SINGLE CALCIUM-DEPENDENT POTASSIUM CHANNELS IN CLONAL ANTERIOR PITUITARY CELLS

BRENDAN S. WONG AND HAROLD LECAR
Laboratory of Biophysics, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205

MICHAEL ADLER
Laboratory of Preclinical Studies, National Institute on Alcohol Abuse and Alcoholism, Rockville, Maryland 20852.

ABSTRACT Single Ca^{2+}-dependent K^+-channel currents were recorded in intact and excised inside-out membrane patches of the anterior pituitary clone AtT-20/D16-16. The frequency of channel openings and lifetimes depends both on membrane potential and on the Ca^{2+} concentrations at the inner membrane surface. The curve of the open-state probability of the channel as a function of membrane potential appears to translate along the voltage axis with changes in internal Ca^{2+} concentration. For Ca^{2+} concentrations between 10^{-7} and 10^{-6} M, the shift is consistent with the hypothesis that three Ca^{2+} ions are required to open a Ca^{2+}-dependent K^+ channel. Single channel conductances are estimated to be 124 pS in patches with normal external K^+ (5.4 mM) and 208 pS in excised patches with symmetrical K^+ (145 mM) across the membrane. Tetraethylammonium (20 mM) added to the cytoplasmic surface reversibly blocks the Ca^{2+}-dependent K^+ channel.

Cultured AtT-20/D16-16 (D16) cells, derived from an anterior pituitary tumor (Yasumura, 1968), synthesize, store, and secrete the corticotropin/endorphin family of peptides (Eipper and Mains, 1980). These cells were recently shown to be electrically excitable (Adler et al., 1980, 1981). For membrane potentials positive to −55 mV, D16 cells generate spontaneous bursts of action potentials, each having one or more Na^+ spikes, followed by a slow Ca^{2+} spike and a prominent Ca^{2+}-dependent afterhyperpolarization (AHP). The AHP reverses near the expected K^+ equilibrium potential, and persists in Cl^--free solution, suggesting that it is carried by K^+ ions (Adler et al., in preparation). The Ca^{2+}-mediated increase in K^+ permeability implies the presence of a Ca^{2+}-dependent K^+ conductance [$g_{K(Ca)}$] in D16 cells that appears to play a key role in the production of slow oscillatory activity (Adler et al., in preparation).

Meech (1972, 1974) postulated the existence of the $g_{K(Ca)}$ based on findings that elevations in internal Ca^{2+} lead to a transient hyperpolarization. Direct evidence for such Ca^{2+}-dependent K^+ channels has very recently been obtained in *Helix* neurons (Lux et al., 1981), chromaffin cells (Marty, 1981), rat myotubes (Pallotta et al., 1981), and sympathetic ganglia (Adams et al., 1981) by use of the patch-clamp technique. Ca^{2+}-dependent K^+ channels isolated from transverse tubule membranes of rabbit skeletal muscle have also been successfully reconstituted in planar lipid bilayers (Latorre et al., 1982).

In the present study, we have applied the patch-clamp technique to probe the Ca^{2+}-dependent K^+ channel in D16 cells. The results indicate that, under physiological conditions, the channel has a very large unit conductance (124 pS) and is gated by both internal Ca^{2+} and membrane potential.

D16 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1 mM dibutyryl cyclic-AMP. The cells were maintained at 37°C in a humidified atmosphere of 10% CO_2 in air for up to 3 wk before use. The patch-clamp circuitry was similar to that described by Neher et al. (1978) and incorporated improvements suggested by Sigworth and Neher (1980) and Hamill et al. (1981) for obtaining seal resistances of >10 GΩ. Excised inside-out membrane patches were formed by briefly raising and lowering the patch electrode through the air-solution interface (Hamill and Sakmann, 1981). The signals were low-pass filtered at 5 kHz (Khron-Hite Inc., Cambridge, MA, model 3202) and sampled at 100 μs/point. A digital oscilloscope (Nicolet Instrument Corp., Madison, WI) was used to record segments of 4096 points which were then stored on magnetic tape for analysis of channel-open-time distributions and current-jump amplitudes by a microcomputer (Plessey Peripheral Sys-

