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Ba^{2+}-Induced Action Potentials in Osteoblastic Cells

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Summary. Trains of long-duration "action potentials" were induced by Ba^{2+} in osteoblast-like rat osteosarcoma cells (ROS 17/2.8), under current clamp and voltage clamp. Large depolarizing pulses were seen in microelectrode measurements at 37°C following the addition of 10 or 20 mM Ba^{2+} to physiological bathing medium. Application of BAY K 8644 resulted in the onset of the pulses at earlier times and at more negative potentials. The pulses were blocked by nifedipine and Cd^{2+}, but not by Ni^{2+}. Large inward current pulses were seen in whole-cell patch technique voltage-clamp measurements at 37°C in the presence of from 10 to 110 mM Ba^{2+} in the bathing medium. The current pulses were not seen at 22°C in the presence of 110 mM Ba^{2+}, but could be induced by BAY K 8644. These pulses were not blocked by TTX, but were blocked by nifedipine, Cd^{2+}, Zn^{2-}, Co^{2+}, and by an increase in bathing $[Ca^{2-}]$. The shape and frequency of the current pulses were the same as for voltage pulses under current clamp.

A model that can explain these observations involves opening of L-type Ca^{2+} channels in a voltage-independent manner by cytosolic Ba^{2+} via a screening of Ca^{2-} from sites that produce either inactivation or a lower probability of opening in the activated state. There would be a closing of these channels at higher $[Ba^{2+}]$ as Ba^{2+} is forced onto these sites. A refractory period is also required to give repeated pulses of openings.

Key Words electrophysiology · action potentials · calcium channels · barium

Introduction

There are ion channels in the cell membrane of osteoblastic cells that, in response to hormones, have an increased probability of being open (Edelman, Fritsch & Balsan, 1986; Ferrier & Ward, 1986; Ferrier et al., 1988; Fritsch, Edelman & Balsan, 1988). However, unravelling the physiological role of ion transport in osteoblasts requires more understanding of the properties of the various channels. For example, it has recently been shown that there are L-type calcium channels in osteoblastic cells, but the physiological processes that could lead to these channels producing a Ca^{2+} influx remain obscure (Grygorczyk, Grygorczyk & Ferrier, 1989).

The present paper is based on a study of a recently discovered aspect of ion transport in an osteoblastic cell line—the occurrence of very large depolarizing "action potentials" that are induced by adding Ba^{2+} to the bathing medium. We investigated this phenomenon with the aim of further characterizing the channel properties.

Materials and Methods

Rat osteosarcoma ROS 17/2.8 cells were used, which have a number of osteoblast-like characteristics (Majeska & Rodan, 1982). They were cultured in α-minimum essential medium (α-MEM) with 15% fetal bovine serum (FBS) and antibiotics (Ferrier et al., 1987).

Experiments were carried out on cells that were well attached to the bottom of tissue culture dishes, in α-MEM with 15% FBS (microelectrode measurements and some of the whole-cell patch-clamp measurements), or in media with either (in mM) 140 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 13 TEA, to which $BaCl_2$ was added during the measurement, or 110 $BaCl_2$ and 10 glucose (whole-cell patch clamp). Temperature was 37 ± 1 or 22 ± 2°C, and pH was held at 7.3 ± 0.1 with HEPES buffer (10 mM in the patch-clamp measurements and 25 mM in the microelectrode ones).

Microelectrodes were pulled out to a tip diameter of about 0.1 μm and filled with 150 mM KCl, with a resistance of 100 to 200 MΩ. Data were recorded on a Gould 110 strip chart recorder at a chart speed of 0.21 mm/sec, and simultaneously on a Gould 2200 high speed strip chart recorder at a chart speed of 2 mm/sec. All other details of the microelectrode measurements were as previously described (Ferrier et al., 1987, 1988).

