# Stretch activation of a toad smooth muscle $K^+$ channel may be mediated by fatty acids

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1. Using standard single channel patch clamp techniques we studied the stretch sensitivity of a 20 pS $K^+$-selective channel which is activated by fatty acids and found in freshly dissociated smooth muscle cells from the stomach of the toad *Bufo marinus*.

2. A pulse of suction applied to the back of the patch pipette in order to stretch the membrane resulted in activation of this $K^+$ channel. A train of suction pulses resulted in a gradually increased level of channel activity during each successive pulse, as well as an increase in baseline activity between pulses. This pattern contrasts markedly with many other stretch-activated channels whose activation is limited to the duration of the suction pulse.

3. Application of fatty acids augmented the response to stretch. In contrast, application of 10 $\mu$M defatted albumin, which removes fatty acids from membranes, rapidly and reversibly decreased the response to stretch.

4. These results are consistent with the hypothesis that fatty acids which are generated by mechanical stimuli, perhaps by mechanically activated phospholipases, are the intermediaries in activation of certain mechanically sensitive ion channels.

We have shown previously that application of arachidonic acid and other fatty acids to toad (*Bufo marinus*) stomach smooth muscle cells activates $K^+$ channels in a direct fashion. By direct we mean a mechanism that does not involve either known second messenger pathways or the conversion of fatty acids to bioactive metabolites (Ordway, Walsh & Singer, 1989). Direct effects of fatty acids on ion channel activity have since been demonstrated for a variety of channel types in a number of different preparations (Ordway, Singer & Walsh, 1991; Kirber, Ordway, Clapp, Walsh & Singer, 1992; Meves, 1994). Furthermore, a study of the structural features required of effective fatty acids disclosed evidence that they may interact with the channel itself, as opposed to affecting general properties of the lipid bilayer (Petrou, Ordway, Hamilton, Walsh & Singer, 1994). Moreover, in the case of one fatty acid-modulated ion channel, the NMDA receptor (Miller, Sarantis, Traynelis & Attwell, 1992), we have detected a putative fatty acid binding site on the basis of sequence homology with cytosolic fatty acid binding proteins (Petrou, Ordway, Singer & Walsh, 1993). Taken together, these findings raise the possibility that endogenous fatty acids regulate certain ion channels, a possibility made more likely by the fact that 'free' or non-esterified fatty acids are liberated from membrane phospholipid in response to a variety of stimuli (Ordway *et al.* 1991).

In the present manuscript we demonstrate that the same $K^+$ channel from toad gastric smooth muscle cells that is activated by fatty acids is also activated by membrane stretch. Moreover, we provide evidence suggesting that endogenous fatty acids and possibly other negatively charged single chain lipids mediate the stretch response.

Although stretch sensitivity has been demonstrated for numerous channel types (Kirber, Walsh & Singer, 1988; Sachs, 1992), the mechanisms underlying mechanical sensitivity and its regulation remain unclear. The results presented here suggest a possible mechanism of stretch activation for at least one type of mechanically sensitive channel. Furthermore, they support a role for endogenous fatty acids in the regulation of ion channel activity. A brief preliminary account of some of this work has been reported elsewhere in abstract form (Ordway, Petrou, Kirber, Walsh & Singer, 1992).

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## METHODS

Ion channels were studied in smooth muscle cells isolated from the stomach of the toad, *Bufo marinus*, using patch clamp techniques described elsewhere (Ordway *et al.* 1989; Petrou *et al.* 1994). Toads were killed by decapitation. All data were single-pole low-pass filtered at 5 kHz and then digitally recorded onto videotapes using a Sony PCM Digital Audio Processor with a sampling frequency of 44 kHz. Data for figures were filtered at 300 Hz and sampled at 1 kHz. For analysis, data were filtered at 1 kHz and sampled at 3 kHz. The Erwin suite of programs (kindly provided by Dr Michel Vivaudou) was used to calculate $NP_o$ (average number of open channels with $N$, the number of channels in the patch, and $P_o$, the probability of a channel being open) over 15 s time periods, by dividing the average current by the single channel current amplitude.

