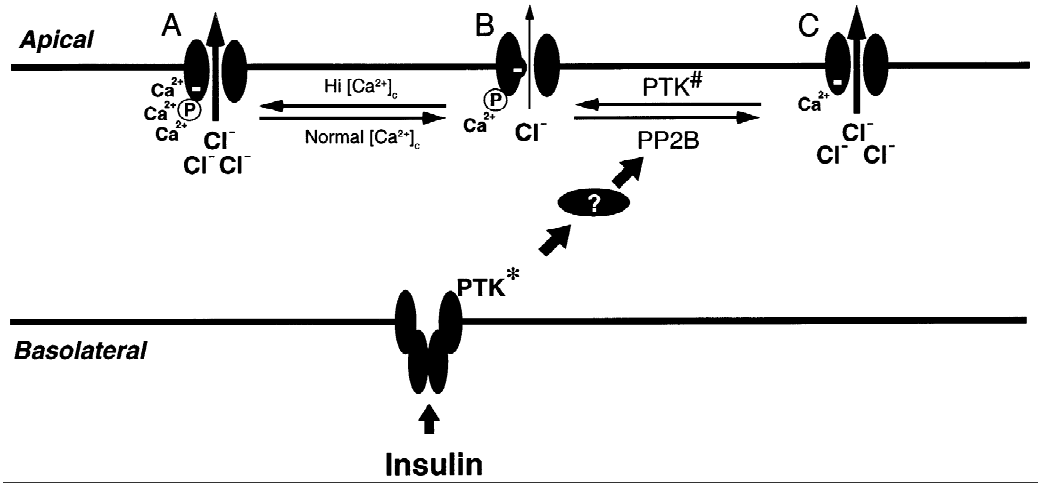

Fig. 10. A model of regulatory pathways by insulin and cytosolic Ca^{2+}. Some negative charge (white bar (−) in the channel protein in figure) interferes accumulation of Cl^-, a charge carrier, into the conductive pore from the cytosolic site, resulting in a low single channel conductance. (*A*) When the channel protein is phosphorylated, high $[Ca^{2+}]_c$ whose positive charge is large enough to keep the negative charge apart from the pore by neutralizing the negative charge of the phosphate causes high accumulation of Cl^- into the pore, leading to the channel with a high conductance. (*B*) When the channel protein is phosphorylated, normal $[Ca^{2+}]_c$ whose positive charge is not large enough to keep the negative charge apart from the pore due to the interfere by the negative charge of the phosphate causes low accumulation of Cl^- into the pore, leading to the channel with a low conductance. (*C*) When the channel protein is dephosphorylated by PP2B activated by insulin, normal $[Ca^{2+}]_c$ whose positive charge is large enough to keep the negative charge apart form the pore without the negative charge of the phosphate causes high accumulation of Cl^- into the pore, leading to the channel with a high conductance. These mean that the $[Ca^{2+}]_c$ required to induce an identical level of the single channel conductance (3 pS) (i.e., accumulation of Cl^- into the channel pore) is much smaller in the dephosphorylated channel than the phosphorylated one. Lavendustin A blocks PTKs associated with the insulin receptor and in the cytosol. Lavendustin A blocks the insulin action by inhibiting the PTK* of the insulin receptor, while lavendustin A mimics the insulin action through an inhibition of the $PTK^{\#}$ in the cytosol which is a similar effect of stimulation of PP2B.

Ca^{2+} through dephosphorylation of phospho-tyrosine of the channel or channel-associated protein in a manner dependent on PP2B and under the basal condition PTK phosphorylates the tyrosine of the channel or channel-associated protein (*see* Fig. 10). In the dephosphorylated channel, more Cl^- could accumulate into the conductive pore from the cytosolic site than in the phosphorylated channel, resulting in an increase in the single channel conductance for inward current (Cl^- efflux). On the other hand, the Cl^- accumulation into the conductive pore from the extracellular site, which causes the outward current (Cl^- influx), is not influenced by the dephosphorylation/phosphorylation of the channel or channel associated protein, resulting in no changes in the single channel conductance for the outward current. The open probability (gating kinetics) of the channel depends upon dephosphorylation/phosphorylation of the channel or channel-associated protein.