Pipettes for whole-cell patch-clamp measurements were prepared from thin-walled borosilicate glass tubes (Rochester Scientific) with an outer diameter of 1.7 mm, inside diameter of 1.3 mm. The pulled pipettes had an outside diameter at the tip of 1.0 μm and an inside diameter of 0.6 to 0.7 μm. The pipette resistance was 2.5 to 2.9 MΩ. Various pipette filling solutions were used. Most measurements were carried out with (in mM):

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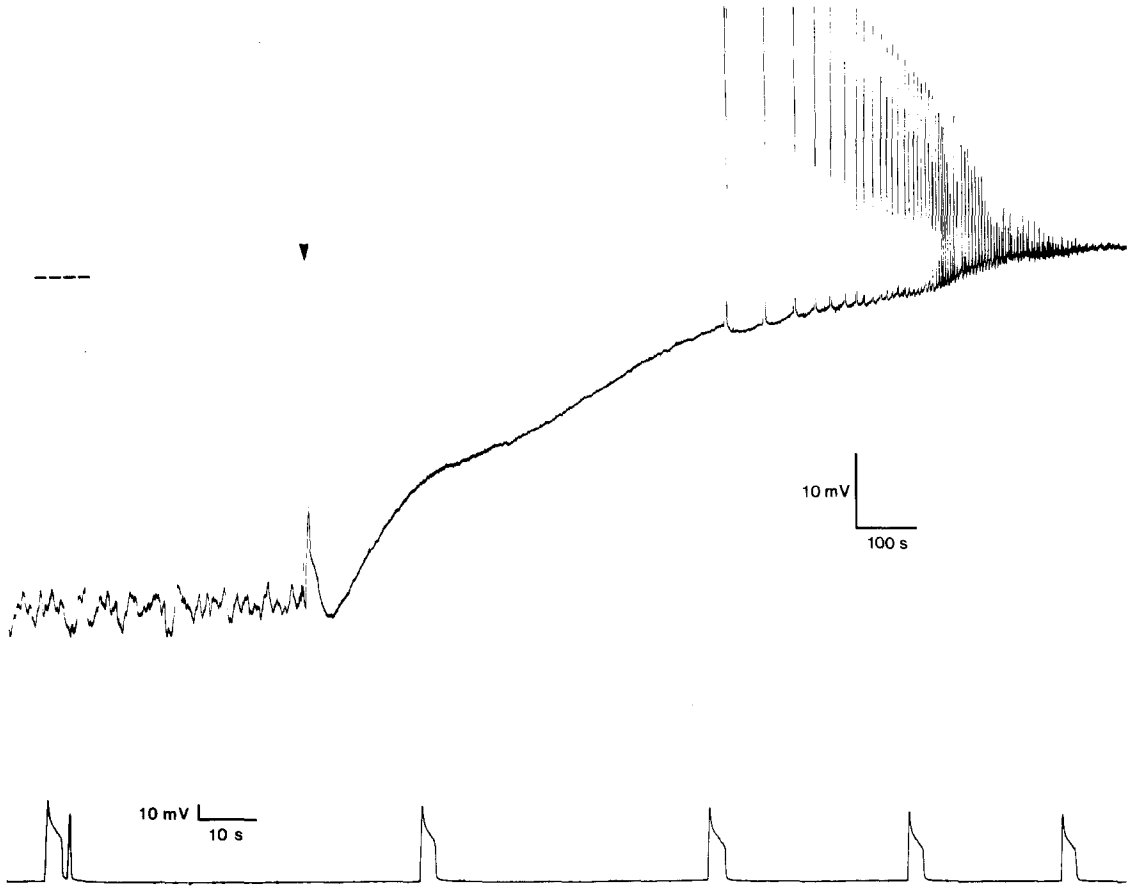

Fig. 1. A microelectrode current clamp recording showing depolarizing pulses following application of 20 mM Ba^{2+} (applied at the arrowhead). The dashed line shows measured potential before microelectrode insertion. Some initial depolarizing pulses in the upper record have gone off scale. The lower record shows the first few pulses on an expanded time scale and a compressed voltage scale

130 CsCl, 1 MgCl, 1 ATP, 10 HEPES, 13 TEA, and 10 to 40 EGTA. Some measurements were with 140 KCl, 20 NaCl, 2 $MgCl_2$, 10 HEPES, 1 $CaCl_2$, and 11 EGTA. Other details are as previously reported (Ferrier et al., 1987; Grygorczyk et al., 1989).

BAY K 8644 was a gift from Miles Pharmaceutical. Nifedipine was obtained from Sigma. $BaCl_2$ was obtained from Baker Chemical.

