Membrane-Pipette Interactions Underlie Delayed Voltage Activation of Mechanosensitive Channels in *Xenopus* Oocytes

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ABSTRACT To investigate the mechanism for the delayed activation by voltage of the predominant mechanosensitive (MS) channel in *Xenopus* oocytes, currents were recorded from on-cell and excised patches of membrane with the patch clamp technique and from intact oocytes with the two-electrode voltage clamp technique. MS channels could be activated by stretch in inside-out, on-cell, and outside-out patch configurations, using pipettes formed of either borosilicate or soft glass. In inside-out patches formed with borosilicate glass pipettes, depolarizing voltage steps activated MS channels in a cooperative manner after delays of seconds. This voltage-dependent activation was not observed for outside-out patches. Voltage-dependent activation was also not observed when the borosilicate pipettes were either replaced with soft glass pipettes or coated with soft glass. When depolarizing voltage steps were applied to the whole oocyte with a two-electrode voltage clamp, currents that could be attributed to MS channels were not observed. Yet the same depolarizing steps activated MS channels in on-cell patches formed with borosilicate pipettes on the same oocyte. These observations suggest that the delayed cooperative activation of MS channels by depolarization is not an intrinsic property of the channels, but requires interaction between the membrane and patch pipette.

INTRODUCTION

Mechanosensitive (MS) channels play a central role in the senses of touch, hearing, balance, and proprioception, as well as being involved in cell volume regulation (for reviews see Morris, 1990; Sackin, 1995; Hamill and McBride, 1996). A number of MS channels are modulated by voltage once they are activated by stretch or are activated by voltage in the absence of stretch. The predominant MS channel in *Xenopus* oocytes is also voltage-sensitive (Methfessel et al., 1986; Yang and Sachs, 1990; Hamill and McBride, 1992), and recent experiments on this MS channel in excised patches of membrane have revealed an unusually slow voltage-dependent activation and deactivation (Silberberg and Magleby, 1997). Stepping the membrane potential from -50 to $+50$ mV maximally activated the MS channels only after delays of typically many seconds and, after the step back to -50 mV, the MS channels deactivated over about 10 s. Interestingly, if the depolarizing voltage step was briefer than the delay to MS channel activation, then upon repolarization the channels transiently activated, giving the appearance that the MS channels were activated by hyperpolarization.

The delay to activation of the channels during the depolarizing step varied substantially from patch to patch, ranging from <0.1 to >20 s. Yet in about 70% of patches that contained multiple MS channels, the channels appeared to activate cooperatively. The similar delay to activation for all MS channels in a patch, despite large variations in the delay to activation among patches, suggests that voltage indirectly activates the MS channels via a common factor. Indirect effects of voltage on the MS channels may be mediated by structures associated with the channels, by second messengers, or more simply by voltage-induced changes in membrane tension. Movement of the membrane up and down the pipette in response to large voltage steps has been reported (Hörber et al., 1995). If these voltage-induced movements result in changes in membrane tension sufficient to activate MS channels, then such a mechanism could explain the apparent cooperative activation of MS channels in inside-out patches of membrane.

The purpose of the present study was to investigate whether the delayed cooperative activation of MS channels with depolarization is an intrinsic property of the channels or whether extrinsic factors, such as membrane tension, are involved. If the MS channels in *Xenopus* oocytes have an intrinsic voltage-sensitive gating mechanism, as in classical voltage-sensitive channels (Papazian and Bezanilla, 1997), then it might be expected that delayed cooperative activation of MS channels by voltage would be the same for inside-out and outside-out patches. This was not the case, as delayed activation by voltage was not observed in outside-out patches of membrane. Furthermore, when inside-out patches were formed either with soft-glass pipettes or with borosilicate glass pipettes coated with soft glass, rather than with borosilicate glass pipettes, MS channels could no longer be activated by voltage.

Finally, if the delayed activation of MS channels by depolarization is an intrinsic property of the channels, then a slowly increasing current with depolarization that can be attributed to MS channels should be present when currents

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are recorded from whole oocytes with the two-electrode voltage clamp. The recent observation of Zhang et al. (1998) that flufenamic acid can block I_c, the predominant membrane conductance in oocytes (Arellano et al., 1995; Ebihara, 1996), allowed us to perform these whole-cell experiments. Currents that could be attributed to MS channels were not observed when depolarizing steps were applied to the whole oocyte with a two-electrode voltage clamp. Yet, the same depolarizing steps activated MS channels in on-cell patches of membrane on the same oocyte.

The finding that delayed cooperative activation of MS channels by voltage in the absence of stretch was observed only in patched membranes of a specific configuration formed with a particular type of glass pipette suggests that the delayed cooperative activation of MS channels by voltage is not an intrinsic property of the channels, but requires interactions between the patch of membrane and the patch pipette. Patch electrode glass is known to release ions that can influence ion channel behavior (Cota and Armstrong, 1988; Furman and Tanaka, 1988; Zuazaga and Steinacker, 1990; Copollo et al., 1991). The release of such ions from the glass does not appear to account for the different results with borosilicate and soft glass pipettes, because similar findings were obtained when high concentrations of chelators were included in the pipette solutions. Thus, the proposed membrane-pipette interactions are thought to reflect direct contact between the patch of membrane and the patch pipette. If such contact produces voltage-dependent tension in the patch of membrane, then this could produce cooperative activation of the MS channels with voltage.

METHODS

Oocyte isolation and single channel recording

The methods used for isolation of oocytes, single-channel recording, and data analysis were performed as previously described (Silberberg and Magleby, 1997). Briefly, adult *Xenopus laevis* frogs were anesthetized by immersion in a solution containing 1.5 g/l of ethyl m-aminobenzoate (methanesulphonate salt, Tricaine, Sigma, St. Louis, MO) and oocytes were removed as described previously (Stühmer and Parekh, 1995). Stage V-VI oocytes were then enzymatically separated and used within 5 days of isolation. The vitelline membrane was removed with forceps from the oocytes just before the experiments. Currents from MS channels were recorded using the patch-clamp technique (Hamill et al., 1981). Patch pipettes were fabricated from borosilicate glass (Corning No. 7740; Clark Electromedical Instruments, Reading, UK) or from soft glass (Corning No. 8161; Garner Glass Company, Claremont, CA) and were coated with Sylgard 184 (Dow Corning).

