Delayed activation of large-conductance Ca^{2+}-activated K channels in hippocampal neurons of the rat

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ABSTRACT We applied a fast concentration jump system to produce step changes in Ca^{2+} concentration ($[Ca^{2+}]_i$) on the cytoplasmic side of the inside-out membrane patch, excised from isolated rat hippocampal pyramidal neurons, and examined the time course of the activation phase of the large-conductance K channel (the BK channel; ~266 pS) after a step rise in $[Ca^{2+}]_i$. Diffusion of Ca^{2+} from the electrode tip to the cytoplasmic surface of the patch was estimated to be almost completed in 10 ms. After a step increase in $[Ca^{2+}]_i$ from 0.04 to 3.2–1,000 μM, the activation of the K channel started after a clear latency of 280–18 ms and proceeded along a sigmoidal function. This was in sharp contrast with the rapid deactivation that began without delay and that was completed within 50 ms. The latency in activation was not accounted for by the binding of Ca^{2+} to EGTA in unstirred layers in the patch, since this binding was reported to be slow, taking up to seconds at physiological pH. Calmodulin (1 μM) did not affect the delay, the activation rate, or the steady-state current level. The calmodulin inhibitors W-7 and W-5 caused flickering of the single-channel current. These results indicate a delayed activation of the BK channel after a step rise in $[Ca^{2+}]_i$, suggesting that the BK current does not contribute to the repolarization of the action potential. Calmodulin is probably not involved in the activation process of the channel.

INTRODUCTION

Single channel experiments showed that an increase in the intracellular Ca^{2+} concentration induces K currents ($I_{K(Ca)}$) in various tissues (1–3). In central neurons, however, few single channel data were reported concerning $I_{K(Ca)}$, mainly because of difficulties in obtaining preparations. The afterhyperpolarization of the action potential, therefore, has been used to study the kinetic and pharmacological properties of $I_{K(Ca)}$. It has been suggested that the TEA-sensitive large-conductance K channel (the so-called BK channel) contributes to the repolarization of the action potential (4–7). This notion was based on the implicit assumption of instantaneous activation of the channel by Ca^{2+}, because the repolarization completes in a few milliseconds after Ca^{2+} entry into the cell. On the other hand, Zhang and Krnjević (1988) reported that $I_{K(Ca)}$ is not a major mechanism of spike repolarization in cat spinal motoneurons (8). Recently, it became possible to isolate pyramidal neurons from rat hippocampal slices after enzymatic treatment (9). We tested the assumption of instantaneous activation of $I_{K(Ca)}$ by giving a step rise in $[Ca^{2+}]_i$ to inside-out membrane patches excised from freshly dispersed pyramidal cells, and found that activation of the channel is not instantaneous after a step increase in $[Ca^{2+}]_i$, but shows a delay and follows a sigmoidal time course.

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MATERIALS AND METHODS

Brains were rapidly excised from 5 to 10 day-old Wistar rats of both sexes, and sliced with a razor blade into ~500-μm thick pieces. Enzyme treatment and mechanical procedure for isolation of hippocampal pyramidal neurons have been described before (9). For ~1 h before use, the isolated neurons were kept in Tyrode solution consisting of (millimolar): NaCl 150, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 0.5, glucose 10 and Hepes 10 with pH adjusted to 7.4 with Tris base. The pipette solution (i.e., the external solution in inside-out patches) contained (millimolar): KCl 150, $CaCl_2$ 1, and Hepes 5 with pH adjusted to 7.4. The standard internal solution consisted of (millimolar): KCl 150, $CaCl_2$ 1, $MgCl_2$ 1, EGTA 1, and Hepes 10 with pH adjusted to 7.2. The Ca^{2+} concentration in the internal solution was controlled by adding EGTA or $CaCl_2$ to the standard solution (Fabiato and Fabiato, 1979) (10).

