

# Effects of Insulin and Phosphatase on a $\text{Ca}^{2+}$ -dependent $\text{Cl}^-$ Channel in a Distal Nephron Cell Line (A6)

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**ABSTRACT** A  $\text{Cl}^-$  channel with a small single-channel conductance (3 pS) was observed in cell-attached patches formed on the apical membrane of cells from the distal nephron cell line (A6) cultured on permeable supports. The current-voltage ( $I$ - $V$ ) relationship from cell-attached patches or inside-out patches with 1  $\mu\text{M}$  cytosolic  $\text{Ca}^{2+}$  strongly rectified with no inward current at potentials more negative than  $E_{\text{Cl}}$ . However, the rectification decreased (i.e., inward current increased) when the cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) was increased above 1  $\mu\text{M}$ . If  $[\text{Ca}^{2+}]_i$  is increased to 800  $\mu\text{M}$ , the  $I$ - $V$  relationship became linear. Besides the change in the  $I$ - $V$  relationship, an increase in  $[\text{Ca}^{2+}]_i$  also increases the open probability of the channel. Regardless of the recording condition, the channel has one open and one closed state. Both closing and opening rates were dependent on  $[\text{Ca}^{2+}]_i$ ; an increase of  $[\text{Ca}^{2+}]_i$  decreased the closing rate and increased the opening rate. The  $\text{Ca}^{2+}$  dependence of transition rates at positive membrane potentials (cell interior with respect to external surface) were much larger than the dependence at negative intracellular potentials. The  $I$ - $V$  relationship of chloride channels in inside-out patches from cells pretreated with insulin was linear even with 1  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$ , while channel currents from cells under similar conditions but without insulin still strongly rectified. Alkaline phosphatase applied to the intracellular surface of inside-out patches altered the outward rectification of single channels in a manner qualitatively similar to that of insulin pretreatment. These observations suggest that phosphorylation/dephosphorylation of the channel modulates the sensitivity of the  $\text{Cl}^-$  channel to cytosolic  $\text{Ca}^{2+}$  and that insulin produces its effect by promoting dephosphorylation of the channel.

## INTRODUCTION

There have been only a few reports of the properties of single channels in the apical membranes of distal nephron cells and most of these reports have concentrated on the properties of cation channels (Palmer and Frindt, 1986; Hamilton and Eaton, 1986). The very few reports of single chloride channels or conductive movements of chloride have concentrated on the chloride permeability of the basolateral mem-

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brane (Koeppen et al., 1983; O'Neil and Sansom, 1984; Sansom et al., 1984). In contrast, little attention has been given to Cl<sup>-</sup> channels in the apical membranes of renal cells presumably because it is generally believed that transepithelial chloride transport is via paracellular pathways (Schuster and Stokes, 1987). However, there have been some reports that suggest that there may be some conductive movement of chloride across the apical membrane (Gross et al., 1986; Schuster, 1986). In support of this idea, several types of single chloride channels have been described in cells from a distal nephron cell line (A6) (Nelson et al., 1984; Marunaka and Eaton, 1989c). In our previous work (Marunaka and Eaton, 1989c), we described the properties of two types of single Cl<sup>-</sup> channels (3 and 8 pS) which may play a physiological role in chloride transport in transporting monolayers of A6 cells. While this previous report described the fundamental properties of the channels, one of the channels (3 pS) appeared to be regulated by several intracellular factors, which suggested that the modulation could be involved in normal tissue control of chloride transport. In particular, the present study shows that the characteristics of the 3-pS chloride channel can be altered by changes in intracellular Ca<sup>2+</sup> concentration and that insulin is capable of changing the sensitivity of the channel to calcium. In addition, the action of insulin can be mimicked by application of exogenous phosphatase. Some of these results have previously been described in preliminary reports (Marunaka and Eaton, 1989a, b).

#### METHODS AND MATERIALS

The methods and materials were the same as those used in a previous study (Marunaka and Eaton, 1989c). In brief, they were as follows.

##### *Cell Line and Culture Methods*

A6 cells were purchased from American Type Culture Collection (Rockville, MD) in the 68th plating (Rafferty, 1969). All experiments were carried out on platings 71–83 with no discernible differences between cells from different platings. Cells were maintained in plastic tissue culture flasks (Corning Glass Works, Corning, NY) at 27°C in humidified incubator with 4% CO<sub>2</sub> in the air. The culture medium was a mixture of Coon's medium F-12 (three parts) and Leibovitz's medium L-15 (seven parts) modified for amphibian cells with 104 mM NaCl, 25 mM NaHCO<sub>3</sub>, and pH 7.4. Besides these components, 10% fetal bovine serum (Gibco, Grand Island, NY), 1.0% streptomycin, and 0.6% penicillin (Irvine Scientific, Santa Ana, CA) were added. When cells were used for patch-clamp experiments, cells from the flasks were subcultured on permeable, glutaraldehyde-fixed collagen films attached to the bottoms of small plastic inserts for 35-mm petri plates. The inserts were made from lucite disks 3 cm in diameter and 1 cm thick. The disks had a centered conical hole that tapered from 2.5 cm in diameter at the top of the disk to 2 mm in diameter on the lower surface. It was on the lower surface that the collagen films were attached. When these disks were placed in 35-mm petri dishes and supported 1 mm off the bottom with glass spacers, the collagen film formed a permeable support that separated the two compartments, a basolateral compartment below the disk in the petri plate and an apical compartment above the collagen in the conical hole within the lucite disk. Cells plated in the conical hole above the collagen film formed polarized monolayers with their apical surfaces upward and transported sodium from the upper surface into the solution in the petri plate. To stimulate transport, 500 nM aldosterone (Sigma Chemical Co., St. Louis, MO) was added to the basolateral culture medium 48 h before experiments.

### *Patch Recording*

Patch pipettes were made from World Precision Instruments TW150 glass (New Haven, CT) and fired-polished to produce tip diameters of roughly 0.5–1.0  $\mu\text{m}$  (Hamill et al., 1981). Single-channel currents from cell-attached and inside-out (50–200  $\text{G}\Omega$ ) were obtained at 22–23°C with List EPC-7 patch-clamp amplifier (Guilford, CT). Only currents recorded from patches that had 50–200  $\text{G}\Omega$  seals were used for analysis. Current signals were recorded on a digital video recorder (SL-HF750 and PCM-501ES; Sony, Tokyo, Japan) and then digitized on a PDP-11/73 computer (at 0.5 ms per point) using a continuous data acquisition program developed by us specifically to acquire data from epithelial tissues.