Three configurations of the patch clamp technique were used in this study: on-cell, inside-out, and outside-out patch recordings. As detailed in Hamill et al. (1981), on-cell patches were formed by pressing the tip of a heat-polished patch pipette against the membrane of the cell and then applying slight negative pressure (< -1 kPa). Inside-out patches were formed by establishing an on-cell patch and then drawing the patch pipette away from the cell surface, exposing the cytoplasmic face of the patch to the bath solution. (Note that the patch of membrane is drawn into the patch pipette for both on-cell and inside-out patches, as the patch is initially formed in the same manner). Outside-out patches were formed by rupturing (with pressure) the membrane patch underlying the patch pipette in the on-cell configuration and then withdrawing the patch pipette. Membrane from the cell surface then formed the outside-out patch, in which the extracellular face of the membrane was exposed to the bath solution.

Experiments with inside-out patches were performed in either NaCl or KCl solutions with similar findings for each. The pipette (extracellular) solution and the bath (intracellular) solution contained: NaCl (or KCl), 150 mM; Tes, 5 mM; and EGTA, 2 mM. In some experiments the EGTA was increased to 10 mM and BAPTA (10 mM) was also included. For experiments with outside-out patches and some inside-out patches the pipette solution contained KF in place of NaCl. All solutions were adjusted to pH 7.0. Step changes in membrane potential were controlled using pClamp 6.0.2 software (Axon Instruments Inc., Foster City, CA). Current traces were initially low-pass filtered at 2 kHz (−3dB), using the integral 4-pole Bessel filter of the Axopatch 200B patch clamp amplifier (Axon Instruments) and stored on VCR tape (Instrutech Corp., Great Neck, NY) for subsequent analysis. Experiments were performed at room temperature, 19–23°C.

The patch of membrane could be stretched by applying negative pressure pneumatically to the back of the pipette through a port on the pipette holder. It is the membrane tension resulting from the pressure rather than the pressure per se that activates MS channels (Gustin et al., 1988; Sokabe et al., 1991). Step changes in pressure were produced with an electronic valve system that switched the pipette holder between the pressure reservoir and room air (atmospheric pressure). The electronic valve and controller (General Valve Corp., Fairfield, NJ) were controlled by computer. Pressure changes are expressed in kPa, where 1.0 kPa = 10.2 cm H_2O. To estimate the number of MS channels in a patch, the channels were maximally activated by stretch at −50 mV and then the magnitude of the stretch-activated current was divided by the unitary current. Experiments were performed on patches of membrane containing from 1 to ~40 MS channels.

Recording of current from whole oocytes

Whole-cell currents were measured using a conventional double-electrode oocyte voltage-clamp (OC-725C; Warner Instruments Corp., Hamden, CT). In some experiments, the vitelline membrane of the oocyte was removed with fine forceps before the recording. No differences in whole-cell currents were observed between oocytes with and without the vitelline membrane. The microelectrodes for voltage recording and for current passing had tip diameters of 2–3 μm and were filed with 3 M KCl. The bath solutions that were used for whole-cell recordings contained NaCl (or KCl), 120 mM; Tes, 5 mM; and EGTA, 2 mM, adjusted to pH 7.4. When flufenamic acid or gadolinium was added, EGTA was omitted from the bath solution.

For simultaneous whole-cell and cell-attached recordings, the resistive feedback configuration of the Axopatch 200B amplifier was used in order to eliminate large current artifacts that could be associated with the simultaneous recording. Current traces were initially low-pass filtered at 1 kHz, using the integral filter of the voltage clamp amplifier and stored on VCR tape for subsequent analysis.

RESULTS

Depolarization activated MS channels in inside-out patches formed with borosilicate glass pipettes

Fig. 1 presents current records from inside-out patches of membrane excised from *Xenopus* oocytes with borosilicate glass pipettes. Fig. 1 *A* is from a patch containing three MS channels, and Fig. 1 *B* is from a patch containing >15 MS channels. Channel opening is indicated by downward deflections at −50 mV and by upward deflections at +50 mV due to the symmetrical 150-mM NaCl solutions. Typical of MS channels (Methfessel et al., 1986; Morris, 1990; Sackin,

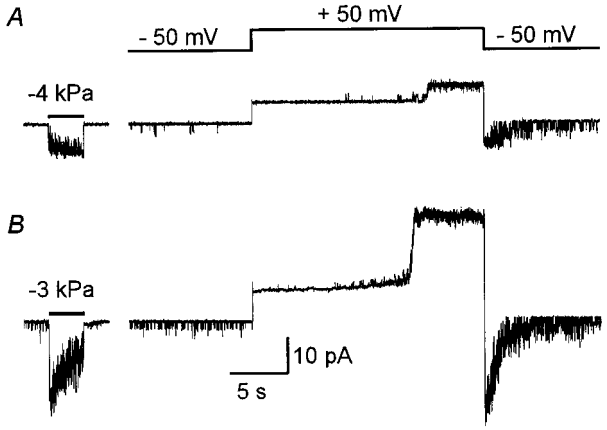

FIGURE 1 Depolarizing voltage steps activate MS channels after a delay of seconds in inside-out patches formed with borosilicate glass pipettes. Current records from excised inside-out membrane patches with three MS channels (*A*) and with >15 MS channels (*B*). In this and related figures, channel opening is indicated by downward current deflections at negative potentials and upward current deflections at positive potentials. Stretching the membrane by applying negative pressure to the patch pipette at −50 mV (−4 kPa in *A* and −3 kPa in *B*, indicated by solid bar) rapidly activated the MS channels. In the absence of stretch, the membrane potential was stepped from −50 to +50 mV for 20 s, and then stepped back to the holding potential. Depolarization activated with pronounced delays the MS channels in the patch in an apparent cooperative manner, and the channels deactivated slowly when the depolarization was removed. Symmetrical 150-mM NaCl solutions are described in the Methods section. The leak currents resulting from step changes in voltage were not subtracted from the current records.