Membrane stretch was elicited by applying suction (negative pressure) to the back end of the patch pipette. Reproducible pulses of negative pressure were generated using a simple open-loop pressure-generating system, and pipette pressure was monitored by a calibrated pressure sensor (Sensym, Milpitas, CA, USA). Figure 1*A* displays a schematic diagram of the open-loop pressure-generating system used to produce suction pulses. While this simple system does not have the absolute control of a closed-loop actively regulated pressure clamp system (McBride & Hamill, 1992), it does provide stable pressure pulses (to within 2–3 mmHg) at a fraction of the cost and development effort of a closed-loop system. Essentially, an aspirator attached to a faucet was set so as to produce a vacuum. Suction levels were kept relatively constant despite small changes in aspirator flow by employing a large flask as a buffer. With valve V2 open (General Valve Corporation 3-287-900, Fairfield, NJ, USA) and valve

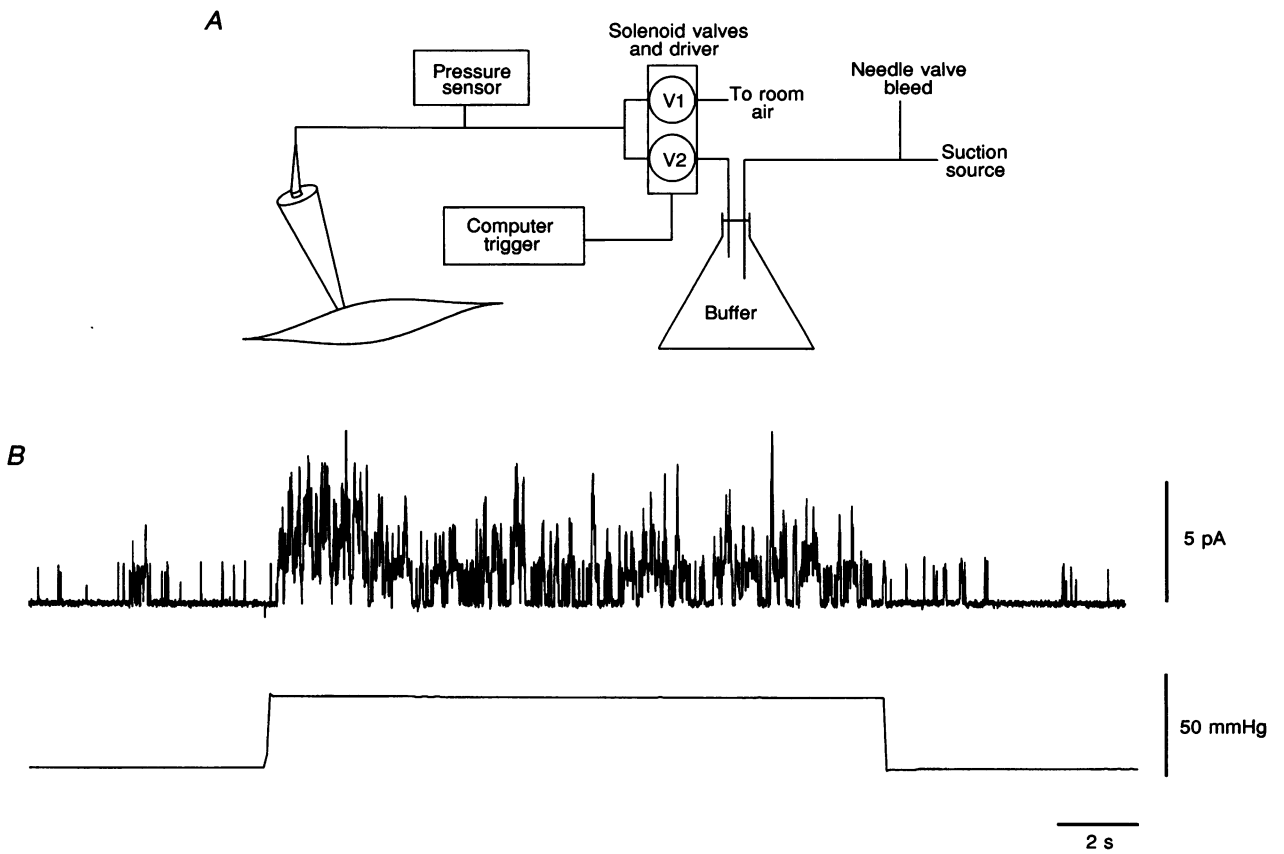


**Figure 1. Typical response of $K^+$ channels to a single pulse of suction produced by a simple open-loop pressure-generating system**