### *Data Analysis*

Data records were low-pass filtered at 200 Hz using a software Gaussian filter. Events were detected by setting a threshold level between 50 and 75% of the estimated single-channel current amplitude. Because of the necessity for analyzing long, continuous records, no commercial patch-clamp analysis software was suitable. Therefore, programs that closely follow the strategy of Colquhoun and Sigworth (1983) were written for use on the VAX family of computers. These programs specifically allow the analysis of long continuous records which are necessary for successful single-channel recording from epithelial tissue. Patches were selected for low baseline noise levels with no drift of the baseline current (in general, low noise was associated with high seal resistances).

### *Solutions*

Normal Ringer's solution contained 130 mM NaCl, 3.4 mM KCl, 0.8 mM  $\text{CaCl}_2$ , 0.8 mM  $\text{MgCl}_2$ , and 10 mM HEPES with pH 7.4 was used. The composition of other solutions used in the present study is described in the text.

### *Chemicals*

Insulin and alkaline phosphatase were purchased from Sigma Chemical Co.

## RESULTS

### *Effects of Cytosolic Ca<sup>2+</sup> Concentrations*

Fig. 1 shows  $\text{Cl}^-$  channel activity in cell-attached and inside-out patches formed on the apical surface of A6 cells. About 80% of the patches (265 out of 332) examined in the present study had one or more channels of the type shown in Fig. 1. For cell-attached patches, there was outward current for potentials more positive than the resting membrane potential (cell interior positive with respect to the interior of the patch pipette at ground), while no current could be observed for potentials more negative than the resting membrane potential (Fig. 1 A). The extent of the outward rectification depends upon the calcium concentration at the intracellular surface of the patch. Channels in inside-out patches exposed to 1  $\mu\text{M}$   $\text{Ca}^{2+}$  on their intracellular surface appeared very similar to cell-attached patches; i.e., there was no measurable inward current at hyperpolarized potentials. There was measurable inward current when inside-out patches were exposed to 10  $\mu\text{M}$   $\text{Ca}^{2+}$  on their intracellular surface, but the inward current was much smaller than that with 800  $\mu\text{M}$  intracellular  $\text{Ca}^{2+}$  (Fig. 1 B). This observation suggests that with the lowest levels of intracellular  $\text{Ca}^{2+}$  we examined (1  $\mu\text{M}$ ), the channel outwardly rectifies in a manner

similar to the current in cell-attached patches. This implied that the outward rectification of the channel depended on the level of intracellular calcium.

The effect of calcium on channel rectification is readily apparent from examination of the single-channel current-voltage relationship. The current-voltage relationship of channels from cell-attached (Fig. 2 *A*) and inside-out patches in the presence of 1  $\mu\text{M}$  intracellular calcium (Fig. 2 *B*, *open diamonds*) strongly rectifies. When intracellular calcium is increased to 800  $\mu\text{M}$ , the current-voltage relationship becomes essentially linear. For intracellular calcium concentrations that are intermediate

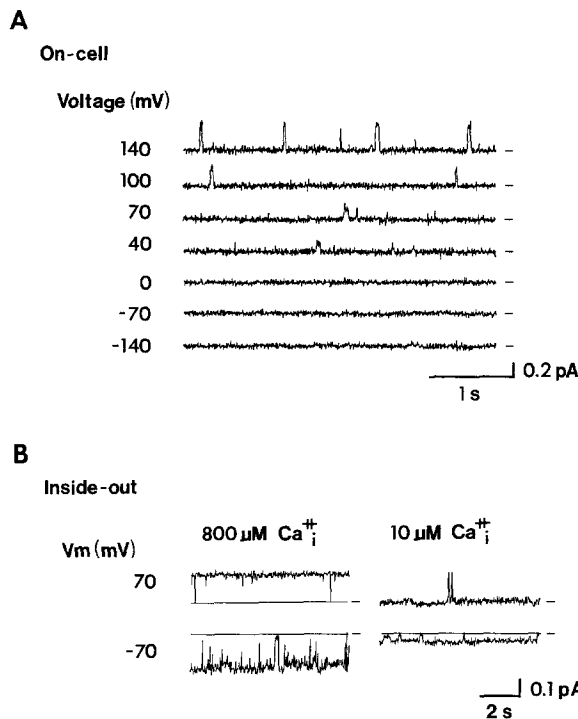


FIGURE 1. The  $\text{Cl}^-$  channel activity in an on-cell patch (*A*) and an inside-out patch (*B*). The solid line to the right of and occasionally through individual current traces indicates the level of the closed state. Outward current is upward. Voltages to the left of the traces in *A* are the displacement (in millivolts) of the intracellular potential from the apical membrane potential. In *B*, the potentials are the potentials of the bath with respect to the pipette interior (at ground). For the cell-attached patch in *A*, outward current events are present at depolarizing potentials, but no inward current is measurable when the patch is hyperpolarized. For the inside-out patch in *B*, intracellular  $\text{Ca}^{2+}$  affects the amplitude of inward current

and the open probability of the channel. For a depolarizing potential (+70 mV), the amplitude of the outward current in either 10 or 800  $\mu\text{M}$  intracellular  $\text{Ca}^{2+}$  is the same (even though the open probability is much larger in 800  $\mu\text{M}$   $\text{Ca}^{2+}$ ). At -70 mV the amplitude of the inward current in 10  $\mu\text{M}$   $\text{Ca}^{2+}$  is much smaller than the amplitude in 800  $\mu\text{M}$   $\text{Ca}^{2+}$  (although the open probability is comparable).

between 1 and 800  $\mu\text{M}$ , there are intermediate amounts of outward rectification (Fig. 2 *B*). In all cases, the single-channel conductance at depolarized potentials is  $\sim 3$  pS. These results suggest that the amplitude of the inward current is dependent on cytosolic  $\text{Ca}^{2+}$  concentration, while the amplitude of the outward current is independent of the  $\text{Ca}^{2+}$  concentration. This effect of intracellular calcium on the amplitude of the inward current is shown in Fig. 2 *C*.