Calmodulin was prepared from whole bovine brain according to the procedures of Yazawa et al. (11). *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and *N*-(6-aminohexyl)-1-naphthalene-sulfon-

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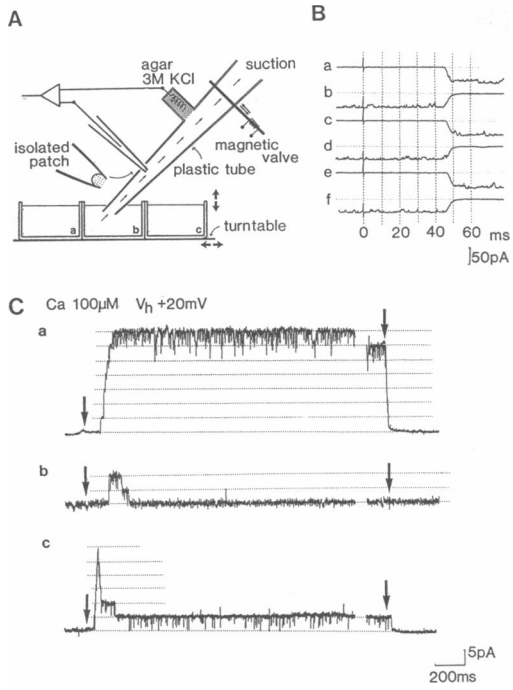

FIGURE 1 The concentration jump system and activation profiles of the K channel by Ca^{2+}. (*A*) A gigaohm seal was formed in the cell-attached mode with a pipette electrode containing 150 mM KCl and 1 mM $CaCl_2$, and a membrane patch was excised in the inside-out configuration in Tyrode solution, containing 5 mM KCl and 1.8 mM $CaCl_2$. An inward current was observed in this situation, indicating activation of K channels by Ca^{2+} (1.8 mM) in the solution. Then, (with the patch continuously in the solution) the tip of the electrode was inserted into the plastic tube through a side hole of ~0.6 mm diam. A negative pressure of ~ -10 cmH_2O was applied at the upper end of the tube and the electromagnetic valve was opened for 1 s to suck up solution from a dish on the turntable. With the valve shut, the turntable was moved down and turned around to change the solution. (*B*) At the beginning of (*a*), an inside-out patch was set in symmetrical solutions of 150 mM KCl and 1 mM $CaCl_2$, yielding no current at a holding potential of 0 mV due to a lack of driving force for K^+ ions. The magnetic valve was opened at the time point indicated zero to replace the internal solution with one containing no K^+ (consisting of 150 mM NaCl and 1 mM $CaCl_2$). An inward current began to flow through several K channels 46 ms after the opening of the valve. The interval between the opening of the valve and the first appearance of the current (the arrival time) is attributed to the travel time of the solution along the distance between the lower end of the tube and the tip of the pipette electrode. The current grew rapidly and reached a steady level within 10 ms. Then, in (*b*), the K^+-containing internal solution was re-applied. After the same arrival time, the current began to disappear due to loss of the driving force. Application (*c* and *e*) and removal (*d* and *f*) of 150 mM K^+ were repeated. Each trace had the same arrival time, and the time courses of the appearance and disappearance of the current did not differ, thereby making it possible to calculate ensemble means of $I_{K(Ca)}$ after a step change in $[Ca^{2+}]_i$. The profile of the current change after the arrival time reflects the time course of K^+ diffusion in the unstirred layers in the pipette tip at the internal surface of the membrane (shaded area in insert of *A*). Horizontal dotted lines indicate the zero current level. (*C*) Different activation profiles of K(Ca) channels. With internal solution containing 150 mM K^+ and 0.04 μM Ca^{2+}, no channel openings were recorded at +40 mV. When $[Ca^{2+}]_i$ was increased to 100 μM (*left-side arrows*, indicating the arrival time of the solution), K channels were activated after a clear latency. The channels were quickly deactivated by reducing $[Ca^{2+}]_i$ to 0.04 μM (*right-side arrows*). Three types of activation profile were observed: (*a*) sustained type (81.1%), (*b*) transient type (5.1%), and (*c*) mixed type (13.8%).

amide (W-5) were purchased from Seikagaku Kogyo Co., Ltd. (Japan).

