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External Calcium Ions Are Required for Potassium Channel Gating in Squid Neurons

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The effects of calcium removal on the voltage-dependent potassium channels of isolated squid neurons were studied with whole cell patch-clamp techniques. When the calcium ion concentration was lowered from 10 to 0 millimolar (that is, no added calcium), potassium channel activity, identified from its characteristic time course, disappeared within a few seconds and there was a parallel increase in resting membrane conductance and in the holding current. The close temporal correlation of the changes in the three parameters suggests that potassium channels lose their ability to close in the absence of calcium and simultaneously lose their selectivity. If potassium channels were blocked by barium ion before calcium ion was removed, the increases in membrane conductance and holding current were delayed or prevented. Thus calcium is an essential cofactor in the gating of potassium channels in squid neurons.

CALCIUM IONS HAVE PRONOUNCED effects on the gating properties of ionic channels. Frankenhaeuser and Hodgkin (*1*) examined the hypothesis that Ca^{2+} ions serve as the gating particles in sodium channels and are expelled from their blocking positions by depolarization. The hypothesis could not completely explain gating, but these researchers noted that the idea was worth remembering. Subsequent experiments failed to show a direct role for Ca^{2+} in channel gating (*2–6*), and it is now clear that the major element in the voltage sensitivity of the Na^+ channel results from mobile charges intrinsic to the protein [see (*7*) for a review]. Potassium channel gating is presumably similar, but recently it has been suggested that Ca^{2+} may play a more direct role and that occupation of a K^+ channel by Ca^{2+} is obligatory for normal closing (*8*). We have now tested this hypothesis by removing all the external Ca^{2+}. The results show that K^+ channels in squid neurons lose their ability to close in the absence of external Ca^{2+} and also lose their selectivity, leading to a large drop in membrane resistance. Thus Ca^{2+} is an essential cofactor in the gating of these and possibly other channels.

We performed our experiments on isolated cells from the giant fiber lobe (GFL) of the stellate ganglion of squid (*Loligo pealei*), using methods described by Llano and Bookman (*9*). We subjected the cells to whole cell patch clamp (*10, 11*) 1 to 5 days after isolation, utilizing patch electrodes with resistances of 0.3 to 1 megohm. Access resistance was compensated for electronically. Experiments were performed in a small chamber that had continuous flow of solution. Solutions could be changed within about 20 seconds.

The membrane current from a typical cell in medium that contained Ca^{2+} is shown in Fig. 1A for a depolarization from −80 to +40 mV. These cells are advantageous for our purposes because, as the trace shows, there is a large K^+ current and essentially no inward current. When Ca^{2+} (or, in the case illustrated, Ca^{2+} and Mg^{2+}) was removed from the external medium, changes in the current pattern occurred roughly with the time course of bath exchange (Fig. 1, B to D).

The sequence of events was as follows. (i) An inward holding current appeared and grew larger from 21 to 53 seconds. (ii)