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References

1. Begum, N. 1994. Phenylarsine oxide inhibits insulin-stimulated protein phosphatase 1 activity and GLUT-4 translocation. *Am. J. Physiol.* **267:**E14–E23
2. Begum, N. 1995. Stimulation of protein phosphatase-1 activity by insulin in rat adipocytes. Evaluation of the role of mitogen-activated protein kinase pathway. *J. Biol. Chem.* **270:**709–714
3. Begum, N., Robinson, L.J., Draznin, B., Heidenreich, K.A. 1993. Protein phosphatase-1 and -2a activities in cultured fetal chick neurons: differential regulation by insulin and insulin-like growth factor-I. *Endocrinol.* **133:**2085–2090
4. Chan, C.P., Gallis, B., Blumenthal, D.K., Pallen, C.J., Wang, J.H., Krebs, E.G. 1986. Characterization of the phosphotyrosyl protein phosphatase activity of calmodulin-dependent protein phosphatase. *J. Biol. Chem.* **261:**9890–9895
5. Chan, C.P., McNall, S.J., Krebs, E.G., Fischer, E.H. 1988. Stimulation of protein phosphatase activity by insulin and growth factors in 3T3 cells. *Proc. Natl. Acad. Sci. USA* **85:**6257–6261
6. Cohen, P. 1989. The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* **58:**453–508
7. Colca, J.R., DeWald, D.B., Pearson, J.D., Palazuk, B.J., Laurino, J.P., McDonald, J.M. 1987. Insulin stimulates the phosphorylation of calmodulin in intact adipocytes. *J. Biol. Chem.* **262:**11399–11402
8. Donella-Deana, A., Krinks, M.H., Ruzzene, M., Klee, C., Pinna, L.A. 1994. Dephosphorylation of phosphopeptides by calcineurin (protein phosphatase 2B). *Eur. J. Biochem.* **219:**109–117
9. Erlij, D., De Smet, P., Van Driessche, W. 1994. Effect of insulin on