TABLE I
COMPOSITION OF SOLUTIONS*

Solution	NaCl	KCl	$CaCl_2$	EGTA	$MgCl_2$	$CoCl_2$
External						
(*a*) Control	140	5.4	1.8	0	2	0
(*b*) High Na^+	140	5.4	0	0	2	1
(*c*) High K^+	0	140	0	0	2	1
Internal						
(*d*) 10^{-8} M Ca	0	140	0.1	1.1	2	0
(*e*) 10^{-7} M Ca	0	140	0.55	1.1	2	0
(*f*) 5×10^{-7} MCa	0	140	0.92	1.1	2	0
(*g*) 10^{-6} M Ca	0	140	1	1.1	2	0

All solutions were buffered to pH 7.3–7.4 by 10 mM HEPES and either 5 mM NaOH (*a*, *b*) or 5 mM KOH (*c–g*). The patch electrode was filled with one of the three external solutions as appropriate.
*Concentrations are millimolar.

tems, Irvine, CA, Micro II). Experiments were performed at room temperature (23°–25°C) to improve the time resolution of fast channel events.

The patch electrode and bath initially contained one of the external solutions listed in Table I. After the formation of "gigaseals" on intact cells, the bath was perfused with one of the internal solutions containing the desired Ca^{2+} concentration and inside-out membrane patches were formed as described above. The channel properties were not appreciably affected by excision, in agreement with the results of previous investigators (Horn and Patlak, 1980; Hamill and Sakmann, 1981). For seal resistances >30 GΩ, excised membrane patches remained functional for as long as 4 h. Ionized Ca^{2+} concentrations between 10^{-8} and 10^{-6} M were obtained by use of the appropriate Ca-EGTA buffers taking 10^{-7} M as the apparent dissociation constant of the complex (Marty, 1981). The bath volume was maintained at 1.5 ml and the solutions were perfused continuously at 1–3 ml/min. Equilibration of the bath was complete in 3–5 min as revealed by phenol red dilutions.

Fig. 1 shows single Ca^{2+}-dependent K^+-channel fluctuations from an excised inside-out membrane patch recorded at a membrane potential depolarized 55 mV from rest. The resting potential was estimated to be −50 mV from intracellular recordings, making the effective membrane potential 5 mV. The patch electrode was filled with a physiological saline in which Ca^{2+} was replaced by Co^{2+} (solution *b*) and the bath was superfused with internal solution *f* containing 5×10^{-7} M free Ca^{2+}. The trace in Fig. 1 *A* is displayed on a slow time base and shows that the channel fluctuations undergo periods of high activity alternating with periods of quiescence. The quiescent periods usually lasted for several seconds and persisted even at higher Ca^{2+} concentrations and more positive membrane potentials. Similar intermittent channel events were observed by Latorre et al. (1982) on Ca^{2+}-dependent K^+ channels reconstituted from skeletal muscle. The initial burst segment in Fig. 1 *A* is shown on an expanded time base in Fig. 1 *B*. In this record, individual channel openings and closings can be resolved. Multiple fast flickers can be seen, especially during long openings. Channel fluctuations similar to these were observed in approximately one-third of the patches examined. The properties of these large unitary outward currents resemble closely those reported for the Ca^{2+}-dependent K^+ channels of chromaffin cells (Marty, 1981) and myotubes (Pallotta et al., 1981), having similar amplitudes, open times, and flickering characteristics.

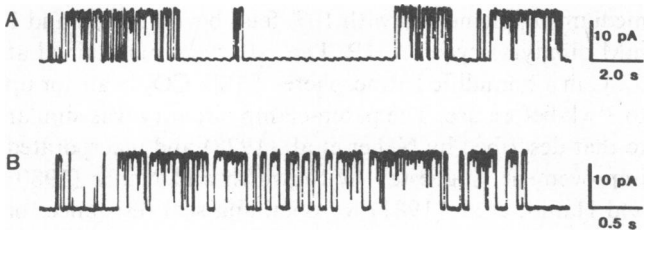

FIGURE 1 Single Ca^{2+}-dependent K^+ channel currents recorded at 5 mV in an excised inside-out patch with a physiological K^+ gradient across the membrane; the patch electrode was filled with solution *b* and the internal perfusate contained 5×10^{-7} M ionized Ca^{2+} (solution *f*). (*A*) Single channel current fluctuations displayed on a slow time scale showing alternating periods of activity and quiescence. (*B*) An expanded record of the first active region in *A* illustrating discrete channel openings and closings. Signals were recorded unfiltered on an FM tape recorder (frequency response 0–4.5 kHz) and displayed on a chart recorder. All unitary events are upward deflections and represent outward current through the channels.