1995; McBride and Hamill, 1993), stretching the membrane patch by applying suction to the patch pipette (Sokabe et al., 1991) at the holding potential of –50 mV rapidly activated the MS channels and the termination of stretch resulted in rapid deactivation (left traces in Fig. 1, *A* and *B*).

MS channels can also be activated in the absence of stretch by applying prolonged depolarizing voltage steps (Silberberg and Magleby, 1997). This is shown in the right traces in Fig. 1, where stepping the membrane potential from −50 to + 50 mV activated the MS channels to high levels of activity after delays of seconds. Upon repolarization, the channels then deactivated slowly over several seconds. The channels activated by depolarization were previously shown to be MS channels based on single channel conductance, ionic selectivity, and on the maximal number of channels per patch that could be activated by voltage or stretch (Silberberg and Magleby, 1997).

The delayed activation of MS channels by depolarization in the absence of stretch was consistently seen in inside-out patches formed with either small tip (≤1 μm) or large tip (1–3 μm) borosilicate glass pipettes (n = 42, examples in Figs. 1, 4, and 5 *A* and in Silberberg and Magleby, 1997). The delay to half-maximal activation of the channels following depolarization to +50 mV varied substantially from patch to patch. In 18 experiments analyzed in detail, the delay ranged from <0.1 to 25.8 s. Nevertheless, in most inside-out patches containing two or more MS channels, the channels appeared to activate in a cooperative manner in response to depolarizing voltage steps. This cooperative activation is clearly seen for the patch containing more than 15 channels (Fig. 1 *B*), where most of the channels activated within 0.6 s after a delay of 14 s. The similar delay to activation for the MS channels in a patch, despite large variations in the delay to activation among patches, raises the possibility that activation of MS channels by voltage is controlled by a factor common to all the MS channels in the patch. The aim of the present study is to determine whether the unusual delayed voltage dependence of the MS channels in *Xenopus* oocytes is an intrinsic property of the channels or whether an extrinsic factor, such as membrane tension, might be involved.

MS channels are not activated by depolarization in outside-out patches

If the delayed cooperative activation of MS channels by voltage were an intrinsic property of the channels, then depolarization might be expected to activate MS channels in outside-out patches as well. This was not the case. Depolarization did not activate MS channels in 17 of 17 outside-out patches containing MS channels. In the example shown in Fig. 2, depolarizing the membrane from −50 to +50 mV for 40 s did not activate MS channels in the outside-out patch (Fig. 2 *A*). Yet 2–3 MS channels could be activated by applying negative pressure to the patch pipette either at −50 or +50 mV (Fig. 2, *B* and *C*, respectively). Another example of the inability to activate MS channels by depolarization in outside-out patches will be presented in Fig. 3 *A* in the next section.

Activation of MS channels by pressure in outside-out patches typically required more pressure (6–10 kPa) than was needed to activate MS channels in inside-out patches

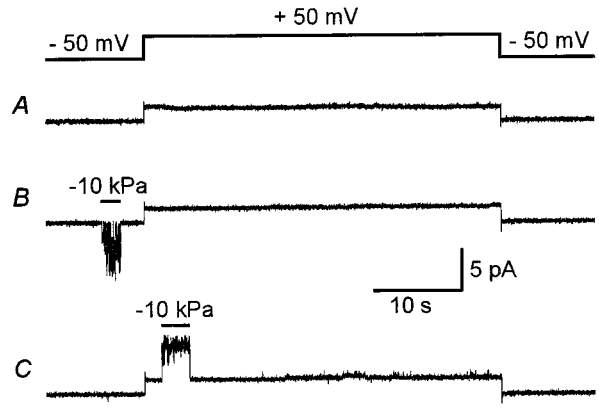

FIGURE 2 Depolarization-induced MS channel activation is not observed in outside-out patches. (*A*) Current record from an excised outside-out membrane patch with three MS channels. From a holding potential of −50 mV the patch was depolarized to +50 mV for 40 s. No MS channel activity was observed at either −50 or +50 mV. The MS channels in the patch could be activated by negative pressure applied either at −50 mV (*B*) or at +50 mV (*C*). The records are from the same membrane patch.

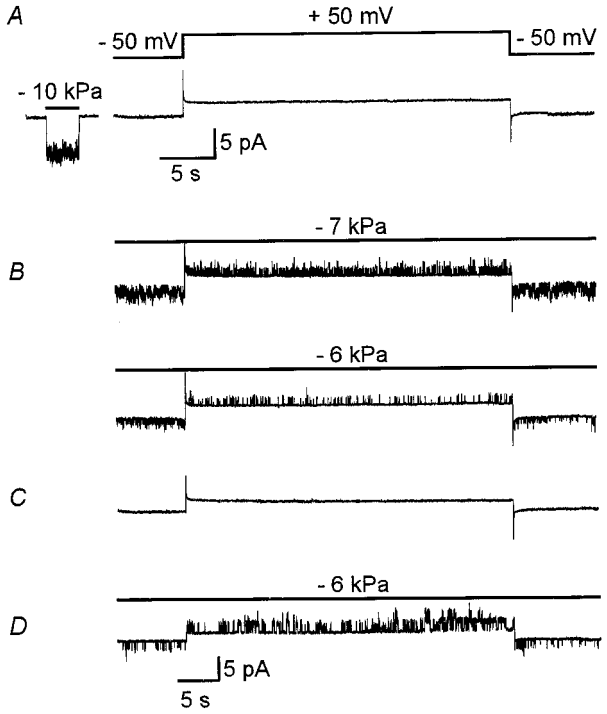

FIGURE 3 Continuous membrane tension does not enable delayed cooperative activation of MS channels by depolarization in outside-out patches. (*A-C*) Current records from an excised outside-out membrane patch. Stretching the membrane by applying −10 kPa to the patch pipette at −50 mV rapidly activated 6–7 MS channels (*A, left trace*). In the absence of stretch, no MS channel activity was observed at either −50 or +50 mV (*A, right trace*). (*B*) With continuous stretch sufficient to induce a low level of basal MS channel activity, depolarization to +50 mV did not maximally activate the MS channels cooperatively after a delay. The stretch was induced by applying negative pressure to the pipette. (*C*) MS channel activity was not observed at either −50 or +50 mV when the continuous stretch was removed. (*D*) The effects of continuous stretch on the outside-out patch presented in Fig. 2. Stretch induced a low level of MS channel activity but did not maximally activate the MS channels cooperatively after a delay. Stretch was applied at the times indicated by thick lines above the current traces.