area and Na^+ channel density of apical membrane of cultured toad kidney cells. *J. Physiol.* **481:**533–542
10. Fischer, E.H., Charbonneau, H., Tonks, N.K. 1991. Protein tyrosine phosphatases: A diverse family of intracellular and transmembrane enzymes. *Science* **253:**401–406
11. Fujii, Y., Kaizuka, M., Hashida, F., Maruo, J., Sato, E., Yasuda, H., Kurokawa T., Ishibashi, S. 1991. Insulin regulates Na^+/glucose cotransporter activity in rat small intestine. *Biochem. Biophys. Acta* **1063:**90–94
12. Goldstein, B.J. 1992. Protein-tyrosine phosphatases and the regulation of insulin action. *J. Cell. Biochem.* **48:**33–42
13. Guarner, V., Hernandez, E.H., Huerto, R., Gorostiza, P., Valenzuela, F. 1993. Trifluoperazine inhibition of insulin-induced increase in skeletal muscle glucose uptake. *Eur. J. Pharmacol.* **237:**139–141
14. Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100
15. Hunter, T., Cooper, J.A.A. 1985. Protein-tyrosine kinases. *Annu. Rev. Biochem.* **54:**897–930
16. Ishibashi, O., Kobayashi, M., Sasaoka, T., Sugibayashi, M., Shigeta, Y. 1989. W-7 specifically inhibits insulin-induced increase in glucose transport. *Diabet. Res. Clin. Exp.* **6:**109–113
17. Ito, O., Kondo, Y., Takahashi, N., Omata, K., Abe, K. 1995. Role of calcium in insulin-stimulated NaCl transport in medullary thick ascending limb. *Am. J. Physiol.* **269:**F236–F241
18. Joyal, J.L., Sacks, D.B. 1994. Insulin-dependent phosphorylation of calmodulin in rat hepatocytes. *J. Biol. Chem.* **269:**30039–30048
19. Kubokawa, M., McNicholas, C.M., Higgins, M.A., Wang, W.H., Giebisch, G. 1995. Regulation of ATP-sensitive K^+ channel by membrane-bound protein phosphatases in rat principal tubule cell. *Am. J. Physiol.* **269:**F355–F362
20. Kubokawa, M., Wang, W., McNicholas, C.M., Giebisch, G. 1995. Role of Ca^{2+}/CaMK II in Ca^{2+}-induced K^+ channel inhibition in rat CCD principal cell. *Am. J. Physiol.* **268:**F211–F219
21. Lawrence, J.C., Jr. 1992. Signal transduction and protein phosphorylation in the regulation of cellular metabolism by insulin. *Annu. Rev. Physiol.* **54:**177–193
22. Lee, J., Pilch, P.F. 1994. The insulin receptor: structure, function, and signaling. *Am. J. Physiol.* **266:**C319–C334
23. Levitan, I.B. 1994. Modulation of ion channels by protein phosphorylation and dephosphorylation. *Annu. Rev. Physiol.* **56:**193–212
24. Lopez-Alarcon, L., Mojena, M., Monge, L., Feliu, J.E. 1986. Stimulation of pyruvate kinase phosphatase activity by insulin in isolated rat hepatocytes. *Biochem. Biophys. Res. Commun.* **134:**292–298
25. Marom, S., Goldstein, S.A., Kupper, J., Levitan, I.B. 1993. Mechanism and modulation of inactivation of the Kv3 potassium channel. *Receptors & Channels* **1:**81–88
26. Marunaka, Y., Eaton, D.C. 1990. Effects of insulin and phosphatase on a Ca^{2+} dependent Cl^- channel in a distal nephron cell line (A6). *J. Gen. Physiol.* **95:**773–789
27. Marunaka, Y., Eaton, D.C. 1990. Chloride channels in the apical membrane of a distal nephron A6 cell line. *Am. J. Physiol.* **258:**C352–C368
28. Marunaka, Y., Eaton, D.C. 1991. Effects of vasopressin and cAMP on single amiloride-blockable Na channels. *Am. J. Physiol.* **260:**C1071–C1084
29. Marunaka, Y., Hagiwara, N., Tohda, H. 1992. Insulin activates single amiloride-blockable Na channels in a distal nephron cell line (A6). *Am. J. Physiol.* **263:**F392–F400
30. Marunaka, Y., Shintani, Y., Downey, G.P., Niisato, N. 1997. Activation of Na^+-permeant cation channel by stretch and cAMP-dependent phosphorylation in renal epithelial A6 cells. *J. Gen. Physiol.* **110:**327–336
31. Marunaka, Y., Tohda, H., Hagiwara, N., Nakahari, T. 1994. Antidiuretic hormone-responding non-selective cation channel in distal nephron epithelium (A6). *Am. J. Physiol.* **266:**C1513–C1522
32. Matsumoto, P.S., Ohara, A., Duchatelle, P., Eaton, D.C. 1993. Tyrosine kinase regulates epithelial sodium transport in A6 cells. *Am. J. Physiol.* **264:**C246–C250
33. Murakami, M.S., Rosen, O.M. 1991. The role of insulin receptor autophosphorylation in signal transduction. *J. Biol. Chem.* **266:**22653–22660
34. Myers, M.G., Jr., White, M.F. 1993. The new elements of insulin signaling. Insulin receptor substrate-1 and proteins with SH2 domains. *Diabetes* **42:**643–650
35. Niisato, N., Marunaka, Y. 1997. Cross talk of bumetanide-sensitive and HCO_3^--dependent transporters activated by IBMX in renal epithelial A6 cells. *J. Membrane Biol.* **157:**53–61
36. Niisato, N., Marunaka, Y. 1997. Hyposmolality-induced enhancement of ADH action on amiloride-sensitive Isc in renal epithelial A6 cells. *Jpn. J. Physiol.* **47:**131–137
37. Niisato, N., Marunaka, Y. 1997. Regulation of Cl^- transport by IBMX in renal A6 epithelium. *Pfluegers Arch.* **434:**227–233
38. Pallen, C.J., Valentine, K.A., Wang, J.H., Hollenberg, M.D. 1985. Calcieneurin-mediated dephosphorylation of the human placental membrane receptor for epidermal growth factor urogastrone. *Biochemistry* **24:**4727–4730
39. Palmer, L.G., Frindt, G. 1987. Effects of cell Ca and pH on Na channels from rat cortical collecting tubule. *Am. J. Physiol.* **253:**F333–F339
40. Rosen, O.M. 1987. After insulin binds. *Science* **237:**1452–1458
41. Sacks, D.B., McDonald, J.M. 1988. Insulin-stimulated phosphorylation of calmodulin by rat liver insulin receptor preparations. *J. Biol. Chem.* **263:**2377–2383
42. Shenolikar, S. 1994. Protein serine/threonine phosphatases—New avenues for cell regulation. *Annu. Rev. Cell Biol.* **10:**55–86
43. Shintani, Y., Marunaka, Y. 1996. Regulation of chloride channel trafficking by cyclic AMP via protein kinase A-independent pathway in A6 renal epithelial cells. *Biochem. Biophys. Res. Commun.* **223:**234–239
44. Smit, L.S., Wilkinson, D.J., Mansoura, M.K., Collins, F.S., Dawson, D.C. 1993. Functional roles of the nucleotide-binding folds in the activation of the cystic fibrosis transmembrane conductance regulator. *Proc. Natl. Acad. Sci. USA* **90:**9963–9967
45. Sokabe, M., Fujitsuka, N., Kori, A.A., Ito, F. 1988. Effects of cyclic nucleotides and calcium on transduction and encoding processes in frog muscle spindle. *Brain Res.* **443:**254–260
46. Villa-Moruzzi, E. 1989. Stimulation of FA and phosphatase-1 activities by insulin in 3T3-L1 cells. *FEBS Lett.* **258:**208–210
47. Weil-Maslansky, E., Gutman, Y., Sasson, S. 1994. Insulin activates furosemide-sensitive K^+ and Cl^- uptake system in BC_3H1 cells. *Am. J. Physiol.* **267:**C932–C939
48. Wilkinson, D.J., Mansoura, M.K., Watson, P.Y., Smit, L.S., Collins, F.S., Dawson, D.C. 1996. CFTR: the nucleotide binding folds regulate the accessibility and stability of the activated state. *J. Gen. Physiol.* **107:**103–119
49. Zierler, K., Wu, F.-S. 1992. An early outward transient K^+ current that depends on a preceding Na^+ current and is enhanced by insulin. *Pfluegers Arch.* **422:**267–272