Besides its effect on the magnitude of the inward current, intracellular calcium also affects the open probability of the channel. At a patch potential of -70 mV

(bath potential with respect to the pipette interior at ground; i.e., inward current) or +70 mV (outward current), the open probability of the channel decreased when the cytosolic Ca<sup>2+</sup> concentration was reduced (Fig. 3). However, the reduction in open probability was much larger for channels held at +70 mV than for those held at -70 mV.

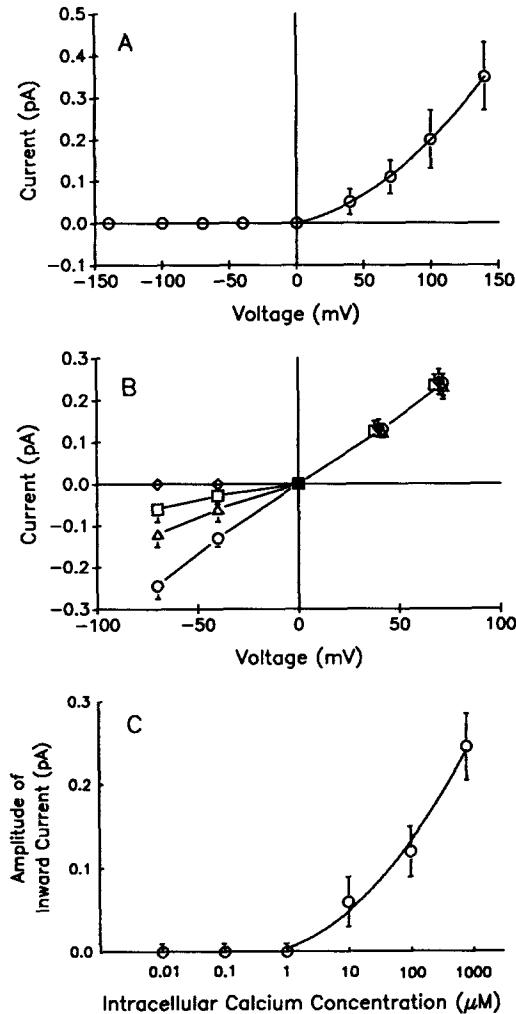


FIGURE 2. The current-voltage relationship of on-cell and inside-out patches. In *A*, the current-voltage relationship of cell-attached patches with normal saline in the pipette and bath has strong outward rectification; there is no measurable inward current at potentials less than the resting potential (values on the voltage axis are the displacement of the intracellular potential from the apical membrane potential). In *B*, for inside-out patches with symmetric chloride concentrations in the bath and pipette, the outward rectification is reduced by increasing intracellular Ca<sup>2+</sup>; i.e., the amplitude of the inward current at  $V_m$  of -70 mV is increased by raising intracellular Ca<sup>2+</sup>. Diamonds, squares, triangles, and circles are the current-voltage relationships with free intracellular Ca<sup>2+</sup> concentrations of 1, 10, 100, and 800  $\mu$ M, respectively. (The values on the potential axis is the bath potential with respect to the pipette at ground.) In *C*, the amplitude of the inward currents at -70 mV from the inside-out patch shown in *B* increases as the intracellular calcium increases. The symbols and vertical bars are mean values and standard deviations (in *A*,  $n = 10$ ; in *B* and *C*,  $n = 3-6$ ). Curves are drawn by eye to simplify data comparison and have no specific significance.

The change in open probability must be due either to a change in the mean open time or the mean closed time or, possibly, both. Interval histograms of channel open and closed durations indicate that the channel has one open state and one closed state (whether measured in inside-out or cell-attached configuration; Fig. 4 and Marunaka and Eaton, 1989c). At depolarized potentials (+70 mV), the closing rate was decreased and the opening rate was increased when the cytosolic Ca<sup>2+</sup> concen-

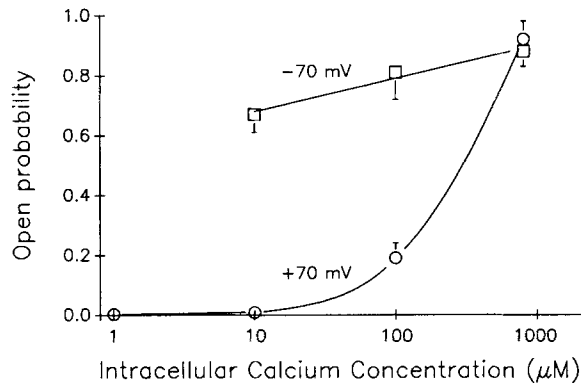


FIGURE 3. Calcium dependence of the open probability. In the same inside-out patch as Fig. 2 with symmetric chloride concentrations in the bath and pipette, the open probability of the 3-pS chloride channel depends upon intracellular  $\text{Ca}^{2+}$  concentration both when the patch is depolarized to +70 mV (circles) and when it is hyperpolarized to -70 mV (squares). However, the calcium dependence is much more pronounced when the patch is depolarized (compare the individual data records in Fig. 1 B). The symbols and vertical bars are mean values and standard deviations ( $n = 3-5$ ).

tration was increased (Fig. 5 A). In contrast, for hyperpolarized holding potentials (-70 mV), the closing rate was slightly decreased but the opening rate was not significantly changed when the cytosolic  $\text{Ca}^{2+}$  was increased (Fig. 5 B). However, the magnitude of the change in the transition rates is much smaller at -70 mV than the change at +70 mV (Fig. 5). Examination of Figs. 3 and 5 shows that the difference in the effect of calcium on the opening rates at different membrane potentials is the main reason for the difference in the effect of  $\text{Ca}^{2+}$  on the open probability at positive and negative membrane potentials.