*A*, schematic diagram of the open-loop pressure-generating system used to produce suction pulses (see Methods). *B*, detailed view of the suction pulse and resultant increase in $K^+$ channel activity in a cell-attached patch. Similar results were seen in excised inside-out and outside-out patches. Note the stable pressure setting of the pulse. In this and all other traces indicating pressure gradients across the patch, the onset of the trace denotes zero pressure difference and upward deflections denote suction at the back of the patch pipette. All experiments were carried out with the membrane potential set at ~0 mV which is close to the zero current potential of the stretch-activated non-selective cation channel found in these cells (Kirber *et al.* 1988) which also would have been activated by membrane stretch. Thus, the current records of the $K^+$ channel under study are not contaminated with cation channel currents.

V1 closed, negative pressures can be applied to the interior of the patch pipette. The absolute level of the suction was set by operating the bleed valve and reading the value on the pressure sensor. Once a stable suction level was set, valve V2 was closed and then valve V1 briefly opened to equalize pressure on both sides of the membrane. To jump to a pre-determined suction level valve V1 was closed and valve V2 briefly opened (~500 ms) to 'sample' the pressure in the buffer. To terminate a suction pulse, valve V2 was kept closed and valve V1 was briefly opened to bring the pressure levels back to zero. We found that brief valve openings produced more stable pressure pulses than those obtained by leaving the valve open to the buffer reservoir for the duration of the pulse. Pulse-to-pulse variation in pressure was usually less than 2 or 3 mmHg. Even more stable pulses can be produced by providing a regulated suction source (such as a regulated compressed air source blowing through a venturi) or by increasing the size of the buffer (although we found that a 4 l flask was a good compromise between stable pressure and the response time of the system to changes in the needle valve setting, the latter getting progressively worse as the buffer volume increases). The valve operation sequence can be performed manually, or if more accurately timed pulses were required, the valves could be triggered by a computer or dedicated triggering device (Model D4030 Digitimer Ltd, Welwyn Garden City, UK). Not all experiments were performed using the solenoid valve apparatus; some simply used hand-operated stopcocks instead of the electrical valves. Full schematics of the valve driver circuit can be obtained on request.

Agents were applied either by bath superfusion or by pressure ejection (Picospritizer II, General Valve Corporation) from a micropipette (puffer pipette). Control applications of solutions lacking these agents applied at approximately the same flow were without effect.

Fatty acid-depleted bovine serum albumin (66 kDa) was obtained from Sigma (USA). Myristic acid and oleic acid were obtained from Nu Check Prep (Elysian, MN, USA). Fatty acids were loaded onto albumin using a procedure described in detail elsewhere (Parks, Cistola, Small & Hamilton, 1983). Briefly, potassium soaps were first made by mixing concentrated fatty acids (1000 times final concentration) in a warmed KOH solution at pH 10. After this solution cooled, albumin was added to give a fatty acid to albumin mole ratio of 8:1. This solution was kept at room temperature (~21 °C) and was stirred constantly for at least 1 h. For all other experiments, fatty acids were dissolved in dimethyl sulphoxide (DMSO; Sigma); the same DMSO concentration was also present in all control solutions.

The bathing solution usually contained (mM): 130 $K^+$, 1 $Mg^{2+}$, 132 $Cl^-$, 5 EGTA, 10 Hepes, 10 glucose, at pH 7·2. The pipette solution usually contained (mM): 3 $K^+$, 127 $Na^+$, 1 $Mg^{2+}$, 132 $Cl^-$, 5 EGTA, 10 Hepes, at pH 7·8. For some experiments symmetrical pH conditions were used (pH 7·4 in the bath and pipette). For outside-out patches, the pipette and bathing solutions were reversed, with the exception of glucose which was again present only in the bathing solution. Experiments were carried out in the absence of $Ca^{2+}$, with 5 mM EGTA on both sides of the membrane to eliminate any possible effects of $Ca^{2+}$ and other heavy metals.