(0.5–5 kPa). This difference may be due to differences in the areas and geometry of the patches and the indirect relationship between applied pressure and the resulting stretch of the membrane patch (Sokabe and Sachs, 1990; Sokabe et al., 1991; Sukharev et al., 1997) or to differences in membrane-cytoskeleton interactions (Sokabe et al., 1991; Hamill and McBride, 1997) that may be associated with the different configurations of inside-out and outside-out patches.

Adding basal membrane tension to outside-out patches cannot induce delayed cooperative activation of MS channels by depolarization

In all outside-out patches examined there was no basal activity of MS channels at the holding potential of −50 mV and also no delayed activation by depolarization (Figs. 2 and 3 *A*). In contrast, basal activity was evident in most (but not all) inside-out patches, and depolarization maximally activated the MS channels in all of the inside-out patches (examples in Fig. 1, *A* and *B*, for the presence of basal activity and in Fig. 4 *A* for the absence of basal activity). If basal activity of MS channels gives a measure of the resting tension in the membrane patch (i.e., the tension in the lipid bilayer and/or in associated structures such as the cytoskeleton), then perhaps some basal membrane tension is needed for the delayed cooperative activation of MS channels by voltage.

To test this possibility, depolarizing voltage steps were applied to outside-out patches in which basal membrane tension was induced by applying continuous negative pressure to the patch pipette. Sufficient negative pressure was applied to induce a basal MS channel open probability, *Po*, in the range of 0.007–0.14 at −50 mV. The number of MS channels in each of the outside-out patches was estimated

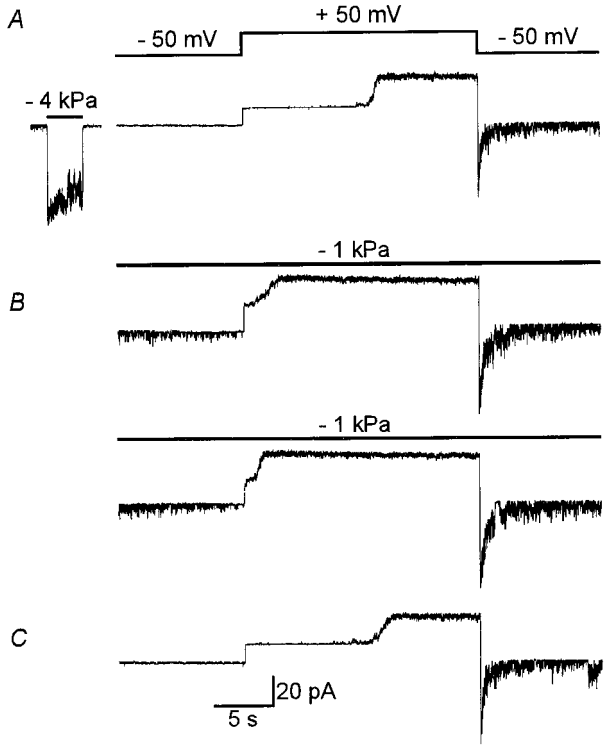

FIGURE 4 In inside-out patches formed with borosilicate glass pipettes continuous membrane tension shortens the delay to cooperative activation of the MS channels by depolarization. Current records from an excised inside-out membrane patch formed with a borosilicate glass pipette. (*A*) Stretching the membrane by applying −4 kPa to the patch pipette at −50 mV rapidly activated >20 MS channels (*A, left trace*). In the absence of stretch, stepping the membrane potential from −50 to +50 mV maximally activated the MS channels in the patch with a pronounced delay in an apparently cooperative manner. The channels deactivated slowly when the depolarization was removed. (*B*) The delay to MS channel activation by depolarization was greatly reduced in the presence of continuous stretch induced by applying negative pressure to the patch pipette. Slow deactivation was still observed in the presence of stretch. (*C*) The long delay to MS channel activation was restored when the continuous stretch was removed. Stretch was applied at the times indicated by thick lines above the current traces.

by applying −10 kPa to the patch pipette at −50 mV. In the example shown in Fig. 3 *A*, 6–7 MS channels were activated by the pressure step but not by a depolarizing voltage step to +50 mV.

Fig. 3 *B* shows that when membrane tension was induced by applying continuous negative pressure, as indicated by the basal activity of MS channels, stepping the membrane potential to +50 mV did not activate the MS channels in the patch cooperatively to high levels of activity after a delay. Instead, a low level of MS channel activity was evident both at −50 mV and throughout the depolarizing voltage step (compare the two examples in Fig. 3 *B* to those in Fig. 1). When the continuous negative pressure was removed, no basal MS channel activity was observed, and there was still no activation by depolarization (Fig. 3 *C*).

A lack of delayed cooperative activation of MS channels by depolarization in outside-out patches was observed in nine of nine experiments in which sufficient membrane tension was applied to induce basal MS channel activity. In two of these experiments, MS channel activity appeared to increase somewhat during the depolarizing voltage steps. An example of a record from one of these experiments is presented in Fig. 3 *D*. Nevertheless, in these two experiments the apparent increase in activity was slight compared to the maximal activation that occurred when depolarizing voltage steps were applied to inside-out patches, and activation was not cooperative (compare to Fig. 1).

Adding basal membrane tension to inside-out patches shortened the delay to cooperative activation of MS channels by depolarization

The previous section explored whether delayed activation of MS channels by depolarization might be related to basal membrane tension. Adding basal membrane tension to outside-out patches did not enable such delayed activation, suggesting either that basal tension was not related to delayed activation or that the outside-out patches were incapable of delayed activation. To differentiate between these possibilities, the effects of basal membrane tension on the depolarization-induced delay to activation of MS channels in inside-out patches was examined. Results are shown in Fig. 4.