Single-channel currents were measured using a patch-clamp amplifier (model EPC-7; List Electronic, FRG) and monitored on an oscilloscope (model 5113; Tektronix, Inc., Beaverton, OR) and a chart recorder (WR 3101; Graphtec, Japan). The data were digitized and stored on video tapes for later analysis, which was performed with a microcomputer (PC-98XL; NEC, Japan). The current signal was filtered at 2 KHz (FV-665; NF Electronic Instruments, Japan) before sampling at 0.5 ms per point. All experiments were carried out at room temperature (23° to 25°C).

RESULTS

Fig. 1 *A* illustrates the concentration jump system we used.

This approach is similar to that reported by Kakei and Ashcroft (12) and a modification of the concentration clamp technique described by us (13, 14). After obtaining an excised membrane patch in inside-out configuration, the tip of the patch electrode was inserted into a plastic tube through a hole at ~8 mm from the tip. The solution at the electrode tip could be exchanged rapidly by sucking up a new test solution by opening of an electromagnetic valve. The time required for this solution exchange was measured by recording the appearance and disappearance of the multi-K channel current on switching between K-rich and Na-rich solution (Fig. 1 *B*). The interval between the opening of the valve and the beginning of the current change (the arrival time) was attributed to the travel time of the solution from the lower end of the tube to the tip of the patch pipette. The arrival time was measured in every experiment and appeared fairly constant (~50 ms). The time course of current change after the arrival time reflects the diffusion of K^+ and Na^+ ions in the unstirred layers between the tip opening and the cytoplasmic surface of the patch membrane, because the exchange of solution at the electrode tip is rapid (complete in 0.5 ms). The time course of diffusion is affected by the thickness and geometry of the unstirred layers on the cytoplasmic side of patch. In Fig. 1 *B*, the current changed within 10 ms after the arrival of the new solution, indicating that the diffusion of K^+ was completed within 10 ms. The thickness of the unstirred layer was estimated

to be ~2 μm, according to the compartment model described by Kameyama et al. (15) (see also Fig. 4 *A*).

With several channels in the patch, three types of activation profile were observed after a step rise in $[Ca^{2+}]_i$ (Fig. 1 *C*). In 81% of the patches, the K channel showed no inactivation in the continuous presence of raised $[Ca^{2+}]_i$ (sustained type; $n = 129$ out of 159 patches). In 5% of the patches, activation was followed by inactivation (transient type; $n = 8$). The remaining 14% of recordings was of a mixed type, as shown in Fig. 1 *Cc* ($n = 22$), probably because both sustained and transient channels were present in the patch. The amplitude of the unitary current was the same for all types. The delay in activation by 100 μM Ca^{2+} at +20 mV of the transient and mixed types was 86 ± 26 ms (mean ± SEM, $n = 4$), which was not significantly different from that of the sustained type (104 ± 11 ms, $n = 7$; student *t*-test). It is not clear whether the sustained and transient types represent different classes of the K(Ca) channel.

Because the sustained type was present in the majority of the recordings, we confined our study to the activation phase of the sustained type. The open probability of the sustained type of the channel was dependent on $[Ca^{2+}]_i$ and on membrane potential (see also Fig. 2 *B*). The single channel conductance was 266 ± 7.9 pS (mean ± SEM, $n = 8$). Tetraethylammonium (5 to 30 mM) on the cytoplasmic side reduced the amplitude of the single-channel current presumably by causing the current to flicker. These findings indicate that the sustained type of the K channel is the BK channel noted in varius tissues (16).