The effects of raising intracellular  $\text{Ca}^{2+}$  concentration were reversible. That is,

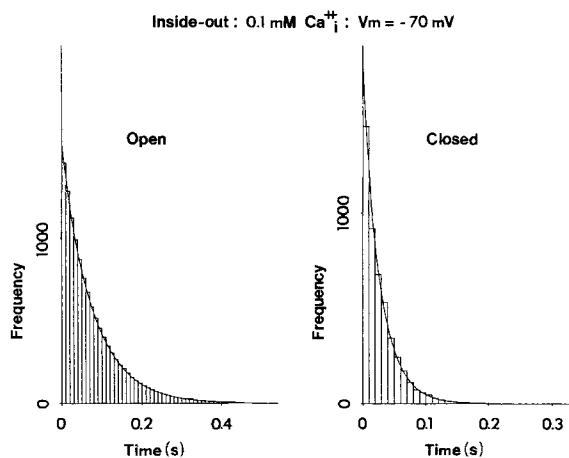


FIGURE 4. The open- and closed-time histograms of an inside-out patch. The  $\text{Cl}^-$  channel has one open and one closed state since each histogram can be fit by single exponential. The mean open time is 74 ms and the mean closed time is 27 ms. The patch has symmetric chloride concentrations in the bath and pipette with 100  $\mu\text{M}$   $\text{Ca}^{2+}$  in the bath and 800  $\mu\text{M}$   $\text{Ca}^{2+}$  in the pipette. Histograms at other potentials and  $\text{Ca}^{2+}$  concentrations were similar in that they could be fit well by single exponential functions.

even when the intracellular Ca<sup>2+</sup> concentration was changed from 1 to 800  $\mu$ M and then back to 1  $\mu$ M, the currents before and after treatment with high Ca<sup>2+</sup> were the same.

Thus, cytosolic Ca<sup>2+</sup> concentration has two distinct effects on the 3-pS Cl<sup>-</sup> channel: one effect is to increase open probability and the other is to decrease the extent of outward rectification of channel currents.

#### *Effects of Insulin on the Cl<sup>-</sup> Channel*

Insulin is known to increase transepithelial Na transport in A6 monolayers (Walker et al., 1984). For insulin to significantly enhance sodium transport in native tissue might also require an enhancement of co-ion transport. Therefore, we examined

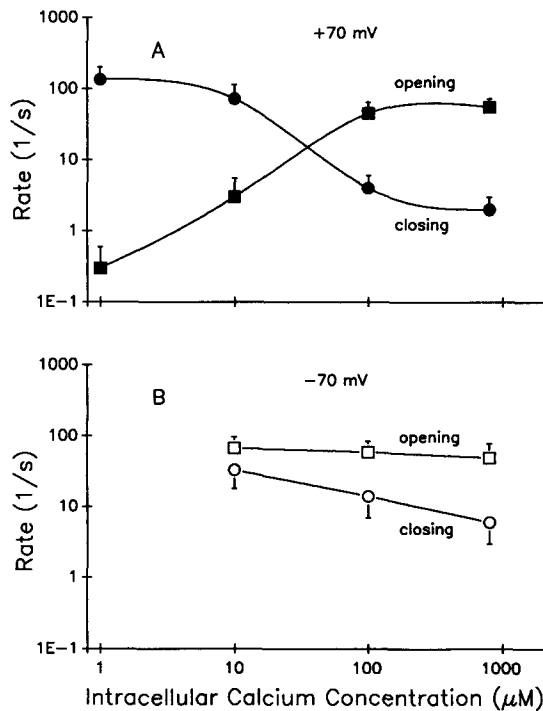


FIGURE 5. The effect of intracellular Ca<sup>2+</sup> on closing and opening rates. In inside-out patches with symmetric chloride concentrations in the bath and pipette held at 70 mV (A) and -70 mV (B), the closing rate at both potentials is decreased by increasing intracellular Ca<sup>2+</sup>. On the other hand, the opening rate at +70 mV is increased but the opening rate at -70 mV is not significantly changed by increasing intracellular Ca<sup>2+</sup>. The lines through the data are drawn by eye for convenience in identifying similar data and have no other significance. The symbols and vertical bars are the mean values and standard deviations ( $n = 3-5$ ).

the effect of insulin on single 3-pS Cl<sup>-</sup> channels. After Cl<sup>-</sup> channel activity was observed in cell-attached patches, insulin (100 mU/ml) was applied from the basolateral side of the cells. As expected from the results described above, no inward current was observable before application of insulin, but 10 min after applying insulin inward currents were apparent at an applied potential of -140 mV (Fig. 6 A). This observation suggests that insulin can reduce the outward rectification that is normally present in the *I-V* relationship of untreated channels in cell-attached patches. Examination of the *I-V* relationship of a cell-attached patch before and after insulin treatment (Fig. 7 A) confirms that there is less outward rectification after application of insulin, but that the single-channel conductance at depolarized

potentials is not changed by insulin. However, unlike treatment with high concentrations of intracellular calcium (Fig. 2 *B*), some outward rectification remains after applying insulin. The chord conductance is 2.5 pS at an applied potential of 140 mV, but only 0.9 pS at  $-140$  mV. To clarify the mechanism of the insulin action, we also formed cell-attached patches on cells that were then treated with insulin until inward chloride channel current was observable. The insulin was then washed off and the patches were ripped off to form inside-out patches. Fig. 6 *B* shows for one