Results

In the microelectrode measurements, application of $BaCl_2$ to the bathing medium (final $[Ba^{2+}] = 10$ or 20 mM) resulted in a slow depolarization in most cells and a rapid depolarization in others. In every case (28 cells with 15% FBS, and five cells without FBS), slowly depolarizing cells showed a train of depolarizing spikes, each spike of 0.5 to 3.5 sec duration, with some greater than 60 mV in amplitude (Fig. 1). The interval between spikes was from 1 to 200 sec. These cells normally exhibit low frequency membrane potential fluctuations under the conditions obtaining before the addition of Ba^{2+}, in medium with FBS, as seen in Fig. 1.

With 20 mM Ba^{2+} added to cells in medium with 15% FBS, the time from application of Ba^{2+} to onset of the action potentials was 10.4 ± 0.5 min (mean ± SEM, 10 cells), and the threshold for onset was −8.0 ± 0.8 mV. Mean membrane potential before Ba^{2+} application was −37 ± 3 mV. When BAY K 8644 (2 μM) was applied to slowly depolarizing cells following 20 mM Ba^{2+} application in medium with 15% FBS, the time from application of Ba^{2+} to onset of action potentials was 7.7 ± 0.5 min (five cells), and threshold for onset was −16.7 ± 0.9 mV. Mean membrane potential before Ba^{2+} application in these cells was also −37 ± 3 mV.

Application of nifedipine (5 μM) or Cd^{2+} (50 μM) prior to application of Ba^{2+} completely eliminated depolarizing spikes in slowly depolarizing cells (four and five cells, respectively). Ni^{2+} (100 μM) applied before the Ba^{2+} had no effect on the action potentials in slowly depolarizing cells (four cells).

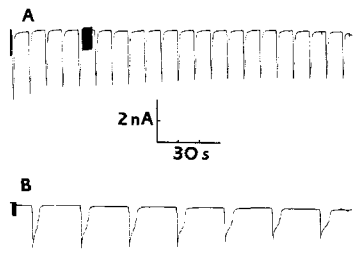

Fig. 2. Whole-cell patch technique voltage-clamp recordings of inward current pulses, in the presence of 110 mM Ba^{2+} in the bathing medium, at 37°C with a holding potential of −20 mV. In the pipette is (*A*) 40 mM EGTA and (*B*) 20 mM EGTA

In the whole-cell patch technique measurements, inward current pulses were seen under voltage clamp (Fig. 2). These pulses were observed as soon as the patch was broken in the presence of 110 mM Ba^{2+} in the bathing medium, at 37°C (38 cells). The holding potentials during patch breaking ranged from −50 to +50 mV (−50 mV: 1 cell; −30: 12; −20: 6; 0: 18; +50: 1). The magnitude of the pulses could be as high as 5 nA, but was as low as 0.4 nA in a number of cells. The duration of each pulse was in the range of 1 to 60 sec, with the time between pulses in the range of 1 to 200 sec. The shape of these inward current pulses was the same as the depolarizing membrane potential pulses seen in the microelectrode measurements: a fast rise, then a slow decline, followed by a rapid drop to baseline. The voltage clamp was also switched to current clamp, giving depolarizing pulses similar in shape to the current pulses and to the voltage pulses in the microelectrode measurements. These inward current pulses could also be induced by adding 10 mM Ba^{2+} to cells in Ba^{2+} free medium (four cells). The current pulses were completely blocked by Cd^{2+} (10 μM, four cells) and Zn^{2+} (20 μM, two cells).

Experiments were carried out in which the holding potential was varied between −120 and +140 mV, in steps of 10 mV, with 30 to 100 sec of recording at each value (six cells). Holding potential affected only the amplitude of the spikes, but had no effect on the spike duration or frequency. In a typical run, the amplitude of the pulses varied approximately linearly from 5 nA at −120 mV to 0.2 nA at +140 mV.

The amplitude, time width and repeat frequency of the inward pulses were affected by the EGTA concentration in the pipette, at 37°C with 110 mM Ba^{2+} in the bathing medium (Fig. 2). With 20 mM EGTA in the pipette, amplitude, time width and frequency were 2.7 ± 0.4 nA, 8.4 ± 1.4 sec and 0.07 ± 0.02 Hz (10 cells), while with 40 mM EGTA, they were 4.1 ± 0.5 nA, 3.9 ± 0.8 sec and 0.17 ± 0.03 Hz (13 cells).

An increase in bathing $[Ca^{2+}]$ by 1 or 2 mM, starting either from a nominal value of 0 (three cells) or from a value as high as 10 mM (two cells), could block the current pulses. This block could then be removed by application of 6 to 10 mM EGTA to the bathing medium.