Six traces of the K current activated by 12.6 μM of $[Ca^{2+}]_i$ are depicted in Fig. 2 *A*. There is a clear delay and a gradual build up of activation. When $[Ca^{2+}]_i$ was lowered to 0.04 μM, deactivation of the channel began without any delay and proceeded at a much higher rate than the activation. Ensemble means were obtained for currents activated by various $[Ca^{2+}]_i$ at −20 and +40 mV (Fig. 2 *B*). At +40 mV, increasing $[Ca^{2+}]_i$ reduced the latency and increased the rate of activation. We tentatively determined the beginning of activation by extrapolating the current trace to null level, by eye. The latencies at +40 mV were 238, 200, 113, and 18 ms and the half activation times 100, 87, 29, and 13 ms at 3.2, 12.6, 100, and 1,000 μM $[Ca^{2+}]_i$, respectively. At −20 mV, the latencies were 278 and 149 ms and the half activation times 174 and 48 ms at 12.6 and 100 μM $[Ca^{2+}]_i$, respectively. The ensemble mean at +40 mV reached a similar steady level with 12.6, 100 and 1,000 μM $[Ca^{2+}]_i$, a finding in agreement with the observations of Barrett et al. (17).

In the present experiments we used excised membrane patches which presumably lacked organelles. The delay and the low rate of activation may, therefore, be ascribed in the lack of intracellular substances which may facilitate the activation. Calmodulin plays an important role in cell functions, particularly those related to Ca^{2+}, and binds to membrane proteins in the presence of Ca^{2+} (18). Calmodulin (1 μM) given intracellularly exerted no effects on the activation (Fig. 3 *A*), thereby suggesting that calmodulin-Ca^{2+} complexes did not affect the K channel protein.

Alternatively, the BK channel protein, in analogy to the Ca^{2+}-dependent protease (19), may have a domain similar to calmodulin-like Ca^{2+}-binding proteins, because the channel is activated by Ca^{2+}. To test this hypothesis, we applied calmodulin inhibitors, W-7 and W-5, on the

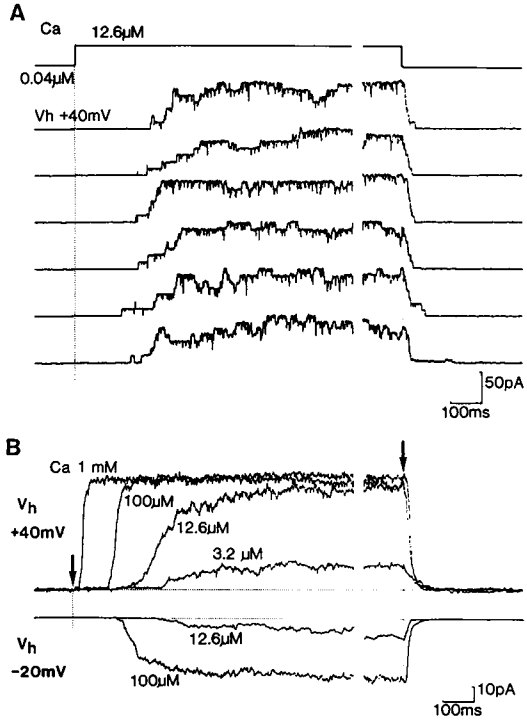

FIGURE 2 Dependence on Ca^{2+}-concentration and voltage of the delay and the activation time course of the sustained K(Ca) channel. (*A*) $[Ca^{2+}]_i$ of 12.6 μM activated the K channel in a membrane patch held at +40 mV in symmetrical 150 mM K^+ solutions. Six traces of the activation and deactivation phases in the same patch are shown. Vertical dotted lines indicate the arrival time of the applied internal solution. The channels were activated with a clear delay, whereas the deactivation after a reduction of $[Ca^{2+}]_i$ occurred quickly without latency. The interval between successive applications was ~30 s. (*B*) An ensemble mean of 10 records was obtained for various concentrations of Ca^{2+} at two membrane potentials. The activation proceeded along a sigmoidal function in all traces. The delay became smaller and the activation rate larger with increasing $[Ca^{2+}]_i$ and with increased depolarization. The amplitude of the steady-state current was also dependent on $[Ca^{2+}]_i$ and the membrane potential, until saturation occurred. The deactivation of the channel was rapid, when $[Ca^{2+}]_i$ was lowered to 0.04 μM. The arrows and vertical dotted lines indicate the arrival time of the internal solution. All records were from the same patch as in *A*. Similar results were obtained in four other experiments.