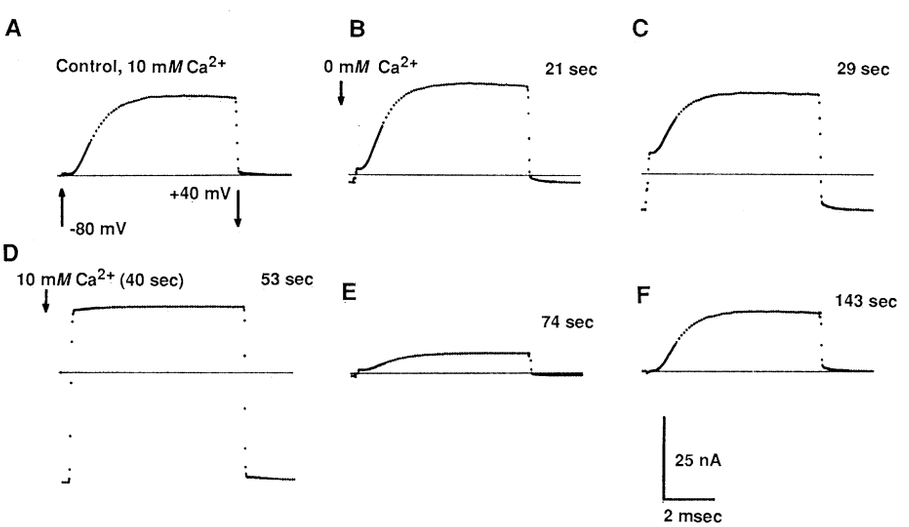

Fig. 1. Potassium current and the effect of reducing the Ca^{2+} concentration. (**A**) In the presence of Ca^{2+}, membrane current has the sigmoid time course characteristic of K^+ channel current (I_K). The membrane potential was stepped from −80 to +40 mV at the first arrow and returned to −80 mV at the second arrow. (**B** to **D**) When Na^+ was substituted for external Ca^{2+} and Mg^{2+}, an inward current developed, there was an instantaneous jump in current on application of the step, and K^+ channel gating activity disappeared. (**E** and **F**) With the restoration of Ca^{2+}, the changes were reversed. The time after the change to 0 m*M* calcium is indicated. Solutions were as follows: external: 10 m*M* $CaCl_2$, 50 m*M* $MgCl_2$ plus 445 m*M* NaCl and 10 m*M* Hepes [4-(2-hydroxyethyl)-1-piperazinethane sulfonic acid]; or 530 m*M* NaCl and 10 m*M* Hepes; internal: 475 m*M* potassium glutamate, 50 m*M* KCl, 25 m*M* KF, and 5 m*M* Hepes. The *p*H of all solutions was 7.3, and the temperature was 15°C.

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When voltage was stepped from −80 to +40 mV, there was an almost instantaneous jump in currents from inward to outward. (iii) K^+ current, which can be recognized by its characteristic sigmoid time course, became progressively smaller as the instantaneous current grew. After 53 seconds, there occurred a square step of instantaneous current, with no indication of K^+ channel gating activity. The instantaneous current disappeared quickly on replacing Ca^{2+}. K^+ current reappeared more slowly after Ca^{2+} restoration but recovered with time to about 75% of its original amplitude (Fig. 1, E and F). Similar results were obtained on every cell (more than 50) subjected to this procedure, though in some cases the cells disintegrated before Ca^{2+} was restored. Calcium removal had no direct effect on the resistance of the tight seal between membrane and pipette, as we ascertained by monitoring the seal resistance of a pipette attached to an intact cell.

Thus K^+ channel gating activity disappears with removal of Ca^{2+}. But do the channels that no longer gate remain open or closed? The instantaneous current suggests that they remain open, but the large inward current is not expected from normally selective K^+ channels. Is the inward current the result of the opening of an independent set of nonselective or inward current channels? Or have K^+ channels lost their selectivity together with their ability to close?

Three observations establish the latter possibility as the most likely.

1) The instantaneous current and the inward current grew with precisely the same time course after K^+ removal, suggesting that the instantaneous current is conducted by a single set of channels that have a reversal potential near 0 mV. This can be seen from the plot of inward current as a function of instantaneous current in Fig. 2A: the two currents are proportional to each other.

2) The instantaneous current grew with the time course of the disappearance of the K^+ channels. This is illustrated by plotting the K^+ channel current (I_K) on one axis and the instantaneous current (I_i) on the other (Fig. 2B). I_K grew slightly with the Ca^{2+} removal (data point 1 to data point 2), because Ca^{2+} has a small inhibitory effect on current through K^+ channels. Thereafter there was an almost perfectly linear relation between the amplitudes of the two currents, with I_i growing at roughly twice the rate at which I_K disappears. Together the two observations suggest that a single set of channels conducts the instantaneous current, and that these channels are K^+ channels that have lost both selectivity and the ability to close.

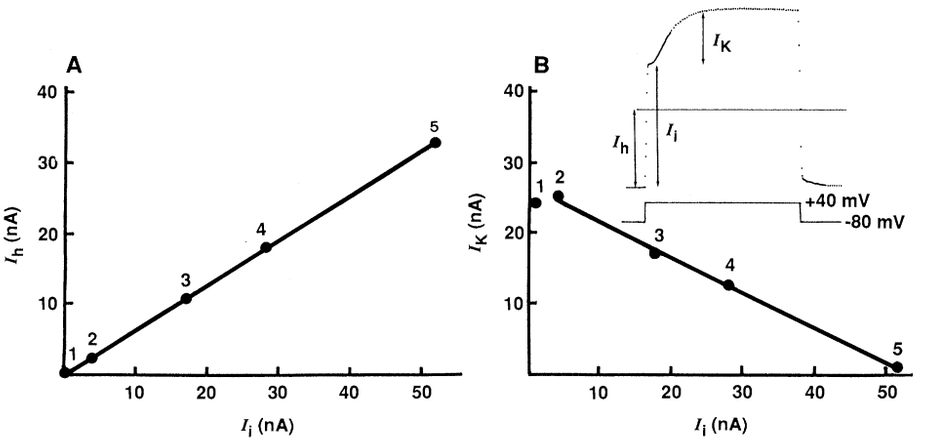

Fig. 2. The relation of potassium current (I_K), instantaneous current (I_i), and holding current (I_h). Currents were measured as indicated in the inset of the figure. After removal of external Ca^{2+}, I_h increased in parallel with I_i (**A**) and the latter grew with the same time course as the disappearance of I_K (**B**). These observations suggest that calcium removal converts K^+ channels into unselective channels that cannot close and that conduct the holding and instantaneous current. The numeral on each data point refers to consecutive current records taken 8, 21, 29, 33, and 53 seconds after removal of Ca^{2+}. The small increase in I_K observed in the first two points in (B) is due to relief of the inhibitory action of Ca^{2+} on K^+ currents. Solutions and other experimental conditions are similar to those for Fig. 1.