## RESULTS

When a single pulse of suction was applied to the back of the patch pipette in order to stretch the membrane of a cell-attached patch, reversible activation of $K^+$ channels was observed (Fig. 1*B*). These appeared to be the same $K^+$ channels previously shown to be activated by fatty acids (Ordway *et al.* 1989) based upon the following shared characteristics: a unitary conductance of approximately 20 pS with 3 mM external $K^+$ and 130 mM internal $K^+$; a lack of appreciable voltage dependence; a mean open time of approximately 20 ms at 0 mV (low-pass filtered with a 1 kHz cut-off frequency); and a reversible 70% reduction in unitary current amplitude at 0 mV by 25 mM external TEA.

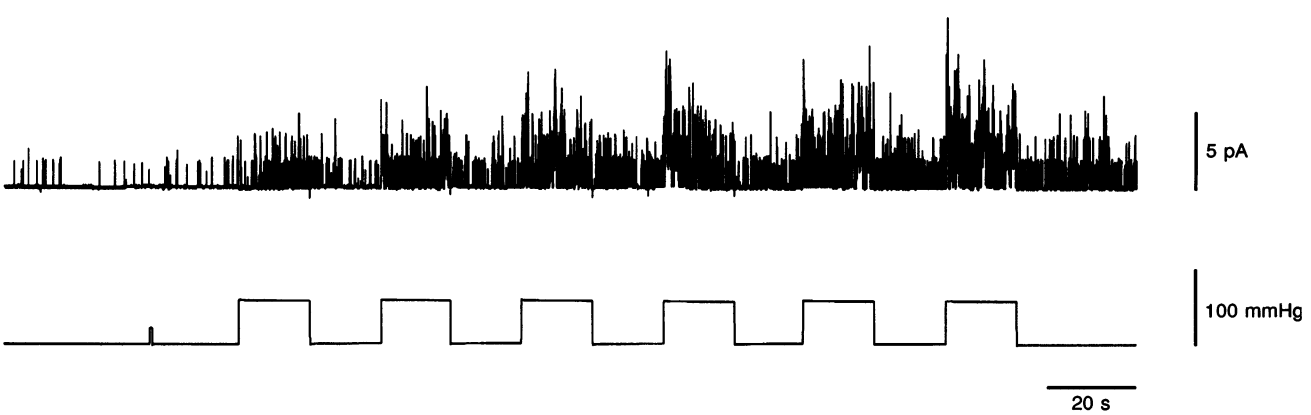


**Figure 2. Stretch activation of $K^+$ channels persists during a train of suction pulses**

The upper trace is a recording of single channel currents showing stretch activation of fatty acid-activated $K^+$ channels in a cell-attached patch. The lower trace shows a train of suction pulses applied to the patch pipette to stretch the membrane.

When a train of suction pulses was applied to a cell-attached patch, there was an increased level of channel activity during each successive pulse, as well as an increase in baseline activity between pulses (Fig. 2). This pattern of activation by stretch stands in marked contrast to that observed for other ion channels. For example, in the same toad stomach smooth muscle cells used in this study, the activation of stretch-sensitive cationic channels is limited to the duration of the suction pulse (Kirber *et al.* 1988). The continued activation of the $K^+$ channel well after termination of suction as seen in Fig. 2 suggested the persistence of a messenger molecule generated by stretch.

The relationship between the channel activation by membrane stretch and that by exogenous fatty acids was examined. When a train of relatively weaker suction pulses was employed to elicit mild activation of the $K^+$ channels in a cell-attached patch (Fig. 3*A*), myristic acid (20 $\mu$M), applied to the cell during the train, reversibly increased both the activity of the $K^+$ channels during suction pulses (Fig. 3*B*) and the level of channel activity between suction pulses (Fig. 3*B*). Similar results were obtained in ten out of twelve patches with myristic acid and in three out of three patches when oleic acid was applied. Thus, stretch activation is augmented by exogenous (applied) fatty acids. Interestingly, the effect seen in Fig. 2 using only a train of relatively strong suction pulses appears to be mimicked by the combination of weaker suction pulses and exogenous fatty acid (Fig. 3).