At 22°C, the inward pulses were not observed in cells with 110 mM Ba^{2+} in the bathing medium. However, the pulses were induced by application of 2 μM BAY K 8644 (Fig. 3). The initial pulse was significantly longer in duration than subsequent pulses (35 ± 8 *vs.* 12 ± 3 sec, 11 cells). These inward current pulses could subsequently be blocked by nifedipine (50 to 70% block with 2 μM, three cells; complete block with 10 μM, two cells), or by Co^{2+} (50 to 100% block with 1 mM, two cells; complete block with 2 mM, three cells). The pulses were not affected by 3 μM tetrodotoxin (TTX; six cells).

The pipette conductances were from 350 to 400 nS. Baseline cell membrane conductances were 6 ± 2 nS with 10 mM EGTA in the pipette (10 cells), 21 ± 4 nS with 20 mM EGTA (12 cells), and 52 ± 6 nS with 40 mM EGTA (13 cells), as measured by 20 mV test pulses. During the Ba^{2+}-induced inward current pulses, the cell membrane conductance typically increased by from 10 to 50 nS, as measured by 20 mV test pulses.

Discussion

The measurements made under current clamp, using the microelectrode technique or the whole-cell patch technique, show repeated depolarizing pulses in the presence of Ba^{2+} in the bathing medium. Since these pulses are not seen in the absence of Ba^{2+}, it may be assumed that Ba^{2+} affects the gating of some ion channels in such a way as to cause these pulses. Based solely on their polarity, such pulses could represent a pulsed increase in Na^+ influx through Na^+-selective channels, or Ba^{2+} influx through Ca^{2+} channels. The effects of the dihydropyridines BAY K 8644 and nifedipine, which have been shown to be fairly specific for L-type Ca^{2+} channels (Fox, Nowycky & Tsien, 1987; Markwardt & Nilius, 1988), as well as those of the various cation blockers, provide evidence that the depolarizing pulses result from Ba^{2+} influx through L-type Ca^{2+} channels. The occurrence of these pulses in bathing medium containing only Ba^{2+} as the cation is further evidence of this. However, the current clamp data leave open the question of whether the gating of this effect is voltage controlled, or whether some other mechanism is at work.

The voltage-clamp results are clear evidence that the depolarizing pulses are not voltage con-

Fig. 3. A whole-cell patch technique voltage-clamp recording of inward current pulses induced at 22°C by the application of 2 μM BAY K 8644, with 110 mM Ba^{2+} in the bathing medium, at 0 mV

trolled. That is, in the whole-cell voltage-clamp measurements we see repeated inward current pulses with the same shape and frequency as the depolarizing pulses seen under current clamp. Although a perfect voltage clamp is impossible, in principle, using the whole cell configuration because of the relatively large currents involved, the magnitude of the current pulses and their similarity in time course to the depolarizing pulses indicate that they are not voltage controlled. That is, given our access conductance through the pipette, the voltage clamp will hold changes in membrane potential during the pulses to less than 3 mV per nA of pulse amplitude. In a previous series of measurements on ROS 17/2.8 cells using depolarizing test pulses, with the same bathing medium and pipette solutions used here, we showed that very little opening of L-type Ca^{2+} channels would be induced by such small changes in membrane potential (Grygorczyk et al., 1989). Moreover, the time required for channel inactivation following openings induced by depolarization at 37°C was considerably less than the time width of the pulses reported here.

The ratio of the pulse amplitude under voltage clamp to that under current clamp is close to the measured cell membrane conductance, as expected for a process that is not voltage gated. If there was a voltage gated step requiring a large depolarization, this ratio should be much lower. This is further supported by our measurements showing that the effect of varying holding potential from -120 to $+140$ mV is one of changing the current amplitude, reflecting the changing driving force on Ba^{2+}, but with no effect on pulse width or frequency. The effect of BAY K 8644 in inducing the current pulses at 22°C, and the blocking effect of nifedipine, Cd^{2+}, Zn^{2+}, and Co^{2+} on the current pulses, are further indications that L-type Ca^{2+} channels are involved. Thus, our results suggest that Ba^{2+} is activating L-type Ca^{2+} channels in a cyclical manner, without the involvement of a voltage-dependent step. However, we cannot rule out the possibility that a relatively small change in membrane potential could facilitate the Ba^{2+}-induced process, perhaps providing a synchronizing role for the membrane potential.