3) The third observation is that Ba^{2+} can prevent the loss of membrane resistance and the development of the instantaneous current. Among the divalent cations that we have tested, Ba^{2+}, which is a potent and long-lived blocker of K^+ channels, is unique in this regard. It is known that Ba^{2+} equilibrates slowly with the channels when applied externally (*12*). Our experiments show that Ba^{2+} prevents development of the instantaneous current only if it is permitted to block the K^+ channels before Ca^{2+} is removed. Thus in one set of experiments Ba^{2+} was added to the external medium at the same time that Ca^{2+} was removed, yielding the results in Fig. 3, A and B. Figure 3A illustrates the control current, recorded in Ca^{2+}-containing medium. Membrane resistance was tested by a negative step that preceded the activating pulse. This pulse

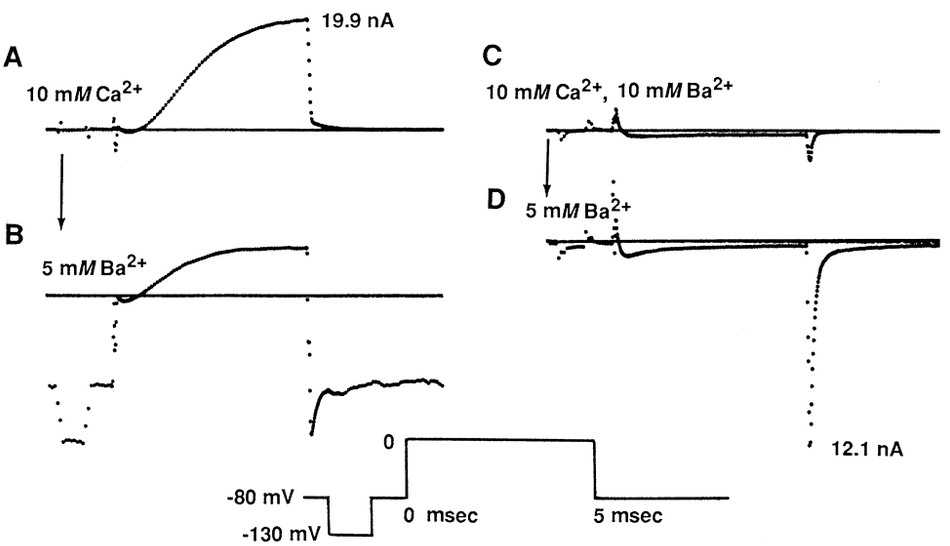

Fig. 3. External Ba^{2+} can prevent the loss of membrane resistance in 0 m*M* Ca^{2+}. The pulse protocol is shown in the inset of the figure. Membrane resistance was monitored by a negative 50-mV pulse. (**A**) In 10 m*M* Ca^{2+} (10 m*M* Ca^{2+}, 50 m*M* Mg^{2+}, 445 m*M* Na^+, 10 m*M* Hepes) membrane resistance is high and instantaneous current is very small. (**B**) Forty-five seconds after changing to 5 m*M* Ba^{2+} (5 m*M* Ba^{2+}, 530 m*M* Na^+, 10 m*M* Hepes) membrane resistance is low, the instantaneous current is large, and I_K is reduced by more than half. Note that the equilibrium potential of the instantaneous current is near 0 mV. (**C**) I_K is totally blocked in 10 m*M* Ca^{2+} and 10 m*M* Ba^{2+} (10 m*M* Ca^{2+}, 10 m*M* Ba^{2+}, 50 m*M* Mg^{2+}, 430 m*M* Na^+, 10 m*M* Hepes), membrane resistance is high, and a small inward current is seen that is due to permeation of Ba^{2+} ions through Ca^{2+} channels. (**D**) After 45 seconds in 5 m*M* Ba^{2+}, this Ba^{2+}-treated cell does not show the large holding and instantaneous currents or the pronounced drop in membrane resistance observed in (B). All external solutions used in these experiments were made with chloride salts. Internal solutions and other experimental conditions are similar to those for Fig. 1.