In some intact and excised patches, smaller K^+ channels with approximately one-fourth of the current amplitude of Ca^{2+}-dependent K^+ channels were also observed. The frequency of openings and lifetimes of these channels were found to be essentially similar in the presence of 10^{-6} M

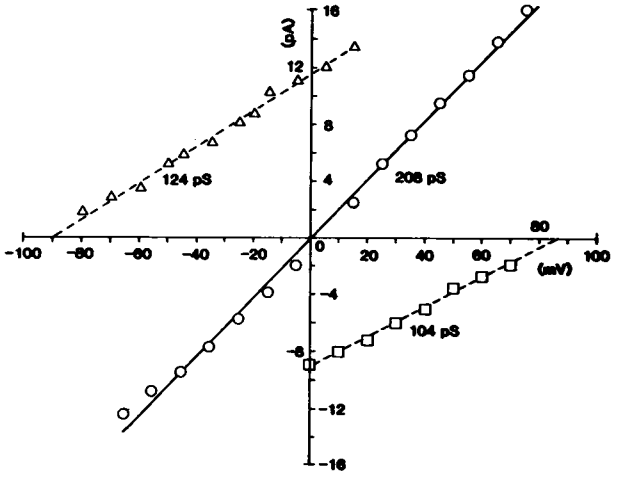

FIGURE 2 Single channel current-voltage curves from excised inside-out membrane patches. The triangles are from a patch with 5.4 mM K^+ (solution *b*) in the patch electrode and 145 mM internal K^+ (solution *e*). The circles are from another inside-out membrane patch bathed in symmetrical solutions containing 145 mM K^+ at the outer (solution *c*) and inner (solution *e*) surfaces and 10^{-7} M internal ionized Ca^{2+}. The squares represent data from the same excised patch but with 5.4 mM internal K^+ (solution *a*).

Ca^{2+} or Ca-free internal solutions. These K^+ channels appear to be activated by voltage alone and their properties will be reported in a future publication.

Current-voltage relationships for single Ca^{2+}-dependent K^+ channels are plotted in Fig. 2. The data were obtained from excised inside-out patches under conditions of normal and reversed physiological K^+ gradients, and symmetrical 145 mM K^+ across the patch. The current-voltage relationships are linear over the range of voltages measured, and yielded slope conductances of 124, 104, and 208 pS, respectively. The corresponding reversal potentials are −85, 89, and 0 mV, respectively. The magnitude of these shifts with different external and internal K^+ is consistent with the Nernst relationship for a K^+ selective channel. Further evidence that the channel is K^+-selective comes from studies with excised patches which show that the reversal potential remains essentially unchanged when all the Cl^- in the internal solution is replaced with isethionate (not shown).

The Ca^{2+}- and voltage-dependences of current-jump lifetimes are illustrated by representative records in Fig. 3. All traces are from the same excised patch with solution *b* in the patch electrode. The top, center, and bottom traces in each panel were obtained in 10^{-8} (solution *d*), 10^{-7} (solution *e*), and 10^{-6} M (solution *g*) internal Ca^{2+}, respectively (see Table I). Multiple channel openings were not observed (even in 10^{-6} M Ca^{2+}) when opening probabilities were very high, suggesting that only one Ca^{2+}-dependent K^+ channel was present in this patch. In the presence of low internal Ca^{2+} concentrations ($\leq 10^{-8}$ M), Ca^{2+}-dependent K^+ channels were rarely observed. Raising the internal Ca^{2+} concentration from 10^{-8} to 10^{-7} M produced only small increases in the frequency of channel openings and channel lifetimes (Fig. 3). However, an additional 10-fold increase in internal Ca^{2+} from 10^{-7} to 10^{-6} M resulted in a dramatic enhancement in the opening probabilities as well as a marked prolongation of the channel open times.