The observation that exogenous fatty acids could mimic the response to membrane stretch suggested that endogenous fatty acids might play a role in mediating the mechanical sensitivity of the channel. We investigated this possibility using fatty acid-depleted albumin, which is known to bind fatty acids and remove them from membranes (Hamilton, 1989; Kamp & Hamilton, 1992). A

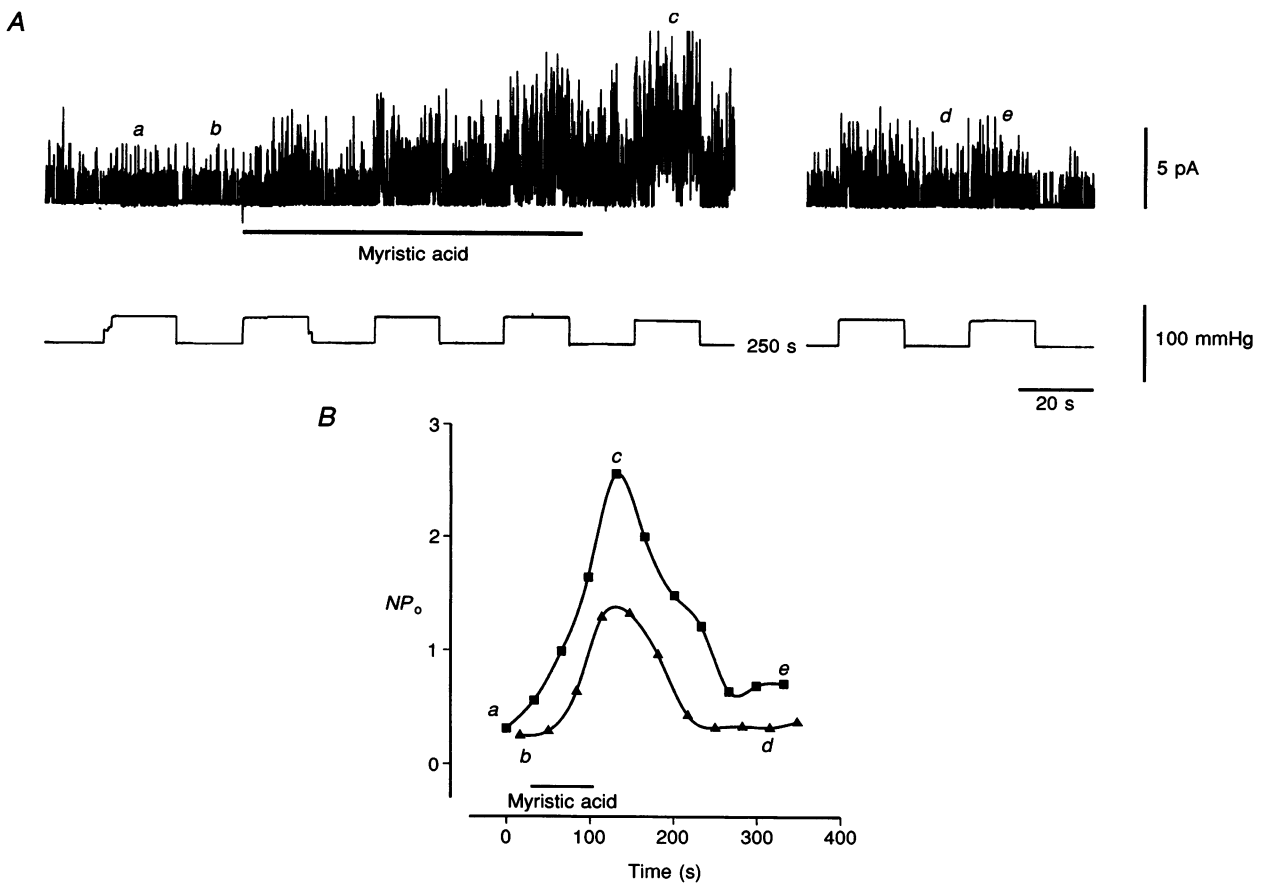


**Figure 3. Exogenous myristic acid increases stretch activation of the $K^+$ channels**

*A*, the effect of myristic acid (20 $\mu$M) on stretch activation of $K^+$ channels in a cell-attached patch using a train of relatively weak suction pulses. Bar under trace indicates the period of application. Note time break in record. The traces are as described in Fig. 2. *B*, the time course of $NP_o$ (the average number of open channels, a measure of channel activity) during the suction pulses (■) and intervening periods without suction (▲) for the experiment shown in *A*. Note that the increase in $NP_o$ elicited by stretch is markedly enhanced by myristic acid. For several $NP_o$ data points shown labelled (*a*–*e*) in *B*, the regions on the current record (*A*) where the measurements were made are labelled correspondingly.

typical experiment is shown in Fig. 4. First, a train of suction pulses was used to elicit a high level of $K^+$ channel activity in a cell-attached patch (Fig. 4*A*). Then, during continued stimulation, albumin (10 $\mu$M) was applied to the cell surface outside the patch pipette and therefore isolated from the cell-attached patch. Albumin dramatically and reversibly decreased both the stretch activation of the channel and the persistent activity of the channel between suction pulses. Similar results were obtained in twenty-nine of thirty-two patches. The absolute decrease in $NP_0$ was greater during the suction pulses than between them (Fig. 4*B*).

The effects of membrane stretch and albumin were also examined in excised, inside-out and outside-out patches where cytosolic factors that might participate in these responses would be absent. Both the stretch activation and the inhibition by albumin were present in both the inside-out (13 out of 13 patches) and outside-out (3 out of 3 patches) configurations. Figure 4*C* illustrates a response from an outside-out patch. In each case the albumin was applied to the surface of the patch facing the bathing solution.