elicited a very small current (Fig. 3A), which shows that the membrane resistance is high. Forty-five seconds after removing Ca^{2+} and adding Ba^{2+}, membrane resistance was very low, and there was a large instantaneous current, the typical effect of Ca^{2+} removal. The result is quite different if Ba^{2+} is added to the medium and allowed to block K^+ channels before Ca^{2+} removal (Fig. 3, C and D). In this case membrane resistance remained high, and current during the pulse was small. Interestingly, in 5 m*M* Ba^{2+} there was somewhat more inward current during the pulse and a very large tail current, which may be current through Ca^{2+} channels. It is known that Ca^{2+} channels in heart and skeletal muscle become abnormally permeable when the Ca^{2+} concentration is very low (*13, 14*), and the tails in Fig. 3D may result from a similar phenomenon. Regardless of the identity of the channels responsible for this current, they do not contribute to the instantaneous current because they close when the cell is polarized to −80 mV. Aside from the tails, we think it unlikely that Ca^{2+} channels play a part in the phenomena observed here. Ca^{2+} channels become unselective for Ca^{2+} in the low micromolar range, but the effect on K^+ channels described here is detectable when Ca^{2+} drops below 4 to 5 m*M*. Furthermore, unlike the abnormal channels described here, Ca^{2+} channels retain their gating properties after losing selectivity.

Our experiments show that K^+ channels in squid GFL neurons maintain their integrity with regard to gating and selectivity properties only in the presence of an adequate concentration of external Ca^{2+}. It is clear from previous work (*7*) that a major element in gating is the movement of charge intrinsic to the channel protein in response to a membrane voltage change. We suggest that a second major factor is the release of a Ca^{2+} ion, perhaps as the last step in opening, and the rebinding of Ca^{2+} as the channel closes. It is unknown whether calcium serves as a cofactor in gating for other channel types as well.

From previous results (*8*), we expected the K^+ channels to lose their ability to close, but their loss of selectivity was unexpected. We speculate that when a K^+ channel remains open for a long time, it slowly assumes an unusual conformation with abnormal selectivity. In the presence of Ca^{2+}, the channel is open so briefly that the probability of assuming this conformation is very small. On restoring Ca^{2+} in Fig. 1 the instantaneous current disappears quickly, but K^+ channel activity returns more slowly. This slow recovery suggests that extensive refolding is required to restore the normal properties of the channel.

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Implantation of Genetically Engineered Fibroblasts into Mice: Implications for Gene Therapy

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In a variety of human genetic diseases, replacement of the absent or defective protein provides significant therapeutic benefits. As a model for a somatic cell gene therapy system, cultured murine fibroblasts were transfected with a human growth hormone (hGH) fusion gene and cells from one of the resulting clonal lines were subsequently implanted into various locations in mice. Such implants synthesized and secreted hGH, which was detectable in the serum. The function of the implants depended on their location and size, and on the histocompatibility of the donor cells with their recipients. The expression of hGH could be modified by addition of regulatory effectors, and, with appropriate immunosuppression, the implants survived for more than 3 months. This approach to gene therapy, here termed "transkaryotic implantation," is potentially applicable to many genetic diseases in that (i) the transfected cell line can be extensively characterized prior to implantation, (ii) several anatomical sites are suitable for implantation, and (iii) regulated expression of the gene of therapeutic interest can be obtained.

BECAUSE IT IS KNOWN THAT AT LEAST some genes of therapeutic value can function normally in heterologous cells, there has been a major effort to develop a gene delivery system during the past few years (*1*). The model system widely used at present is retroviral-mediated genetic transformation of mouse bone marrow cells and subsequent transplantation of the marrow into lethally irradiated mice (*2–6*). The advantages of this approach are that the marrow cells are readily accessible, can be cultured outside the animal, and can be efficiently infected with recombinant retroviruses. However, this approach is limited in that retroviral vectors might activate endogenous retroviral genomes in the target cells by recombining with them (*4*); the batch-infected marrow cells are subject to a large number of random retroviral integration events, some of which might be harmful to the recipient of those cells; expression of the gene inserted into the retroviral vector has proved difficult, particularly in human bone marrow cells (*7*); and the marrow is probably not an acceptable site for production of many proteins of clinical relevance. Although some of these limitations may be eliminated in the future [see (*6*), for example], there are cogent reasons to search for alternative models for gene therapy.

An alternative approach to gene therapy would be to remove and transfect cells from a desired anatomical source in the affected individual, isolate a clone of transfected cells, test the clonal population for proper regulation of the transfected gene and for the absence of deleterious integration events, and finally, reintroduce the cells into the individual in a physiologically suitable location. As a first step toward evaluating this approach, we have introduced a human growth hormone (hGH) fusion gene into cultured mouse Ltk^- (thymidine kinase–deficient) fibroblasts and have subsequently implanted these cells into various locations in mice. We report here that the function of such implanted cells depends on the location and size of the implant, and on the histocompatibility of the donor cells with their

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