A number of studies have shown that Ca^{2+} channels are inactivated (or, alternatively, that the probability of opening in the activated state is decreased) by the binding of Ca^{2+} to a site near to but not immediately adjacent to the pore mouth, on the cytosolic side of the membrane (Belles et al., 1988; Plant, 1988; Gutnick et al., 1989; Yamamoto, Hu & Kao, 1989; Yue, Backx & Imredy, 1990). It is also shown in these studies that Ba^{2+} can greatly reduce this effect. This probably results from Ba^{2+} screening of Ca^{2+} from the "inactivation site." However, inactivation (or else a reduced probability of opening) also occurs with Ba^{2+}, only over a much longer time scale than with Ca^{2+} (Plant, 1988; Gutnick et al., 1989; Yamamoto et al., 1989; Grygorczyk et al., 1989), implying that a higher Ba^{2+} concentration will induce channel closing or inactivation. This may be because a sufficiently high $[Ba^{2+}]$ will provide a large enough chemical potential to force Ba^{2+} onto this site.

With this in mind, a model for Ba^{2+} control of repeated Ca^{2+} channel activation that explains the important features of our data can be developed. In this model we assume (i) a range of cytosolic $[Ba^{2+}]$ over which greatly increased opening of the channels occurs; (ii) a cytosolic $[Ba^{2+}]$ threshold that produces closing or inactivation of the channels; and (iii) a refractory period following Ba^{2+}-induced channel closing.

This model provides for an initial time period of high channel openings following Ba^{2+} application during which there is a buildup of cytosolic $[Ba^{2+}]$ from the lowest level that gives increased channel openings to the threshold for inducing channel closing. This is followed by repeated cycles of cytosolic $[Ba^{2+}]$ decrease during the period when the channels are closed (with cytosolic $[Ba^{2+}]$ decreasing mainly because of diffusion to the pipette), which a subsequent period of channel opening and cytosolic $[Ba^{2+}]$ increase starting at the end of the refractory period (if cytosolic $[Ba^{2+}]$ is still in the range that induces openings). These subsequent periods of increased openings should be of shorter duration than the initial one because less time is required to build up to the closing threshold. This prediction agrees with our results following induction of spiking at 22°C with BAY K 8644. This also fits in with the expectation that there would be less spontaneous channel opening at 22 than 37°C, and thus less Ba^{2+} in the cytosol before application of BAY K 8644.

During the period of increased openings, the cytosolic $[Ba^{2+}]$ increase will decrease the transmembrane driving force on Ba^{2+}, resulting in a continuous decline in current during the pulses, as we observe. The increase in cytosolic $[Ba^{2+}]$ can be calculated (ignoring diffusion to the pipette and buffering by EGTA) to be of the order of tens of mM,

which should have a significant effect on the transmembrane driving force. The inhibition of the current pulses by addition of an extra 1 or 2 mM Ca^{2+} to the bathing medium is also accounted for by the model. The resulting increase in cytosolic [Ca^{2+}] should allow an increased rate of binding of Ca^{2+} to the closing sites, thus counteracting the effect of Ba^{2+} in promoting channel openings.

The EGTA data suggest that the refractory period increases as cytosolic [Ba^{2+}] increases. That is, although EGTA has much less affinity for Ba^{2+} than for Ca^{2+}, an increase in EGTA concentration should lower the cytosolic [Ba^{2+}] resulting from any given course of Ba^{2+} transport across the cell membrane and through the cell. Increased EGTA should also hasten the decline of cytosolic [Ba^{2+}] from a threshold for channel closing. Such an effect of Ba^{2+} on the refractory period could perhaps result from cytosolic Ba^{2+} slowing down the molecular dynamics of the L-type Ca^{2+} channel.

The question of whether the results reported here, and the model put forth to explain them, have any physiological relevance, must be answered by future work. There is some evidence that there may be analogous behavior under physiological conditions in another cell type. In the pancreatic β-cell, repeated Ca^{2+} channel action potentials occur during depolarizing pulses produced by K^+ channel inactivation, which are thought to lead to significant Ca^{2+} influx and insulin release (Perez-Armendariz, Atwater & Rojas, 1985). Although models have been presented representing the Ca^{2+} action potentials as resulting from voltage-dependent opening (e.g., Chay & Keizer, 1983), it is conceivable that an opening/closing cycle similar to that described here could also account for these much shorter Ca^{2+} action potentials. That is, Ca^{2+}-induced channel closing, followed by a refractory period during which cytosolic [Ca^{2+}] could decrease to a level allowing greatly increased channel openings, followed by a period of openings, could occur on a faster time scale than the Ba^{2+}-induced events described in this paper. The requirements for this to occur, that the channel closing threshold with Ca^{2+} be much lower than with Ba^{2+}, and that the refractory period be shorter in the absence of Ba^{2+}, are consistent with the data and the model presented here.