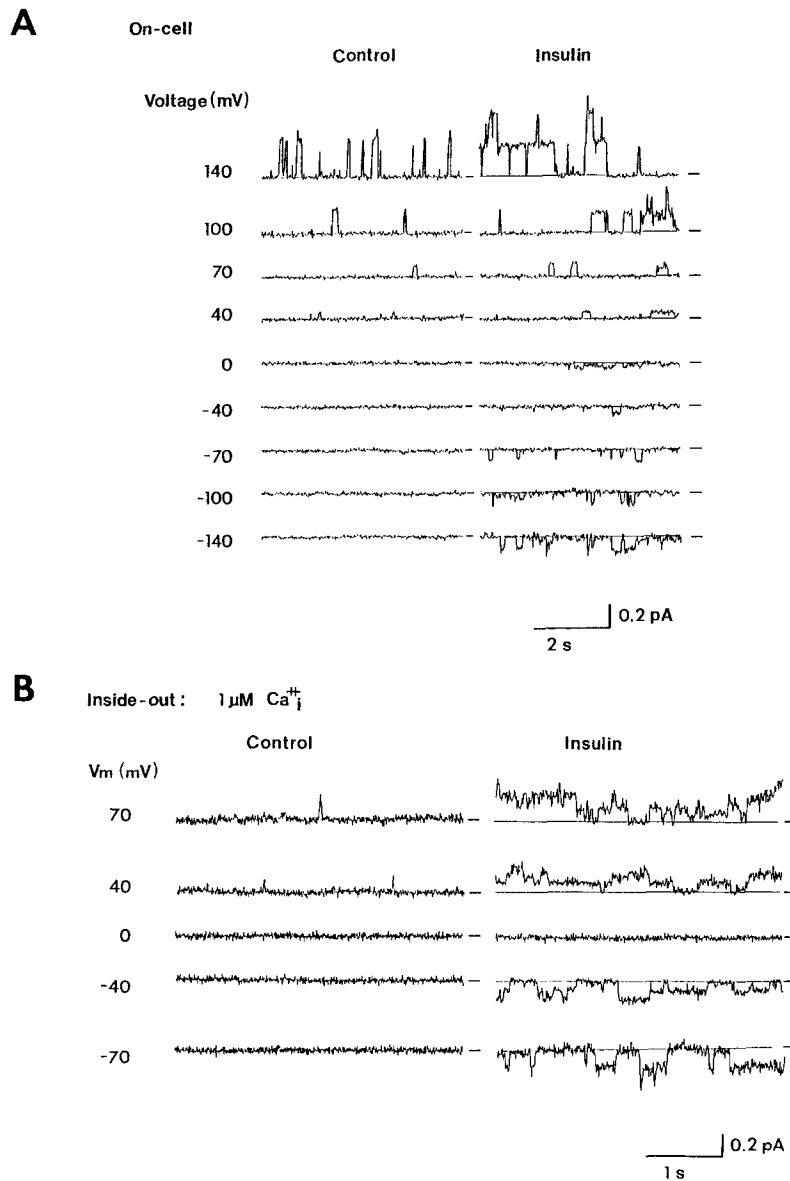


FIGURE 6



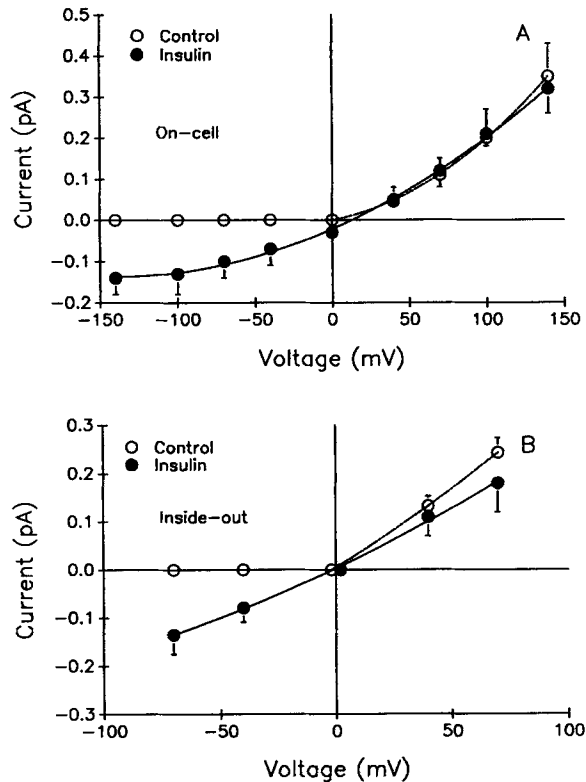


FIGURE 7. The  $I$ - $V$  relationship of  $Cl^-$  channels in the presence and absence of insulin. (A)  $I$ - $V$  relationships of  $Cl^-$  channels in on-cell patches in the presence (filled circles) and absence (open circles) of insulin differ in the amount of inward current present at hyperpolarized potentials; insulin pretreatment induces inward currents. (B) The  $I$ - $V$  relationships of  $Cl^-$  channels in inside-out patches with  $1 \mu M$  intracellular  $Ca^{2+}$  recorded from the cells with (filled circles) and without (open circles) insulin pretreatment also differ in the amount of inward current. In both A and B, insulin was applied before forming the inside-out patch. The lines through the data are drawn by eye for convenience in identifying similar data and have no other significance. The symbols and vertical bars are the mean values and standard deviations ( $n = 3-5$  at each point).

such inside-out patch that, unlike channels from untreated cells, channels from cells that have been pretreated with insulin carried inward current even in  $1 \mu M$  intracellular calcium. This result suggested that insulin might be producing its effect by altering the sensitivity of the channel to intracellular calcium concentration. Examination of the  $I$ - $V$  relationship from this patch also supports this idea; the  $I$ - $V$  rela-

FIGURE 6. (opposite) The effect of insulin on the  $Cl^-$  channel activity. In A, addition of insulin ( $100 \text{ mU/ml}$ ) to a cell on which a cell-attached patch has been formed increases the open probability and increases the amount of inward current measurable at hyperpolarizing potentials. Since both the bath and the pipette contain normal A6 saline, these results show that insulin could have an effect on chloride transport under physiological conditions. In B,  $Cl^-$  channel activity in an inside-out patch with  $1 \mu M$  intracellular  $Ca^{2+}$  increases when cells are pretreated with insulin before forming the inside-out patch. Channels in patches from untreated cells have no measurable inward current at hyperpolarized potentials, while channels from insulin-treated cells have inward currents. Voltages to the left of the traces in A are the displacement (in millivolts) of the intracellular potential from the apical membrane potential. In B, the potentials are the potentials of the bath with respect to the pipette interior (at ground). The horizontal line beside and occasionally through individual current records indicates the level of the closed state. Outward current is upward.

tionships shown in Fig. 7 *B* (inside-out patches from untreated and insulin pretreated cells) look similar to those shown in Fig. 7 *A* (untreated and insulin pretreated cell-attached patches). In inside-out patches the insulin-induced reduction in rectification is even more profound than in the cell-attached patches: the chord conductance is 3 pS at +70 mV and 1.9 pS at -70 mV.