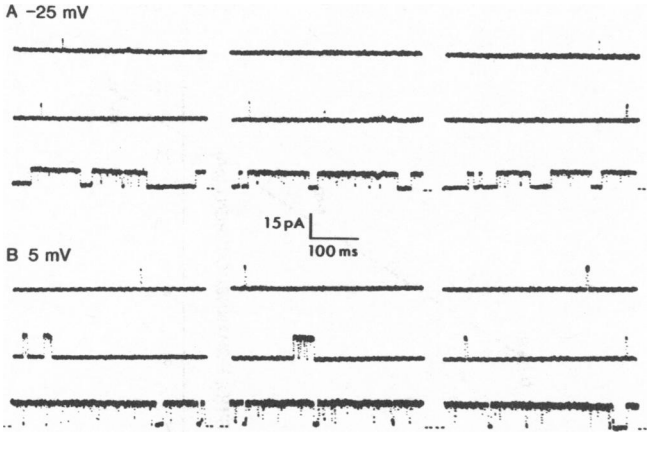

FIGURE 3 Effects of internal Ca^{2+} concentration and voltage on the frequency of channel openings and lifetimes of an excised inside-out membrane patch with 5.4 mM K^+ (solution *b*) in the patch electrode at two different membrane potentials, (*A*) −25 mV and (*B*) 5 mV. The top, center, and bottom traces of each panel are representative records taken in the presence of 10^{-8} M (solution *d*), 10^{-7} M (solution *e*), and 10^{-6} M (solution *g*) Ca^{2+}, respectively. The top middle trace of *A* has no channel opening. The dotted line at the beginning and end of some traces represents the reference base line.

The channel kinetics also exhibited marked voltage dependence. For a fixed Ca^{2+} concentration, channels opened more frequently and remained open for longer periods with increases in the depolarizing membrane electric field (Fig. 3). For an excised membrane patch with 10^{-7} M internal Ca^{2+}, the increase in channel open times at positive membrane potentials had a voltage dependence of 28 mV per *e*-fold change. In some excised patches bathed in symmetrical K^+, increases in activation frequency and channel duration were observed with potentials both positive and negative to the reversal potential.

Fig. 4 shows the probability of a channel being in the open state as a function of membrane potential, $P_o(V)$, for three different internal Ca^{2+} concentrations from the same patch as shown in Fig. 3. If channels act independently, an open-state probability plot, such as that of Fig. 4, corresponds to the macroscopic steady-state voltage-dependent conductance (Ehrenstein et al., 1970; Lecar et al., 1975). For a given Ca^{2+} concentration, $P_o(V)$ was fit to the relation

$$P_o(V) = \{1 + \exp[-A(V - V_o)]\}^{-1}, \quad (1)$$

where the parameter A is 0.11 mV^{-1} and the voltage at which $P_o(V) = 0.5$, (V_o), is a function of Ca^{2+} concentration. The most striking feature of the curves in Fig. 4 is that $P_o(V)$ appears to translate along the voltage axis without change in shape as internal Ca^{2+} concentration is varied. In the range 10^{-7} to 10^{-6} M, this shift is ~65 mV per 10-fold

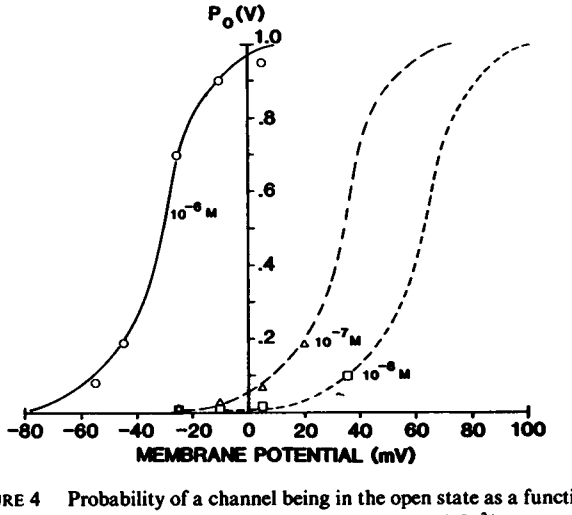

FIGURE 4 Probability of a channel being in the open state as a function of membrane potential, $P_o(V)$, at three different internal Ca^{2+} concentrations. $P_o(V)$ was determined as the sum of open—state dwell times divided by the total time of the single channel records. The solid and dashed lines are best-fit curves to the data points using Eq. 1 as described in the text.

increase in Ca^{2+} concentration. A similar shift in a higher Ca^{2+} concentration range has been found for Ca^{2+}-dependent K^+ channels extracted from skeletal muscle (Latorre et al., 1982).