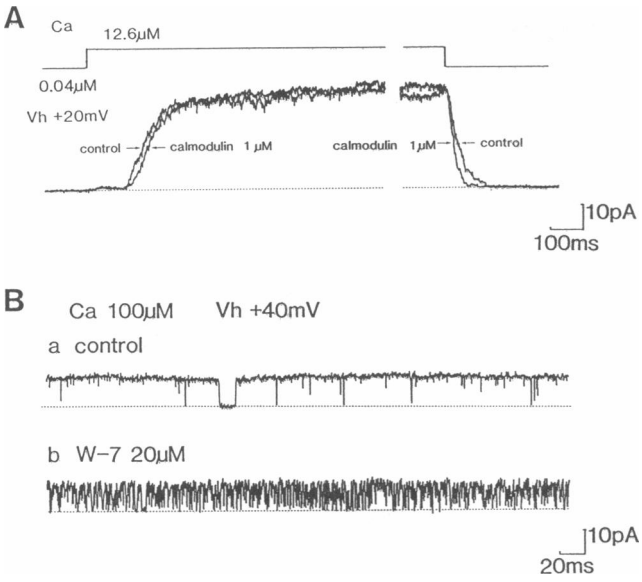

FIGURE 3 Effects of calmodulin and W-7 on the Ca^{2+}-activated K current. (*A*) Ensemble means of 10 records of the K current activated by 12.6 μM Ca^{2+} at +20 mV were obtained in the presence and absence of calmodulin (1 μM) on the cytoplasmic side of the patch. Calmodulin did not affect the delay, the activation rate or the peak current level. The same results were obtained in three other experiments. (*B*) Internally-applied W-7 (20 μM) caused flickering of the single-channel current at +40 mV without producing long closures. The same results were obtained in five other experiments.

cytoplasmic side of the patch. W-7 (1 to 50 μM) caused the outward single-channel current to flicker at +40 mV without producing long closures (Fig. 3 *B*), thereby acting as an open channel blocker. The agent exerted similar effects at −20 mV, where the current was inward (not shown). W-5 (5 to 100 μM) showed similar but weaker effects than W-7. These inhibitors had no effects when applied to the external surface of the membrane of outside-out patches (not shown). These results may indicate that the channel protein has a domain analogous to calmodulin, to the active site of which the inhibitors bind and unbind rapidly resulting in flickering of the current. However, after the findings of McCann and Welsh on smooth muscle K(Ca) channels (20), a different explanation should not be ruled out at the moment.

DISCUSSION

In our experimental set up, the increase in Ca^{2+} concentration just adjacent to the internal surface of the patch membrane might be retarded by two factors: (*a*) the diffusion of Ca^{2+} in the unstirred layers in the electrode tip and (*b*) the binding of Ca^{2+} to EGTA. Calculated profiles of K^+ and Ca^{2+} diffusion are illustrated in Fig. 4 *A*. They show that when the diffusion of K^+ is completed, the Ca^{2+} concentration just adjacent to the patch membrane has reached more than 90% of the eventual value. This estimation rules out the possibility that the slow diffusion of Ca^{2+} causes the latency seen in Fig. 2.

The rate constants of Ca^{2+} binding to EGTA were studied in skinned muscle fibers by Hellam and Podolsky (1969). They reported the forward and reverse rate constants at pH 7.0 to be 2×10^6 $M^{-1}s^{-1}$ and 0.4 s^{-1}, respectively (21). With a Ca^{2+} concentration of 12.6 μM, the time constant of the binding is calculated to be ~40 ms. At physiological pH, EGTA binds Ca^{2+} more slowly, taking up to seconds to buffer a Ca^{2+} transient (22). This implies that Ca^{2+} will not be caught up by EGTA during diffusion to the internal surface of the patch membrane, because the diffusion progresses up to 90% within 10 ms (Fig. 4 *A*). When the applied Ca^{2+} concentration is high, 100 μM for instance, the binding time constant will be as small as 5 ms. The Ca^{2+} diffusion profile in Fig. 4 *A* predicts that it takes <1 ms for Ca^{2+} to exceed the threshold for the activation, which is usually <1 μM at positive membrane potentials. These arguments indicate that the binding of Ca^{2+} to EGTA is not rate limiting in the activation of the channel. This notion was further supported by the finding depicted in Fig. 4 *B*, where no EGTA was used. The latency in the EGTA-free solution was the same as in the solution which contained 1 mM EGTA.