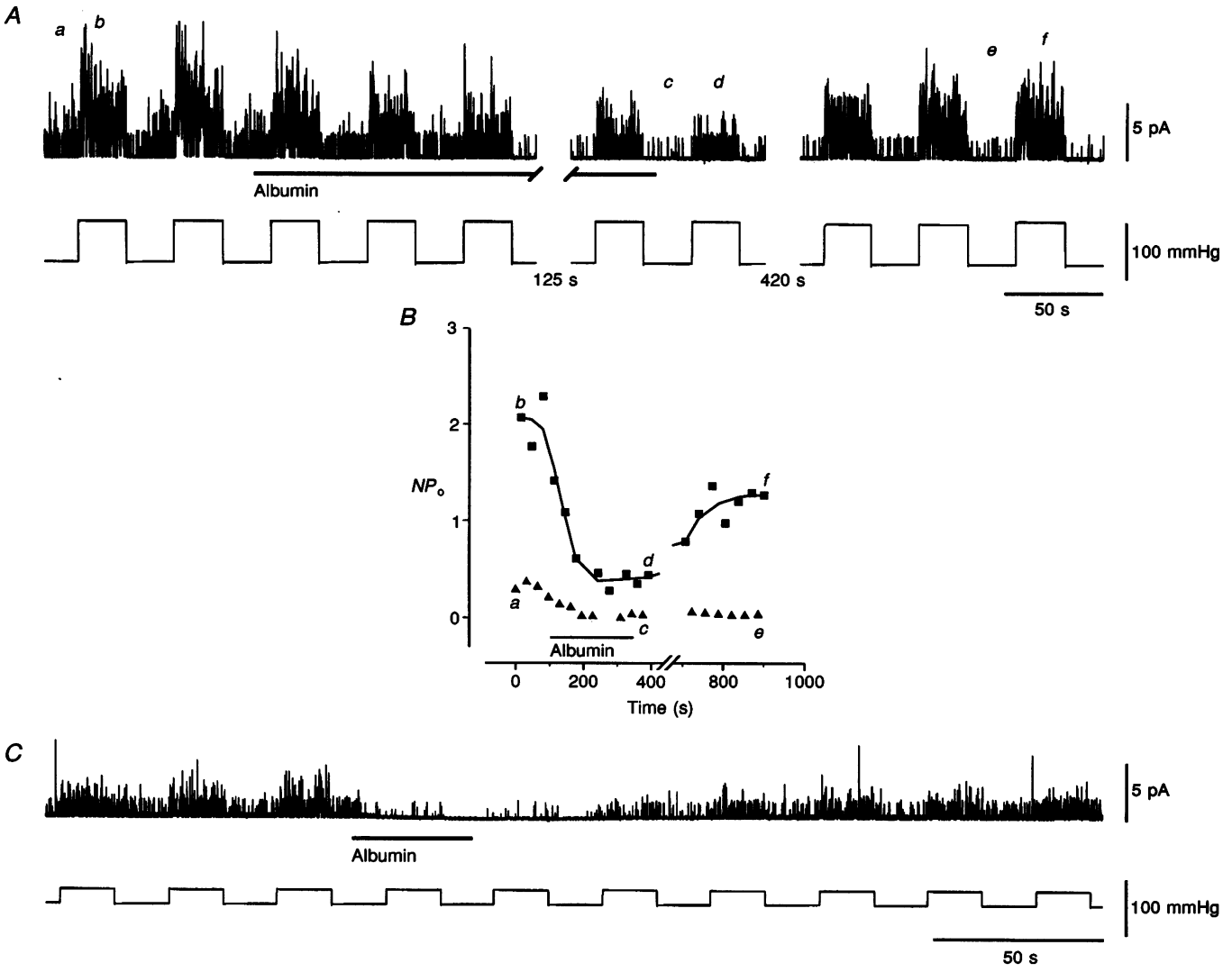


**Figure 4. Albumin decreases stretch-activation of the $K^+$ channels**

*A*, the effect of albumin (10 $\mu$M) on stretch activation of $K^+$ channels in a cell-attached patch during a train of suction pulses. Note time breaks in records. *B*, the time course of $NP_0$ during the suction pulses (■) and during the intervening periods without suction (▲) for the experiment shown in *A*. Note that the increase in $NP_0$ elicited by stretch is markedly suppressed by albumin. *C*, albumin (10 $\mu$M) decreases stretch activation of the $K^+$ channels in an excised outside-out membrane patch. Bar under trace indicates the period of application. The traces are as described for Fig. 2. For several $NP_0$ data points shown labelled (*a–f*) in *B*, the regions on the current record (*A*) where the measurements were made are labelled correspondingly. Similar results were seen for excised inside-out patches.

An effect of albumin itself on the $K^+$ channel activity is unlikely for the following reasons. (1) In the experiments using cell-attached patches, albumin was applied to the cell surface outside the patch pipette. Hence albumin should not have been able to reach the $K^+$ channels. (2) Albumin complexed with myristic acid (at a ratio of 1 : 8) *activated*, rather than inhibited, the $K^+$ channels (4 out of 4 patches). (3) Albumin did not exert its actions through calcium binding since it was effective in the presence of 5 mM EGTA. (4) Soybean trypsin inhibitor (10 $\mu$M) applied as a control for any possible non-specific effects of proteins had no effect on channel activity (3 out of 3 patches).

## DISCUSSION

In previous work (Petrou *et al.* 1994), we determined some of the structural features required of fatty acids in order for them to act on $K^+$ channels in toad stomach smooth muscle cells. We found that both a negatively charged head group and a sufficiently long acyl chain were required for channel activation. Thus, not only fatty acids but also palmitoyl lysophosphatidate and acyl coenzyme As (CoAs) could activate the channels. The first two could activate channels whether applied to the membrane surrounding cell-attached patches, the cytosolic surface of excised inside-out patches, or the extracellular surface of excised outside-out patches. However, acyl CoAs could activate channels only when applied to the cytosolic surface of inside-out patches. We used these results to localize the site where negatively charged lipids might bind so as to activate the channels. Acyl CoAs do not 'flip' across the bilayer and so remain on the side of the bilayer to which they are applied (Boylan & Hamilton, 1992), while the other single chain lipids employed in those studies may readily flip across the bilayer (Kamp & Hamilton, 1992). Thus, if all these lipids are acting at the same site, it would appear that they act on the cytosolic surface of the membrane in these smooth muscle cells.