#### Channel Selectivity after Insulin Treatment

If the anion to cation selectivity of the channel changes to make the channel much less selective after treatment with insulin, then inward current would appear. Fig. 8 shows the ion selectivity of the channel in inside-out patches with and without insulin pretreatment. For both treated and untreated cells, outward currents could be observed at no applied potential when the intracellular  $\text{Cl}^-$  concentration was reduced by replacement with gluconate (the bathing solution had 30 mM Cl, 100 mM gluconate, and 130 mM Na, the pipette solution had 130 mM NaCl and the

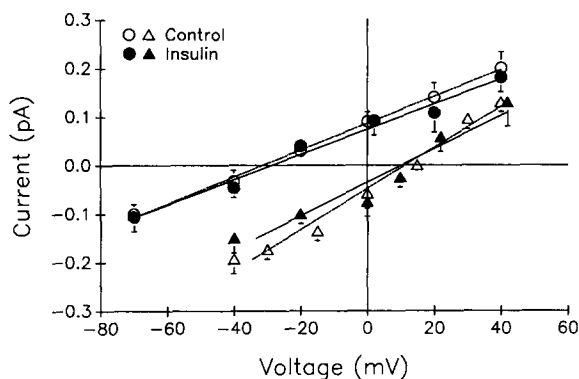


FIGURE 8. The ion selectivity of chloride channels in inside-out patches with (filled symbols) and without (open symbols) insulin pretreatment. Two different solutions were used. Circles show the *I-V* relationship when 30 mM Cl, 100 mM gluconate, and 130 mM Na were used for the bathing solution. Triangles show the *I-V* relationship when the 230 mM NaCl was used for the bathing solution. The pipette

solution contained 130 mM NaCl. Both pipette and bathing solutions contained 800  $\mu\text{M}$   $\text{CaCl}_2$ . Insulin has little if any effect on ion selectivity. The symbols and vertical bars are the mean value and standard deviation ( $n = 3$ ) while the straight lines are the best linear least-squares fit to the data.

concentrations of other ions in the pipette and bathing solutions were symmetrical). In addition, regardless of insulin treatment, inward current could be observed at no applied potential in inside-out patches when the internal (bath) NaCl concentration (230 mM) was higher than the external (pipette) NaCl concentration (130 mM). Taken together, these observations imply that even after applying insulin the channel remains selective for  $\text{Cl}^-$ . We calculated the ion selectivity, assuming three conditions: the channel is impermeable to gluconate, the osmolarity has no effect on the ion selectivity, and the activity coefficient of NaCl is 0.76 for 130 mM and 0.72 for 230 mM. From the reversal potentials, we calculated the ratio of the  $\text{Cl}^-$  to  $\text{Na}^+$  permeability before insulin treatment to be  $\sim 14$ , while after insulin treatment the ratio decreased slightly to  $\sim 8$ . However, since there was no statistically significant difference in current amplitudes before and after insulin treatment, the difference in selectivity is not likely to be significant.

*Sensitivity to Intracellular Calcium after Insulin Treatment*

Another way for insulin to induce inward current in cell-attached patches would be for the channel to be much more sensitive to intracellular calcium concentrations. Such a shift in the calcium sensitivity does appear to be one effect of insulin treatment. The shift in the channel's sensitivity to intracellular calcium is obvious if the amplitude of the inward current (at  $-70$  mV) in inside-out patches obtained from insulin-treated and untreated cells is plotted vs. the cytosolic  $\text{Ca}^{2+}$  concentration (Fig. 9). Insulin shifted the  $\text{Ca}^{2+}$  sensitivity to the left; i.e., lower concentrations of intracellular calcium produce significant inward currents after insulin treatment. This shows that insulin increases the  $\text{Ca}^{2+}$  sensitivity of the channel about 100-fold.

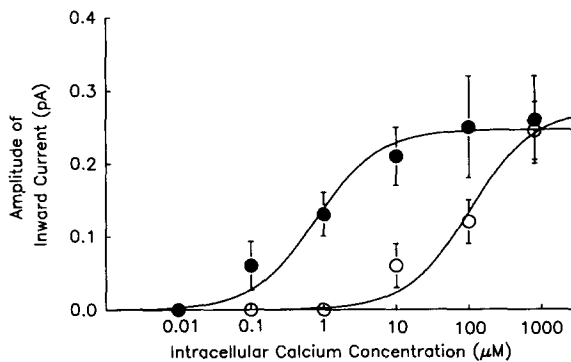


FIGURE 9. The  $\text{Ca}^{2+}$  dependency of inward current amplitude before and after insulin treatment. The magnitude of the inward current at  $V_m$  of  $-70$  mV was measured in untreated and insulin pretreated patches with varied intracellular  $\text{Ca}^{2+}$ . Insulin increases the sensitivity of the channel to intracellular  $\text{Ca}^{2+}$  about 100-fold. The curves drawn through the data points

are the best least-squares fit for a single calcium binding site. In the absence of insulin, the best fit is for a maximum inward current amplitude at high calcium concentrations of  $0.273$  pA and a  $K_{1/2}$  of  $106 \pm 12$   $\mu\text{M}$ . After insulin pretreatment the fit gives a maximum inward current amplitude of  $0.247$  pA and  $K_{1/2}$  of  $0.8 \pm 0.2$   $\mu\text{M}$ . Insulin increases the sensitivity of the channel to intracellular  $\text{Ca}^{2+}$  at least 100-fold. The symbol and vertical bar show the mean value and the standard deviation (each point represents at least three and up to six experiments).

*Effects of Phosphatase on the Cl<sup>-</sup> Channel*

One possible explanation of the effect of intracellular calcium would involve calcium screening a fixed negative charge near the inner mouth of the channel. Such a charge might so reduce the chloride concentration at the inner mouth that there would be little or no outward chloride movement (i.e., inward current). A likely candidate for such a charge would be the phosphate associated with a protein phosphorylation site. Indeed, one reported action of insulin is to promote dephosphorylation (Witters et al., 1988) possibly by stimulating phosphatase activity (Chan et al., 1988). If insulin dephosphorylates a site at the inner mouth of the chloride channel, it might explain the effect of insulin to promote inward current (like high concentrations of intracellular calcium). To test this possibility, we examined the effect of phosphatase applied to the intracellular surface of  $\text{Cl}^-$  channels.