The shift can most readily be explained by attributing the major part of the voltage dependence to the Ca^{2+} binding step in channel activation. Thus, if we assume a highly cooperative scheme for which N Ca^{2+} ions must bind simultaneously to sites on a receptor in order to open a channel, we can examine a simplified two-state scheme,

$$\text{Closed} \underset{k_-(V)}{\overset{N\,Ca^{2+}\ k_+([Ca^{2+}],V)}{\rightleftharpoons}} \text{Open},$$

where the rates k_+ and k_- can be given explicit concentration and voltage dependence. The steady-state probability of being open can be described in terms of a binding constant, $K = k_+/k_-$. For the binding of a charged particle to a site in the membrane, we expect K to show concentration and voltage dependence,

$$K = K'[Ca^{2+}]^N \exp(AV). \tag{2}$$

This assumption would immediately give Eq. 1, but with the explicit relation,

$$V_0 = -(N/A)\ln(K'[Ca^{2+}]). \tag{3}$$

From Eqs. 1 and 3, we can independently fit N and A; Eq. 3 implies that the voltage-shift per 10-fold Ca^{2+} concentration change is $-2.3(N/A)$, and the parameter A can be obtained independently from Eq. 1 as $A = 4\,(dP_0/dV)_{V=V_0}$. For the data of Fig. 4, the best-fit value of N is 3, which is consistent with both the data of Latorre et al. (1982) and with the macroscopic dose-response data on invertebrate neurons requiring three Ca^{2+} ions to open a Ca^{2+}-dependent K^+ channel (Meech and Thomas, 1980).

The shift of V_0 for a 10-fold Ca^{2+} concentration change between 10^{-8} and 10^{-7} M appears smaller than that between 10^{-7} and 10^{-6} M, suggesting a fit to N of 1 or 2 at the lower Ca^{2+} concentrations. Also, Fig. 3 shows an increase in burst length as a function of increased Ca^{2+} concentration. By invoking sequential binding steps, the kinetic scheme can be generalized to predict both the number of bound ions required for opening and the concentration dependence of burst lengths.

More generally, we expect the N ions to bind sequentially, with the last step in the sequence being the open state. This scheme can be represented as

$$C_0 \underset{k_{-1}}{\overset{Ca^{2+}\ k_{+1}}{\rightleftharpoons}} C_1 \underset{k_{-2}}{\overset{Ca^{2+}\ k_{+2}}{\rightleftharpoons}} C_2 \underset{k_{-3}}{\overset{Ca^{2+}\ k_{+3}}{\rightleftharpoons}} \cdots C_{N-1} \underset{k_{-N}}{\overset{Ca^{2+}\ k_{+N}}{\rightleftharpoons}} O_N.$$

If we let the jth binding constant be $K_j = k_{+j}/k_{-j}$, we can obtain the steady-state relation

$$P_o = \left(1 + \sum_{m=1}^{N} \prod_{j=m}^{N} K_j^{-1}\right)^{-1} \tag{4}$$

For the highly cooperative case in which all the intermediate states of binding are so short-lived that the virtually simultaneous binding of N ions is needed (i.e., $K_N >> K_j$ $[j \neq N]$), Eq. 4 reduces to Eq. 1.

However, for the opposite case, in which the N sites are independent, we can show that the same conclusions about the concentration dependence of the shift still follow. In that case, the rates for each sequential step are weighted by the number of independent sites available, so that the products in Eq. 4 can be rewritten in terms of one basic binding constant, K,

$$\prod_{j=m}^{N} K_j^{-1} = \binom{N}{m} K^{-m},$$

and Eq. 4 becomes

$$P_0\,(\text{independent sites}) = \left[1 + \sum_{m=1}^{N} \prod_{j=m}^{N} \binom{N}{m} K^{-m}\right]^{-1} = (1 + K^{-1})^{-N}. \tag{5}$$