In the absence of applied stretch, depolarizing the membrane from −50 to +50 mV, maximally activated the MS channels in the inside-out patch in a cooperative manner after a delay of 11 s (Fig. 4 *A*). When sufficient membrane tension was then applied to raise the average basal *Po* of each channel to ~0.01, the delay to activation of the MS channels was greatly reduced to ~2 s (Fig. 4 *B*). Removing the induced membrane tension restored the longer delay to cooperative activation by depolarization (Fig. 4 *C*). In five of five experiments with inside-out patches of membrane, applying negative pressure sufficient to increase the level of basal activity shortened the delay to activation. Thus, the observation in the previous section that basal tension did not induce delayed activation in outside-out patches suggests that outside-out patches are incapable of delayed activation.

The above results, that MS channels in outside-out patches were not activated by depolarization in either the presence or absence of basal tension, whereas MS channels in inside-out patches were, suggests either that the formation of the outside-out patch configuration damaged the voltage dependence of the MS channels or that the cooperative delayed activation of MS channels is not an intrinsic property of the channels. The following section examines these possibilities.

Currents that could be attributed to MS channels were not observed in recordings from whole oocytes with the two-electrode voltage clamp technique

If cooperative delayed activation by depolarization is an intrinsic property of MS channels, then voltage steps applied to whole oocytes with the two-electrode voltage clamp technique might be expected to activate MS channels. Gadolinium (Gd^{3+}), which is known to block currents through MS channels in oocytes (Yang and Sachs, 1989), was used to examine whether MS channels in whole oocytes could be activated by depolarization. In order to look for currents from MS channels, it was necessary first to block I_c, the predominant membrane conductance in oocytes (Arellano et al., 1995; Ebihara, 1996). Flufenamic acid could be used to block I_c (Zhang et al., 1998) if it did not also block MS channels. Fig. 5 *A* shows that flufenamic acid did not interfere with the activation of MS channels, as MS channels in an inside-out patch were readily activated by depolarizing steps with 300 μM of flufenamic acid both in the bath and in the pipette. A similar delayed activation of MS channels in inside-out patches in the presence of 150–300 μM flufenamic acid was observed in 10 of 10 additional patches.

Fig. 5 *B* presents whole-cell currents recorded from a whole oocyte with the two-electrode voltage clamp. In extracellular solutions containing no divalent cations, a voltage step from −50 to +50 mV rapidly activated a large current that slowly deactivated upon repolarization (control trace). Consistent with the findings of Zhang et al. (1998), flufenamic acid blocked this I_c current (flufenamic acid trace). If depolarization activated MS channels in the whole oocyte after a delay of 0.1–20 s, as seen in inside-out patches, then the depolarizing voltage step to +50 mV should have activated a slowly developing current. Clearly, such a current was not present in the whole oocyte after I_c was blocked (flufenamic acid trace). Furthermore, there was no evidence of a slowly deactivating (tail) current.

Further indication that depolarization did not activate whole-cell currents through MS channels came from the observation that the whole-cell currents measured in flufenamic acid were not reduced when Gd^{3+} replaced the flufenamic acid (Fig. 5 *B*, gadolinium trace). Because Gd^{3+}

blocks both I_c (Zhang et al., 1998) and MS channels (Yang and Sachs, 1989), whereas flufenamic acid blocks only I_c, any whole-cell currents through MS channels should have been evident as a decrease in current amplitude in Gd^{3+} when compared to currents in flufenamic acid. Such a decrease was not seen (gadolinium trace). In a total of 35 of 35 oocytes from eight different frogs, there was no evidence for activation of whole-cell currents from MS channels by voltage steps to +50 mV, even for depolarizing steps lasting as long as 100 s.

Thus, the findings that depolarization does not activate MS channels in whole oocytes lends further support to the suggestion that delayed cooperative activation of MS channels by depolarization is not an intrinsic property of MS channels.

Depolarization of whole oocytes with the two-electrode voltage clamp activated MS channels in on-cell patches but not MS channels in the unpatched membrane

It is unlikely that a soluble component within the oocytes inhibited the MS channels in the whole-oocyte recordings, because activation of MS channels by depolarizing voltage steps has previously been observed in on-oocyte patches (Silberberg and Magleby, 1997). Therefore, the ability of depolarizing voltage steps to activate MS channels in inside-out and on-cell patches but not in whole-cell recordings suggests that activation of MS channels by depolarization may require membrane-pipette interactions. If this is the case, then it might be expected that the same depolarizing voltage steps that failed to active MS channels in voltage-clamped oocytes would activate MS channels in on-cell patches on the same oocyte. Indeed, this was found to be the case. Fig. 6 presents two examples of simultaneous whole-cell and on-cell patch recordings in the presence of flufenamic acid. In both oocytes, voltage steps from −50 to +50 mV did not activate slow currents in the whole oocyte, (top current traces in Fig. 6, *A* and *B*). Yet MS channels did activate within 10 s in the on-cell patches (bottom two traces in Fig. 6, *A* and *B*). Similar results were observed in 11 of 11 experiments of this type.

The predicted magnitude of the whole-oocyte MS current was estimated in order to asses whether the MS channel currents were perhaps too small to detect. Assuming 3×10^6 MS channels per oocyte (Methfessel et al., 1986), a unitary single-channel current of 1.5 pA (the approximate unitary current for MS channels at +50 mV in the on-cell patches), and a *Po* close to 1.0 at the end of a depolarizing step, then a whole-cell current of about 4–5 μA should have been recorded if depolarization activated the MS channels in the whole oocyte. Figs. 5 and 6 clearly show that the observed whole-cell currents after blocking I_c were ~10 to 500-fold less than the magnitude expected if MS channels had been activated. Furthermore, there was no evidence of a slowly activating current, however small, as would be expected for delayed activation of MS channels.