The delay and sigmoidal time course of the activation and the undelayed rapid deactivation may practically be accounted for by a simple sequential model with several closed states, as described by Singer and Walsh (23),

$$C_1 \overset{Ca^{2+}}{\rightleftharpoons} Ca^{2+} \cdot C_2 \overset{Ca^{2+}}{\rightleftharpoons} 2\,Ca^{2+} \cdot C_3 \overset{Ca^{2+}}{\rightleftharpoons} 3\,Ca^{2+} \cdot O,$$

where C_n denotes closed states and O the open state. Studies of the kinetic properties of BK channels suggested that openings can occur with 1, 2, 3, or more Ca^{2+} bound to the channel (16, 23). Preliminary results in the present experiments gave a Hill coefficient of three in our preparation, indicating the involvement of 3 Ca^{2+} ions in the activation process.

The activation delay was clearly observed even with 100 μM of $[Ca^{2+}]_i$ (Fig. 2 *B*). $[Ca^{2+}]_i$ has been reported to increase to several μM after a train of action potentials, as determined using a Ca^{2+}-indicator dye and Ca^{2+}-sensitive microelectrodes (24). The local concentration of Ca^{2+} in the space immediately adjacent to the internal membrane surface is difficult to determine, but an estimation of more than 30 μM was made in a three-dimensional simulation of transmitter release. It was suggested that a local concentration of 50 μM does not significantly affect the driving force for Ca^{2+} (25). If the