It then follows, when Eq. 2 is used, that

$$P_o(\text{independent sites}) = \{1 + \exp[(-A/N)(V - V^*)]\}^{-N}, \tag{6}$$

where $V^* = -(N/A)\ln(K[Ca^{2+}])$, and the half-maximum voltage V_0 becomes

$$V_0 = -(N/A)\ln\{(2^{1/N} - 1)K[Ca^{2+}]\}, \tag{7}$$

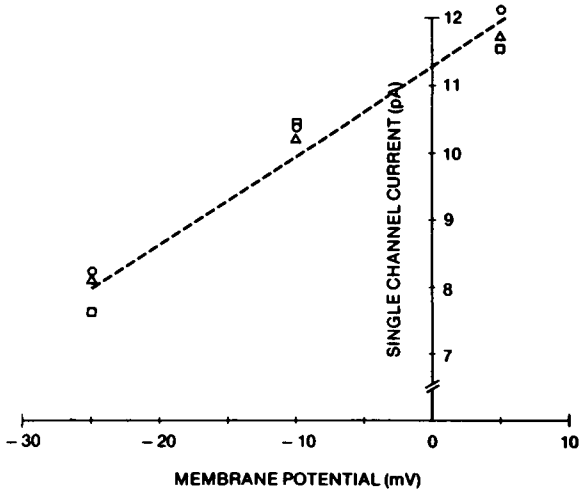

FIGURE 5 Single channel current amplitudes in the presence of 145 mM internal K^+ and 10^{-8} M (□), 10^{-7} M (△), and 10^{-6} M (○) internal Ca^{2+} at three different membrane potentials. The external solution contained 5.4 mM K^+ (solution *b*). The dashed line is the regression line with an ohmic conductance of 131 pS.

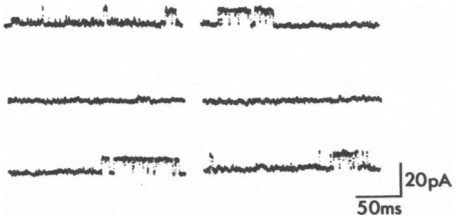

FIGURE 6 Effect of 20 mM internal tetraethylammonium chloride on single Ca^{2+}-dependent K^+ channels of an excised inside-out membrane patch with symmetrical 145 mM K^+ external solution *c* and internal solution *e* at a membrane potential of 35 mV. The top, center, and bottom trace of each panel corresponds to before, during, and after the addition of tetraethylammonium, respectively. Note the presence of small voltage-dependent K^+ channels, which persist in the presence of TEA.

giving the same prediction for the shift as the highly cooperative case. For the independent sites case the shape of the curve given by Eq. 6 is somewhat different than that predicted by Eq. 1, and the slope at half-maximum would now be $(dP_o/dV)_{V=V_o} = A/(2^{N+1})$.

A higher Ca^{2+} concentration than that tested in this study (10^{-3} M) has been reported to abolish the voltage-sensitivity of Ca^{2+}-dependent K^+ channels in chromaffin cells (Marty, 1981). Marty also found a decrease in the unitary current amplitude with increasing Ca^{2+} concentrations, especially at large positive membrane potentials. In these pituitary cells, Ca^{2+} does not seem to significantly affect the single channel current amplitude in the potential range studied (Fig. 5).

Tetraethylammonium (TEA), the well-known blocker of K^+ channels (Armstrong and Binstock, 1965), has also been reported to block the outward current resulting from Ca^{2+} injection in a molluscan neuron (Hermann and Gorman, 1981). It was therefore of interest to examine the effect of TEA on single Ca^{2+}-dependent K^+ channels. As shown in Fig. 6, when 20 mM TEA was added to the internal side of an excised inside-out membrane patch, the Ca^{2+}-dependent K^+ currents were abolished. The inhibition was reversible and independent of membrane potential or direction of current flow. Interestingly, the smaller voltage-dependent K^+ channels could still be detected in the presence of 20 mM TEA.

This study shows that Ca^{2+}-dependent K^+ channels in D16 pituitary cells are activated by the appearance of free Ca^{2+} at the inner membrane surface. The variation of jump frequency and lifetime with membrane potential for fixed Ca^{2+} concentrations demonstrate the dual ability of Ca^{2+} and voltage to control the channel as had been shown by Gorman and Thomas (1980) in molluscan neurons. The relatively large value of unit conductance is consistent with single-channel measurements of Ca^{2+}-dependent K^+ channels in other vertebrate preparations.

Received for publication 6 November 1981 and in revised form 24 March 1982.