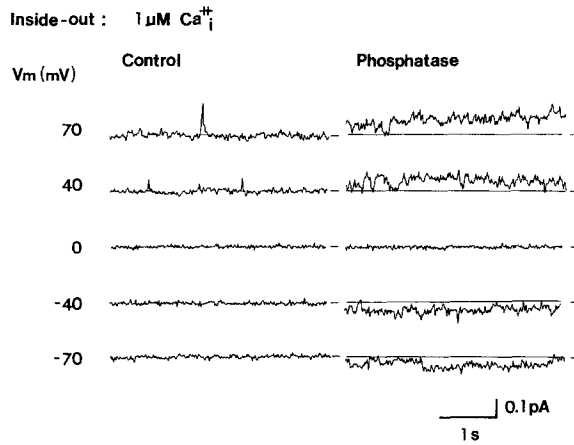


FIGURE 10. Effects of phosphatase on the  $\text{Cl}^-$  channel activity in an inside-out patch. In an untreated patch, no inward current can be seen. About 5–10 min after adding phosphatase inward currents are visible. The voltage values next to individual records are the intracellular potentials of the patch membrane. The horizontal line beside and occasionally through individual current records indicates the level of the closed state. Outward current is upward.

Fig. 10 shows the  $\text{Cl}^-$  channel activity in an inside-out patch exposed to  $1 \mu\text{M}$  cytosolic  $\text{Ca}^{2+}$ . Before adding alkaline phosphatase, outward current was measurable at depolarized potentials ( $+70$  mV) even though the open probability was small with relatively infrequent events. Also, as expected based on the results from our untreated, cell-attached patches, there is no measurable inward current at  $-70$  mV (Fig. 10, *left*). In contrast, inward current appeared and open probability increased after 5–10 min of phosphatase treatment (Fig. 10, *right*; alkaline phosphatase, 10 U/ml). Even if the phosphatase was subsequently washed off the patch the inward current persisted. Fig. 11 shows the current-voltage relationship before and after treating the channel with phosphatase in the presence of  $1 \mu\text{M}$  cytosolic  $\text{Ca}^{2+}$ . As for the case of insulin treatment, phosphatase treatment did not eliminate outward rectification. The chord conductance at holding potentials of  $-70$  and  $+70$  mV is 1.2 and 1.7 pS, respectively. Thus, phosphatase treatment, besides reducing rectification and increasing open probability, also reduces the channel conductance for outward current (chord conductance at  $+70$  mV is decreased from 2.9 to 1.7 pS). As

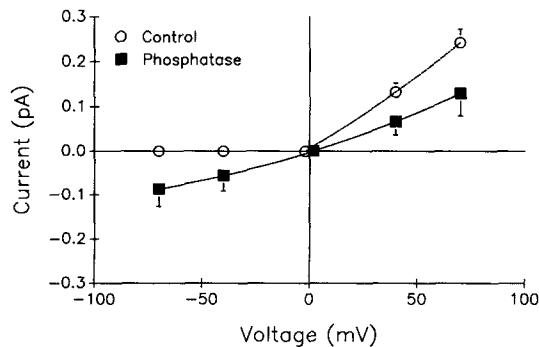


FIGURE 11. The  $I$ - $V$  relationship of  $\text{Cl}^-$  channels in an inside-out patch before (*open circles*) and after (*filled circles*) treatment with phosphatase with  $1 \mu\text{M}$  intracellular  $\text{Ca}^{2+}$ . Outward rectification is reduced after treatment with phosphatase; i.e., inward current increases. The voltage values are the intracellular potentials of the patch membrane. The symbols and vertical bars are the mean value and standard deviation ( $n = 3$ ).

we had done for insulin, we also attempted to measure the magnitude of the inward current in various intracellular calcium concentrations after phosphatase treatment. Unfortunately, phosphatase treatment tended to make inside-out patches unstable so that solution changes after phosphatase treatment were difficult. Nonetheless, it is clear that there is inward current at 1  $\mu\text{M}$  intracellular calcium after phosphatase treatment while there is no measurable inward current in untreated patches. Quantitatively, for 10  $\mu\text{M}$  intracellular  $\text{Ca}^{2+}$ , the inward current amplitude at a potential of  $-70$  mV after phosphatase treatment was  $0.21 \pm 0.04$  pA (mean value  $\pm$  SD,  $n = 3$ ), which is comparable to inward current in untreated cells exposed to 100  $\mu\text{M}$  intracellular  $\text{Ca}^{2+}$  ( $0.21 \pm 0.04$  pA;  $n = 5$ ); for 1  $\mu\text{M}$  intracellular  $\text{Ca}^{2+}$ , the inward current amplitude at  $-70$  mV after treatment of phosphatase was  $0.09 \pm 0.04$  pA ( $n = 3$ ), which is comparable to the inward current from untreated patches when they were exposed to calcium concentrations between 10 and 100  $\mu\text{M}$  ( $\sim 50$   $\mu\text{M}$ ). This suggests that phosphatase treatment increases the  $\text{Ca}^{2+}$  sensitivity of the channel at least 10-fold. So, despite our being unable to generate a complete dose-response curve for calcium after phosphatase treatment, our limited results do suggest that phosphatase treatment increases the sensitivity to cytosolic  $\text{Ca}^{2+}$  in a manner similar to insulin treatment. This observation suggests that the channel undergoes phosphorylation/dephosphorylation which plays a role in modulating the magnitude of the inward current through the channel.

#### DISCUSSION

##### *Other Ca<sup>2+</sup>-dependent Chloride Channels*

In the present study, we describe an outwardly rectifying  $\text{Cl}^-$  channel. The rectification is characterized by there being no measurable inward current but significant outward current in cell-attached patches (see Figs. 1 A and 2 A). In addition, the outward rectification is reduced by increasing the cytosolic  $\text{Ca}^{2+}$  (see Figs. 1 B and 2, B and C). Unfortunately, most other studies that have examined the effect of calcium on chloride conductances or single chloride channels have not examined the properties in a manner that can be directly compared with our results.