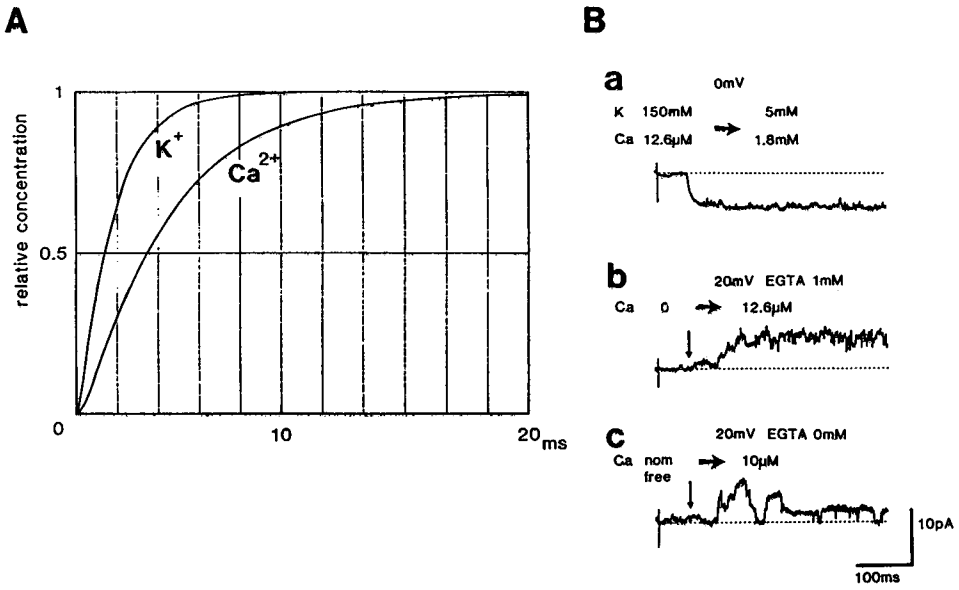

FIGURE 4 (*A*) Diffusion profiles of K^+ and Ca^{2+}. The diffusion profile of K^+ was calculated according to the compartment model described by Kameyama et al. (15) for a tip diameter of 1 μm, a semi-vertical angle of 8° and a diffusion coefficient for K^+ of 1.96×10^{-5} $cm^2 s^{-1}$ (30). Simulation of the K^+ diffusion curve yielded similar diffusion times as in Fig. I *B* when the thickness of the unstirred layer was assumed to be 2 μm. The Ca^{2+}-diffusion profile was obtained using those values and a diffusion coefficient for Ca^{2+} of 0.79×10^{-5} $cm^2 s^{-1}$ (30). The Ca^{2+} concentration at the internal surface of the patch membrane reaches more than 90% of the applied concentration and the diffusion is completed in 20 ms. (*Ordinate*) relative concentration of K^+ or Ca^{2+} at the cytoplasmic surface of the patch membrane. (*Abscissa*) time in millisecond after arrival of a new solution at the electrode tip. (*B*) The same latency of activation in the presence and absence of EGTA. (*a*) Determination of the arrival time by replacing, at a holding potential of 0 mV, a high-K solution ($[K^+]_i$ 150 mM and $[Ca^{2+}]_i$ 12.6 μM) with a low-K solution ($[K^+]_i$ 5 mM and $[Ca^{2+}]_i$ 1.8 mM) (compare Fig. 1 *B*). The arrival time determined this way is indicated by vertical arrows in *b* and *c*. The rising phase of the current was slightly retarded due to a further activation of the channel by the higher concentration of Ca^{2+}. An ensemble mean of five applications. (*b*) Application of Ca^{2+}-free and 12.6 μM Ca^{2+} solutions with 1 mM EGTA. Elevation of $[Ca^{2+}]_i$ activated one channel in the same patch as in *a*, which was voltage-clamped at +20 mV. The activation had a latency of ~50 ms and increased along a sigmoidal function with time. An ensemble mean of 5 applications. (*c*) Application of nominally Ca^{2+}-free and 10 μM Ca^{2+} solutions without EGTA. In our laboratory, the nominally Ca^{2+}-free solution with 150 mM KCl has a Ca^{2+} contamination of ~5 μM, which was determined with a Ca-electrode (expandable ion analyzer, model EA 920; Orion Research Inc., Cambridge, MA). In this patch, the nominally Ca^{2+}-free solution without EGTA did not activate the channel at +20 mV. When $[Ca^{2+}]_i$ was increased to 10 μM, the channel was activated after the same latency as in the presence of EGTA. An ensemble mean of three applications. From the same patch as *a* and *b*.

local concentration rose close to that in the extracellular space, single Ca channel currents recorded by several investigators would have been reduced toward the end of depolarizing pulses due to a decrease in the driving force. Such a reduction has never been observed in cell attached patches (see references 26, 27). These arguments suggest that 100 μM Ca^{2+} is high enough to approximate $[Ca^{2+}]_i$ after an action potential.

Brett et al. (1986) described a similar solution exchange system and examined the process of activation by Ca^{2+} of the large-conductance K channel in cultured rat hippocampal neurons (28). They simultaneously raised K^+ (from 70 to 140 mM) and Ca^{2+} (from nominally free to 1 or 10 μM) concentrations on the cytoplasmic side of the patch. Although they gave no unambiguous estimation of the point in time where Ca^{2+} reached the internal surface of the membrane patch, one can see in their records a clear interval between K^+ arrival and channel activation. It is unclear, however, whether the latency was due to a slow diffusional exchange of Ca^{2+} or to a true delay in activation (see Fig. 3 in reference 28).

In conclusion, the activation of the BK channel is not instantaneous after a step rise in $[Ca^{2+}]_i$ in excised membrane patches from rat hippocampal neurons. This finding suggests that the BK current activated by Ca^{2+} which flows into the cell during an action potential is not causally involved in the repolarization of the action potential. However, resting $[Ca^{2+}]_i$ may be high enough to allow voltage-dependent activation of the channel during the spike. In addition, accumulation of Ca^{2+} during a burst of spikes may facilitate BK channel activation in the later stages of the burst. A Ca^{2+}-related intracellular protein activator, calmodulin, did not affect the activation. It cannot be ruled out, however, that the K(Ca) channel protein has a domain analogous to calmodulin. Finally, the possibility remains that other intra-

cellular substances may facilitate the activation of the channel (29).

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