In the present study we have presented evidence that endogenous fatty acids serve as mediators of activation of a $K^+$ channel by membrane stretch. Both stretch and exogenously applied fatty acids activate the channel, and albumin, a large soluble protein with a high affinity for fatty acids, suppresses stretch activation, presumably by acting as a sink for fatty acids. The mediating fatty acids might be generated in response to stretch by a mechanically sensitive phospholipase such as the stretch-activated phospholipase A enzymes which act on membrane phospholipid (Thuren, Virtanen & Kinnunen, 1987; Sadoshima & Izumo, 1993).

In *excised patches*, albumin inhibited stretch activation of $K^+$ channels when applied either to the cytosolic or the extracellular surfaces of the patch even though the site of fatty acid action on the $K^+$ channel appears to lie exclusively on the cytosolic surface of the membrane (Petrou *et al.* 1994). This is not at all paradoxical since fatty acids are known to 'flip' between leaflets of lipid bilayers (Kamp & Hamilton, 1992). Similarly, in *cell-attached patches*, albumin suppressed stretch-activated $K^+$ channel activity when applied to the surface of the cell lying outside the patch pipette. This is consistent with the ability of fatty acids to pass across and diffuse laterally through the membrane and thus be taken up by the remote albumin 'sink'.

Fatty acids meet all the criteria demanded of a messenger molecule by our observations: (1) they activate the $K^+$ channels; (2) they traverse the plasma membrane; (3) they diffuse laterally in lipid bilayers; (4) they bind to albumin; and (5) they can be generated by membrane stretch. More decisive evidence for a mediating role for fatty acids will require direct measurement of their liberation in response to mechanical stimuli. It is also possible that other lipophilic molecules which meet these criteria might mediate the stretch activation. For example, palmitoyl lysophosphatidic acid increases the activity of the channel (Petrou *et al.* 1994) and may also be produced in the membrane by the action of a mechanically sensitive phospholipase.

There have been four other reports of ion channels (all $K^+$ channels) that are both mechanically and fatty acid activated: a large-conductance calcium-activated $K^+$ channel we described in rabbit pulmonary artery (Kirber, *et al.* 1992) and mesenteric artery (Dopico, Kirber, Singer & Walsh, 1994) smooth muscle cells; the S-channel found in *Aplysia* (Vandorpe, Small, Dabrowski & Morris, 1994); and a $K^+$ channel in rat atrial cells (Kim, 1992). However, albumin had no effect on stretch activation of the atrial $K^+$ channels suggesting that in these cells, membrane stretch and fatty acids are activating the channel by different pathways (Kim, 1992). There is no reported test for an effect of albumin on the *Aplysia* and rabbit channels.

It is also possible that fatty acids *modulate*, not mediate, the stretch-induced activity of the $K^+$ channel. As modulators, fatty acids would enhance the direct effects of membrane stretch on the channel itself. As mediators, fatty acids, liberated from membrane phospholipid in response to membrane stretch, would act as second messengers to activate the $K^+$ channels.

The apparent mediation of membrane stretch effects by endogenous fatty acids suggests that mechanical sensitivity of cells may be regulated by increasing or decreasing fatty acid levels. In this way, a smooth muscle cell might control the amount of hyperpolarizing $K^+$ current elicited by stretch. Stretch-induced contraction of the cells examined in this study is thought to be mediated by activation of cation-selective stretch channels (Kirber

*et al.* 1988). These cation channels are calcium permeant and thus probably carry some calcium into the cell as well as causing depolarization and activation of voltage-gated calcium channels. Since robust activation of the $K^+$ channels in response to stretch occurs with a delay, they might act to generate a repolarizing current causing inhibition of contraction during prolonged stretch. Alternatively, if calcium passing through the cation channels is a major source of calcium for contraction, then $K^+$ channel activation would not be inhibitory but might even enhance contraction by hyperpolarizing the membrane and thereby maintaining a large electrochemical driving force for calcium entry. Our findings strongly suggest a role for endogenous fatty acids as novel physiological second messengers which are generated in response to mechanical stimulation and act to increase $K^+$ channel activity.


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