$\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels have been reported in several epithelial tissues. In particular, such channels have been reported in tracheal epithelial cells (Frizzell et al., 1986; Welsh, 1986; Li et al., 1988). Frizzell et al. (1986) describe two different types of  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels with single-channel conductance of 20 and 50 pS. One of them (50 pS) has single-channel currents in cell-attached and inside-out patches that outwardly rectify in the presence of 0.5  $\mu\text{M}$  cytosolic  $\text{Ca}^{2+}$ . The activity of both of these  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels is dependent on cyclic adenosine monophosphate (cAMP). The chloride channel described in this report is also sensitive to cAMP (Marunaka and Eaton, 1989a, c). Despite the large disparity in unit conductance, the  $\text{Ca}^{2+}$  dependency and cAMP dependency of the  $\text{Cl}^-$  channels in A6 and tracheal cells suggest that at least the regulation of the chloride channels in both tissues may be similar.

##### *Effects of Insulin and Phosphatase*

Insulin's most well known action is a stimulation of glucose transport in muscles and fat cells. But besides insulin's action on glucose transport, the hormone apparently

has a stimulatory action on the Na/K pump and on the Na/H exchange system (Siegel and Civan, 1976). Therefore, the mechanism by which insulin increases transepithelial sodium transport is generally thought to be through activation of either the Na/K pump or Na/H exchange (Siegel and Civan, 1976; Cox and Singer, 1977; Herrera, 1965). However, besides the action on the Na/K pump and Na/H exchange, insulin may have direct effects on ion channels such as the amiloride-blockable Na<sup>+</sup> channel in the apical membrane of epithelial tissues (Walker et al., 1984; Schoen and Erlj, 1987; Baxendale, 1988). The mechanism of the insulin action on the Na<sup>+</sup> channel is not yet clear, but, in the present study, we suggest that insulin stimulates another epithelial ion channel, an apical 3-pS Cl<sup>-</sup> channel, by modifying the open probability of the channel and by modifying the extent of the channel's outward rectification. In addition, insulin's action is mimicked by treatment of the intracellular surface of the channel with phosphatase. The similarities in the action of insulin and phosphatase treatment include reducing the extent of outward rectification, and increasing the open probability of the channel (while leaving the number of open and closed states unchanged). Other reports (Kiechle et al., 1980; Chan et al., 1988; Saltiel and Cuatrecasa, 1988; Witters et al., 1988; Yang et al., 1989) support the idea that insulin can stimulate phosphatase activity; thereby resulting in the dephosphorylation of membrane proteins (such as the Cl<sup>-</sup> channel protein in this report).

One difference in the action of insulin and phosphatase treatment is that, unlike insulin treatment, phosphatase treatment reduced the outward current amplitude (although insulin appeared to reduce the mean value of the outward current, the decrease was not statistically significant). This may mean that, despite the similarities in insulin and phosphatase action, phosphatase may be acting at an additional site which leads to an overall reduction in current. Considering the relatively impure preparation of phosphatase that we used, besides the specific, insulin-mimicking effects, there well might be some nonspecific effects which would appear as reductions in current.

#### *Insulin Action and Intracellular Ca<sup>2+</sup>*

Our results suggest that insulin (by stimulating phosphatase activity) induces inward current through the Cl<sup>-</sup> channels in cell-attached patches by shifting the Ca<sup>2+</sup> sensitivity of the channel so that normal intracellular calcium can stimulate inward current in the presence of insulin but not in its absence. The cytosolic Ca<sup>2+</sup> concentration of A6 cells has not been measured. However, if the cytosolic Ca<sup>2+</sup> concentration of A6 cells is similar to that of collecting tubule cells, the concentration would be 0.1–0.2 μM (Teitelbaum and Berl, 1986). Such a low concentration of intracellular calcium is consistent with our measurements. In cell-attached patches on A6 cells, there is no measurable inward current; in inside-out patches with 1 μM cytosolic Ca<sup>2+</sup>, we also cannot observe any inward current (Figs. 1 and 2). This observation suggests that the cytosolic Ca<sup>2+</sup> concentration of intact A6 cells must be no greater than 1 μM, at least if the Ca<sup>2+</sup> dependency of the Cl<sup>-</sup> channel in cell-attached patches is same as that in inside-out patches. This seems likely considering the similarities of the current-voltage relationships of cell-attached and inside-out patches.

On the other hand, in cell-attached patches, insulin induces inward current. This implies that under physiological conditions after insulin treatment, the cellular calcium is high enough to induce inward current (and an increase in open probability). We have shown that one of the ways that this occurs is through at least a 100-fold shift to lower concentrations of the response of single channels to intracellular calcium (Fig. 9). However, there is a possibility that, besides shifting the sensitivity of the chloride channel to calcium, insulin may also produce an increase in intracellular Ca<sup>2+</sup> concentration as it apparently does in several other preparations (Teitelbaum and Berl, 1986; Pershadsingh et al., 1987; but compare Klip et al., 1984; Cheung et al., 1987). If one of the effects of insulin in A6 cells is to increase intracellular calcium, then this would only serve to accentuate the action of insulin since if the sensitivity to calcium is increased and the intracellular calcium is also increased, the effect on the channel will be to promote more chloride secretion than the change in calcium sensitivity or the change in calcium alone. Regardless of which effect, the change in sensitivity or a possible increase in intracellular calcium, is more important the effect of insulin could be of physiological importance since insulin effects are measurable in cell-attached patches with physiological concentrations of intracellular calcium.

#### *Summary*

We have described a Ca<sup>2+</sup>-dependent Cl<sup>-</sup> channel in the apical membrane of a distal nephron cell line (A6). The single-channel current normally rectifies strongly favoring chloride influx (outward current). The modulation is via two separate mechanisms. Increasing intracellular calcium increases the open probability of the channel, but also increases the amount of inward current thus allowing chloride efflux (inward current). Besides intracellular calcium, insulin also increases the open probability and increases inward current of single channels. Insulin appears to act by promoting phosphatase activity. The action of both insulin and phosphatase is to increase the Ca<sup>2+</sup> sensitivity of the channel. In particular, insulin induces inward current (Cl<sup>-</sup> secretion) through the Cl<sup>-</sup> channel, which is not present in the absence of insulin.

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