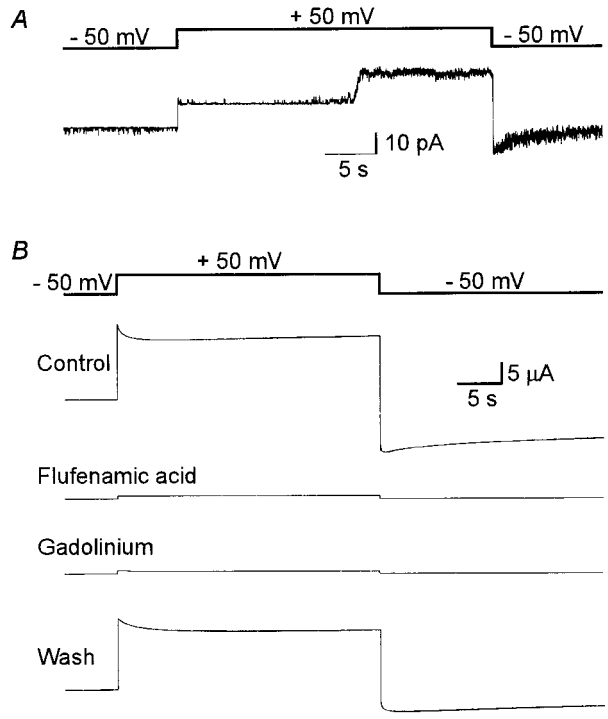

FIGURE 5 Currents from MS channels are not observed in recordings from whole oocytes. (*A*) current record from an excised inside-out membrane patch formed with a borosilicate glass pipette. Flufenamic acid (300 μM) was present in both the bath and pipette solutions. Depolarization activated the MS channels in the patch with a pronounced delay in an apparently cooperative manner, and the channels then deactivated slowly when the depolarization was removed. (*B*) Current records from a whole oocyte measured with a two-electrode voltage clamp. For each of the four traces, the current was stepped from a holding potential of −50 to +50 mV for 30 s and then stepped back. In 120-mM KCl solution (see Methods) large membrane currents were recorded (*Control*) which were then inhibited by flufenamic acid (300 μM). With Gd^{3+} (100 μM) in the bath solution in place of flufenamic acid, there was no further inhibition of the whole-oocyte current. The effects of flufenamic acid and Gd^{3+} could then be washed off (*Wash*).

Activation of MS channels by depolarization was dependent on the type of glass used for the patch pipette

One difference between the recordings from whole oocytes, where MS channels were not activated by depolarization, and the recordings from both on-cell patches and inside-out patches, where MS channels were activated by depolarization, was the association of the patch of membrane with the patch pipette in the on-cell and inside-out recordings. Perhaps the activation of MS channels by depolarizing voltage steps requires some form of membrane-pipette interaction exclusive to both inside-out and on-cell patches. If this were the case, then changing the properties of the patch pipettes might alter the delayed activation of the MS channels.

To examine this possibility, currents were recorded from inside-out patches formed using pipettes constructed of soft glass (Corning No. 8161). In contrast to the findings in Figs. 1 and 4, where depolarization led to delayed cooperative

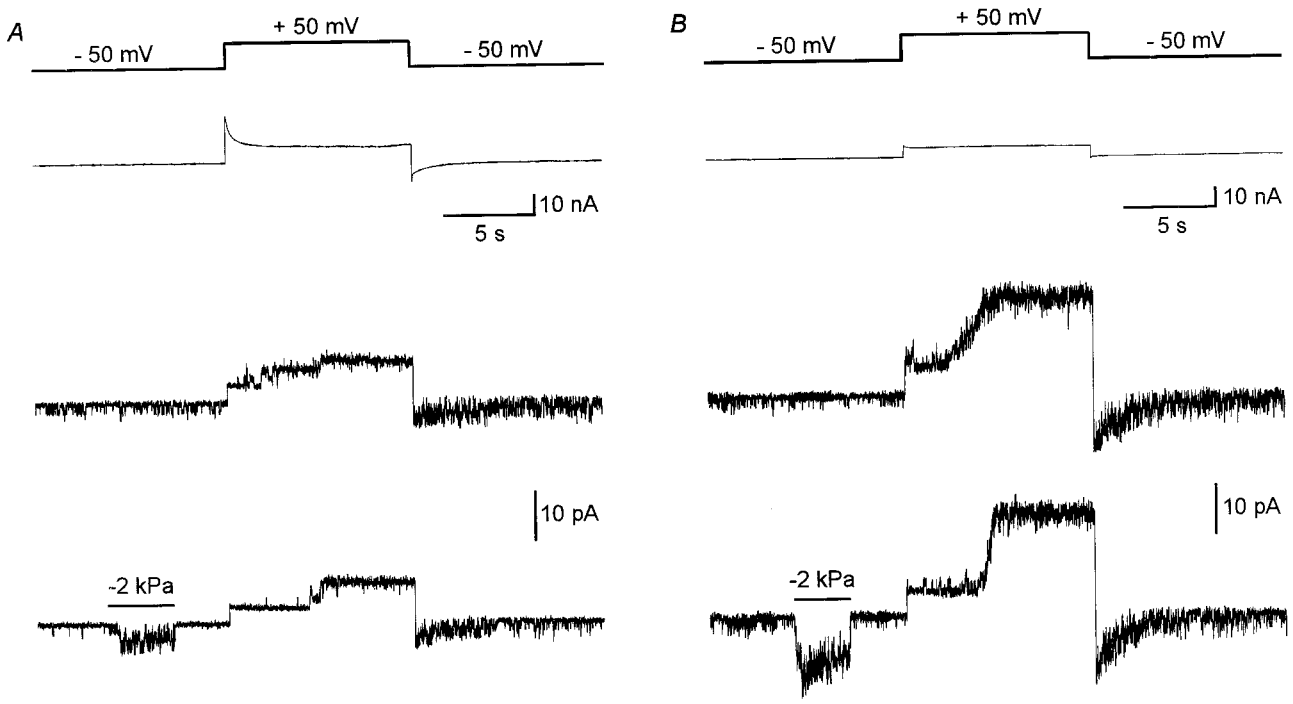

FIGURE 6 Depolarizing voltage steps applied to the whole oocyte activated MS channels in on-cell patches. (*A*) Simultaneous whole-oocyte current records measured with the two-electrode voltage clamp technique (*top current trace*) and on-cell current records measured with the patch clamp technique (*bottom two traces*). (*B*) Same protocol as in *A*, but on a different oocyte. From a holding potential of −50 mV the whole oocytes were depolarized to +50 mV for 10 s with the two-electrode voltage clamp. Depolarizing steps of the whole oocyte with the two-electrode voltage clamp activated MS channels in the on-cell patches without activating currents from MS channels in the unpatched membrane of the whole oocyte. The channels in the on-cell patches could also be activated by pressure (*bottom traces*, indicated by thick lines above the current traces).