REFERENCES

Adams, P. R., A. Constanti, R. B. Clark, C. E. Y. Adams, and D. A. Brown. 1981. Single channel and noise analysis of outward currents in bullfrog ganglion cells. *Soc. Neurosci. (Abstr.).* 7:15.

Adler, M., N. Busis, H. Higashida, S. Sabol, A. Rotter, and M. Nirenberg. 1980. Modulation of rhythmic electrical activity in an anterior pituitary cell line. *Soc. Neurosci. (Abstr.).* 6:300.

Adler, M., S. Sabol, M. Jackson, N. Busis, and M. Nirenberg. 1981. Effect of dibutyryl cyclic-AMP on electrical excitability, $^{45}Ca^{2+}$-uptake and β-endorphin secretion from AtT-20/D16-16 pituitary cells. *Soc. Neurosci. (Abstr.).* 7:515.

Armstrong, C. M., and L. Binstock. 1965. Anomalous rectification in the squid giant axon injected with tetraethylammonium chloride. *J. Gen. Physiol.* 48:859–872.

Ehrenstein, G. M., H. Lecar, and R. Nossal. 1970. The nature of the negative resistance in bimolecular lipid membranes containing excitability-inducing material. *J. Gen. Physiol.* 55:119–133.

Eipper, B. A., and R. E. Mains. 1980. Structure and biosynthesis of proadrenocorticotropin/endorphin related peptides. *Endocr. Rev.* 1:1–27.

Gorman, A. L. F., and M. V. Thomas. 1980. Potassium conductance and internal calcium accumulation in a molluscan neurone. *J. Physiol. (Lond.).* 308:287–313.

Hamill, O. P., and B. Sakmann. 1981. A cell-free method for recording single-channel currents from biological membranes. *J. Physiol. (Lond.).* 312:41–42P.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch. Eur. J. Physiol.* 391:85–100.

Hermann, A., and A. L. F. Gorman. 1981. Effects of tetraethylammonium on potassium currents in a molluscan neuron. *J. Gen. Physiol.* 78:87–110.

Horn, R., and J. B. Patlak. 1980. Single channel currents from excised patches of muscle membrane. *Proc. Natl. Acad. Sci. U. S. A.* 77:6930–6934.

Latorre, R., C. Vergara, and C. Hidalgo. 1982. Reconstitution in planar lipid bilayers of a Ca^{2+}-dependent K^+ channel from transverse tubule membranes isolated from rabbit skeletal muscle. *Proc. Natl. Acad. Sci. U. S. A.* 79:805–809.

Lecar, H., G. Ehrenstein, and R. Latorre. 1975. Mechanism for channel gating in excitable bilayers. *Ann. N. Y. Acad. Sci.* 264:304–317.

Lux, H. D., E. Neher, and A. Marty. 1981. Single channel activity associated with the calcium dependent outward current in *Helix pomatia*. *Pfluegers Arch. Eur. J. Physiol.* 389:293–295.

Marty, A. 1981. Ca-dependent K channels with large unitary conductance in chromaffin cell membranes. *Nature (Lond.).* 291:497–500.

Meech, R. W. 1972. Intracellular calcium injection causes increased potassium conductance in *Aplysia* nerve cells. *Comp. Biochem. Physiol.* 42A:493–499.

Meech, R. W. 1974. The sensitivity of *Helix aspersa* neurones to injected calcium ions. *J. Physiol. (Lond.).* 237:259–277.

Meech, R. W., and R. C. Thomas. 1980. Effect of measured calcium chloride injections on the membrane potential and internal pH of snail neurones. *J. Physiol. (Lond.).* 298:111–129.

Neher, E., B. Sakmann, and J. H. Steinbach. 1978. The extracellular patch clamp: a method for resolving currents through individual open channels in biological membranes. *Pfluegers Arch. Eur. J. Physiol.* 375:219–228.

Pallotta, B. S., K. L. Magleby, and J. N. Barrett. 1981. Single channel recordings of Ca^{2+}-activated K^+ currents in rat muscle cell culture. *Nature (Lond.).* 293:471–474.

Sigworth, F. J., and E. Neher. 1980. Single Na^+ channel currents observed in cultured rat muscle cells. *Nature (Lond.).* 287:447–449.

Yasumura, Y. 1968. Retention of differentiated function in clonal animal cell lines, particularly hormone-secreting cultures. *Am. Zool.* 8:285–305.