In summary, our data show that Ba^{2+} can induce repeated pulses of channel openings in ROS 17/2.8 cells, producing large inward current pulses under voltage clamp, and large depolarizing pulses under current clamp. The channels involved are L-type Ca^{2+} channels. This process does not require voltage-dependent opening of channels (although we cannot rule out a facilitating role for small changes in membrane potential), but rather results from Ba^{2+} "interference" in the Ca^{2+}-inactivation mechanism of the L-type Ca^{2+} channel.

References

Belles, B., Malecot, C.O., Hescheler, J., Trautwein, W. 1988. "Run-down" of the Ca current during long whole-cell recordings in guinea pig heart cells: Role of phosphorylation and intracellular calcium. *Pfluegers Arch. Eur. J. Physiol.* **411:**353–360

Chay, T.R., Keizer, J. 1983. Minimal model for membrane oscillations in the pancreatic β-cell. *Biophys. J.* **42:**181–190

Edelman, A., Fritsch, J., Balsan, S. 1986. Short-term effects of PTH on culture rat osteoblasts: Changes in membrane potential. *Am. J. Physiol.* **251:**C483–490

Ferrier, J., Ward, A. 1986. Electrophysiological differences between bone cell clones: Membrane potential responses to parathyroid hormone and correlation with the cAMP response. *J. Cell. Physiol.* **126:**237–242

Ferrier, J., Ward-Kesthely, A., Heersche, J.N.M., Aubin, J.E. 1988. Membrane potential changes, cAMP stimulation and contraction in osteoblast-like UMR 106 cells in response to calcitonin and parathyroid hormone. *Bone Miner.* **4:**133–145

Ferrier, J., Ward-Kesthely, A., Homble, F., Ross, S. 1987. Further analysis of spontaneous membrane potential activity and the hyperpolarizing response to parathyroid hormone in osteoblastlike cells. *J. Cell. Physiol.* **130:**344–351

Fox, A.P., Nowycky, M.C., Tsien, R.W. 1987. Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurones. *J. Physiol.* **394:**149–172

Fritsch, J., Edelman, A., Balsan, S. 1988. Early effects of parathyroid hormone on membrane potential of rat osteoblasts in culture: Role of cAMP and Ca^{2+}. *J. Bone Miner. Res.* **3:**547–554

Grygorczyk, C., Grygorczyk, R., Ferrier, J. 1989. Osteoblastic cells have L-type calcium channels. *Bone Miner.* **7:**137–148

Gutnick, M.J., Lux, H.D., Swandulla, D., Zucker, H. 1989. Voltage-dependent and calcium-dependent inactivation of calcium channel current in identified snail neurones. *J. Physiol.* **412:**197–220

Majeska, R.J., Rodan, G.A. 1982. The effect of $1,25(OH)_2D_3$ on alkaline phosphatase in osteoblastlike cells. *J. Biol. Chem.* **257:**3362–3365

Markwardt, F., Nilius, B. 1988. Modulation of calcium channel currents in guinea-pig ventricular heart cells by the dihydropyridine Bay K 8644. *J. Physiol.* **399:**559–575

Perez-Armendariz, E., Atwater, I., Rojas, E. 1985. Glucose-induced osctillatory changes in extracellular ionized potassium concentration in mouse islets of langerhans. *Biophys. J.* **48:**741–749

Plant, T.D. 1988. Properties and calcium-dependent inactivation of calcium currents in cultured mouse pancreatic β-cells. *J. Physiol.* **404:**731–747

Yamamoto, Y., Hu, S.L., Kao, C.Y. 1989. Inward current in single smooth muscle cells of the guinea pig taenia coli. *J. Gen. Physiol.* **93:**521–550

Yue, D.T., Backx, P.H., Imredy, J.P. 1990. Calcium-sensitive inactivation in the gating of single calcium channels. *Science* **250:**1735–1738

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