activation of MS channels in patches formed with borosilicate glass pipettes, MS channels could not be activated by depolarization in patches formed with soft glass pipettes (Fig. 7 *A*, right trace). The lack of voltage activation with soft glass pipettes was not due to the absence of functional MS channels, as application of negative pressure could activate MS channels in the patch (Fig. 7 *A*, left trace). In 18 of 18 inside-out patches formed with soft glass pipettes that contained MS channels, as verified by pressure activation, depolarization did not activate the MS channels present in the patch. The lack of depolarization-induced activation of MS channels when patches were formed of soft glass was unlikely to arise from the possible effects of divalent or multivalent cations released by the soft glass (Cota and Armstrong, 1988; Furman and Tanaka, 1988; Zuazaga and Steinacker, 1990; Copollo et al., 1991), since the solutions contained EGTA (2–10 mM, and in some experiments also 10 mM BAPTA), which would chelate these cations.

Fig. 1 showed that delayed cooperative activation of MS channels was present in inside-out patches formed with either small-tip or large-tip borosilicate glass pipettes, suggesting that the delayed cooperative activation of MS channels by depolarization does not require a specific pipette geometry. However, the geometry of soft glass pipettes is very different than the geometry of borosilicate glass pipettes (Sakmann and Neher, 1995). Hence, to investigate whether differences in the pipette geometry that occurred when pipettes were fabricated with soft glass led to the loss of delayed voltage activation of MS channels, we coated borosilicate patch pipettes of standard geometry with soft glass. The heat filament of a microforge was first coated with soft glass, then the tip of a borosilicate pipette was placed 20–30 μm from the filament, and the filament was heated for ~0.5 s. Because soft glass has a substantially lower melting temperature than borosilicate glass (Rae and Levis, 1992), evaporated molecules of soft glass formed a fine coat of soft glass over the tip of the borosilicate pipette. At 600× magnification, the tip geometry of borosilicate pipettes was little affected by the coating. However, if the filament was heated for longer than 1 s, then the coating of soft glass could clearly be seen on the tip of the borosilicate glass pipette.

In 19 of 19 experiments in which borosilicate glass pipettes coated with soft glass were used to form inside-out patches, the MS channels in the patch could be activated by pressure (Fig. 7 *C*, left trace), but not by depolarizing voltage steps (Fig. 7 *C*, right trace). In >70% of patches formed with either soft glass pipettes or with borosilicate glass pipettes coated with soft glass, there was no basal activity of the MS channels at −50 mV. Furthermore, coating borosilicate pipettes with soft glass also prevented activity of MS channels at hyperpolarized potentials in excised inside-out patches. For example, MS channels that displayed basal activity at −20 to −60 mV typically re-

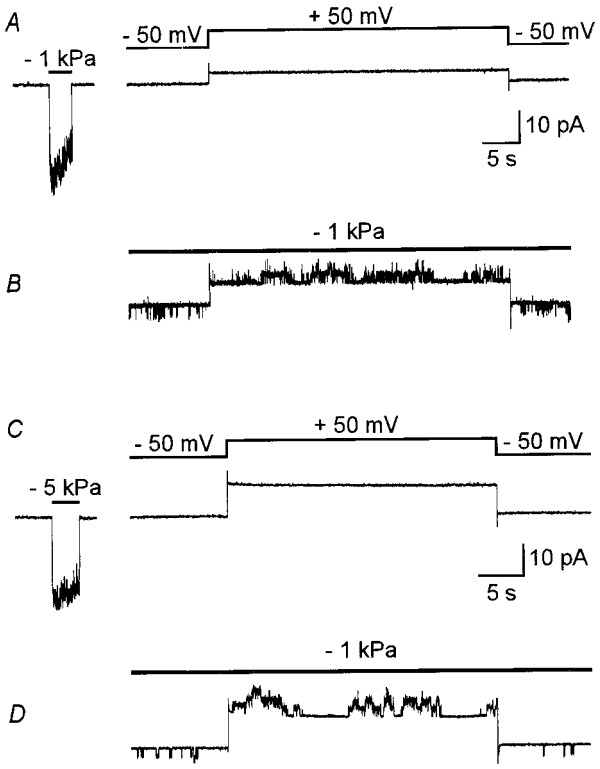

FIGURE 7 Depolarization-induced MS channel activation is not observed with soft-glass pipettes. Current records from an excised inside-out membrane patch formed with a soft-glass patch pipette (*A* and *B*) or with a borosilicate patch pipettes coated with soft glass (*C* and *D*). Stretching the membrane at −50 mV rapidly activated 7–8 MS channels in *A* and 9–10 MS channels in *C*. In the absence of stretch, no MS channel activity was observed at either −50 or +50 mV (*A* and *C, right traces*). (*B* and *D*) With continuous stretch sufficient to induce a low level of basal MS channel activity, voltage steps had little effect on the activity of MS channels. Stretch was applied at the times indicated by thick lines above the current traces.

mained active during prolonged 50 s steps to hyperpolarized potentials of −60 to −90 mV (3 of 3 patches). When the borosilicate pipettes were coated with soft glass, the activity at the holding potentials and during the hyperpolarizing steps was no longer present (3 of 3 patches). Soft glass pipettes may thus provide a means to reduce the basal activity of MS channels in patches of membrane used to study other channels. If basal activity reflects resting tension of the membrane, then the observation of decreased basal activity with soft glass raises the possibility that a delayed activation was not seen with soft glass because the resting tension of the membrane was too low. However, this did not appear to be the case. Increasing the basal level of membrane tension with negative pressure for inside-out patches formed with either soft glass pipettes (Fig. 7 *B*) or with borosilicate glass pipettes coated with soft glass (Fig. 7 *D*), did not lead to delayed cooperative activation or high levels of MS channel activity by depolarizing steps. Results similar to those shown in Fig. 7, *B* and *D*, were observed in 14 of 14 experiments with soft glass pipettes and in 10 of 10 experiments with borosilicate glass pipettes coated with soft glass.

The observation that either replacing or coating borosilicate pipettes with soft glass prevented the delayed cooperative activation of MS channels by depolarization lends further support to the suggestion that interactions between the membrane patch and the glass of the pipette are required for the delayed cooperative activation of MS channels by voltage.

DISCUSSION

In inside-out patches of membrane from *Xenopus* oocytes formed with borosilicate glass pipettes, depolarizing voltage steps lead to a delayed cooperative activation of MS channels in the patch (Silberberg and Magleby, 1997). The delay to activation is unusually slow (typically, several seconds), and deactivation after the depolarizing step also occurs over many seconds. The delayed cooperative activation followed by maintained channel activity is specific for steps to positive rather than to negative potentials (Silberberg and Magleby, 1997). In the present study we found that this delayed cooperative activation of MS channels by depolarization specifically required inside-out and on-cell patches formed with borosilicate glass pipettes. Delayed cooperative activation of MS channels was not seen in outside-out patches, in the intact membrane of whole oocytes, or, surprisingly, when the borosilicate glass pipettes were replaced or coated with soft glass.

Our observations that (*a*) delayed voltage activation of MS channels was seen only for the inside-out and on-cell patch configurations, (*b*) delayed voltage activation was dependent on the composition of the glass in the patch pipette, and (*c*) delayed voltage activation was not seen in membrane outside of the patch pipette collectively suggest that the delayed activation by depolarization requires some form of interaction or contact between the patch of membrane (and/or associated structures) and the patch pipette.

How might membrane-pipette interactions lead to delayed activation of MS channels by voltage? It has been shown that coupling of the lipid bilayer to the adjacent glass of a borosilicate pipette supports tensions on the order of 0.5–4.0 dyn/cm (Opsahl and Webb, 1994; Akinlaja and Sachs, 1998). Perhaps voltage changes the adhesion of the membrane to the borosilicate glass at the site of seal formation or where the patch of membrane contacts the patch pipette, resulting in membrane stretch. Alternatively, voltage-induced flexing of the membrane patch arising from the converse flexoelectric effect (Petrov et al., 1993; Todorov et al., 1994; Mosbacher et al., 1998) might possibly lead to changes in membrane tension, provided that the membrane remains tightly adhered to the pipette. Such voltage-induced changes in patch tension might be expected to be different for inside-out and outside-out patches where the membrane configurations are opposite, for patch pipettes of different glass composition which may have different adhesive prop-

erties with the membrane, and for whole-cell versus patch recordings, where the membrane being recorded from is either outside or inside the patch pipette.

Although it is known that changes in voltage can lead to movement of cell membranes in patch pipettes (Hörber et al., 1995), it is not yet clear whether such voltage-induced movements would produce sufficient mechanical force to activate MS channels. In addition, it is unclear whether such mechanisms could account for the long delay to activation as well as the complex interactions between the inhibition and activation of MS channels induced by both voltage and stretch, as detailed previously (Silberberg and Magleby, 1997).

Nevertheless, support for the notion that voltage acts through changes in membrane tension is the observation that delayed activation of MS channels by depolarization is typically cooperative, with all channels in the patch activating over a brief period of time following a long delay (Silberberg and Magleby, 1997; see Figs. 1 and 4 *A*). Voltage-induced changes in membrane tension would provide a means to synchronize the activity of MS channels, as individual channels in the same patch could all sense the same membrane tension, giving an apparent cooperative activation. If depolarization-induced interactions between the patched membrane and the patch pipette give rise to a change in membrane tension that activates the MS channels, then the delay to MS channel activation upon depolarization may reflect the time required to generate sufficient tension to activate the channels. Depolarization-induced membrane tension might also provide an explanation for the observation that the delay to activation was decreased in the presence of basal membrane tension imposed by negative pressure (Fig. 4). The negative pressure may remove any slack from the membrane patch and add to the tension so that activation would occur more readily. If voltage-induced MS channel activity is a measure of the tension in the patch of membrane, then the slow deactivation of the MS channels upon repolarization may reflect the time course of relaxation of the tension.

The nature of the proposed membrane-pipette interaction that could give rise to the delay in MS channel activation by depolarization is unclear. Voltage might be expected to change the forces between glass and membrane, since alkali ions within the glass have a certain mobility, giving rise to slight electrical conductivity (Scholze, 1990). Ion movements within the glass in response to changes in the electric field could change the surface properties of the glass and hence perhaps also change the adhesive properties of the glass over time. Displacement of a significant amount of charge within glass in response to changes in the electric field can occur with long relaxation times, leading to delayed polarization effects with time courses of many seconds (Figs. 3.21 and 3.22 in Holloway, 1973). Perhaps these slow polarization effects, which can have time courses similar to the delay times for the cooperative activation of MS channels by depolarization, play a role in the delayed activation of the channels by changing the attraction of membrane to pipette over time.

Borosilicate glass and soft glass differ in chemical composition and in electrical properties such as the dielectric constant and the surface charge density (Corey and Stevens, 1983; Rae and Levis, 1992). Possibly these differences give rise to the membrane-pipette interactions that allow the voltage-dependent activation of MS channels in patches formed with borosilicate glass pipettes and not with soft glass pipettes.

The results presented in this study are not sufficient to determine whether the proposed voltage-induced tension activates the MS channels directly through their mechanosensitivity (the simplest hypothesis) or whether the voltage-induced tension acts to facilitate or enable voltage activation of the channels. In either case, the delayed activation of MS channels by depolarization does not appear to have the same type of intrinsic voltage-sensitive gating mechanism as voltage-dependent sodium and potassium channels (see Papazian and Bezanilla, 1997 for a review of such voltage-dependent gating), since voltage activation of the MS channels was observed only in inside-out and on-cell patches formed with borosilicate glass pipettes and was not present in outside-out patches, patches formed with soft glass pipettes, or in membrane outside of patch pipettes.

In the present study, we show that currents attributable to MS channels could not be activated in whole oocytes by voltage alone. MS channels in whole oocytes are also not activated by volume changes, as discussed in Schütt and Sackin (1997), and block of MS channel activity does not prevent the processes of oocyte maturation, fertilization, or tadpole development (Wilkinson et al., 1998). Thus, the physiological role of MS channels in *Xenopus* oocytes remains to be determined.

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