RNA PCR Kit; Perkin-Elmer Cetus) with the following CFTR-specific primers: 5′-GCTGGATCCACTGGAGCAGGCAAG-3′ (exon 9) and 5′-AATTCTTGCTCGTTGACCTCCACT-3′ (exon 11). The size of the PCR product was identical to that predicted (300 bp). The PCR products were transferred to nylon membrane and hybridized with radiolabeled oligonucleotide probes, as described [E. J. Sorscher and Z. Huang, *Lancet* **337**, 1115 (1991)]. We could detect wild-type, but not mutant, mRNA in CFPAC-PLJ-CFTR cells after one round of PCR amplification, which implied that small amounts of the endogenous mutant gene were expressed, as previously reported (*3*).

22. Cell monolayers incorporated HRP by endocytosis as described (*12*). A membrane fraction enriched in endosomes was prepared by sucrose density gradient centrifugation of cell homogenates. We determined HRP activity specifically located within endosomes by monitoring latent peroxidase activity—that is, the increase in measured peroxidase activity that resulted from the release of HRP from endosomes after treatment with detergent. For fluorescent studies, cells were exposed to rhodamine-dextran (formula weight: 10,000), and the average fluorescent intensity of labeled vesicles (200 to 400 vesicles per image) was determined in five randomly selected fields with fluorescent microscopy as described (*12*). In contrast to the HRP enzyme assay, the fluorescent assay sampled the appearance of marker in relatively large, optically detectable compartments. This technical difference likely accounts for the differing degrees of endocytosis inhibited by forskolin observed with the two assays.
23. Cells grown on glass cover slips were exposed (2 hours at 37°C) to bWGA (1 μg/ml). After internalization of this marker, any remaining surface marker was saturated with unlabeled avidin (200 μg/ml for 15 min). Cells were then washed in avidin-free buffer and incubated under the appropriate conditions at 37°C for 30 min, during which they recycled the previously internalized bWGA back to the cell surface where it was detected by exposure of the cells to Texas Red–avidin (2.5 μg/ml for 30 min at 4°C). Fluorescent microscopic images were collected with a Zeiss IM-35 inverted microscope, ×40 objective lens, and cooled charge-coupled device video camera (Photometrics).
24. In each experiment, six images per cover slip were selected at random (one to two islands of cells or 40 to 200 cells per image). The focal plane was selected so that the apical cell surfaces (identified by the appearance of microvilli) were in focus. For each island, the fluorescence on the cell surface was quantified with interactive software that permitted tracing the edge of the island and calculating the mean fluorescence intensity within the traced island. Background fluorescence (mean fluorescent intensity of a measurement window located off the island) was subtracted to give a single value for each cover slip, which represented the mean intensity of several hundred cells within six randomly selected fields.
25. Of the two CFPAC-PLJ-CFTR clones that were assayed for membrane recycling as well as for endocytosis, clone 10 exhibited a 3.18 ± 1.66–fold increase in marker recycling over untreated controls in response to cAMP treatment (n = 2; compare to Fig. 2A). Clone 20 exhibited a 3.14 ± 0.84–fold increase after forskolin treatment (n = 5). None of the mock-transfected clones exhibited any cAMP responsiveness with any of the three assays of endocytosis or exocytosis.
26. We thank J. Wilson, F. Collins, and R. A. Frizzell for retrovirally transfected cells, and T. Elton for help with RNA PCR. Supported by the NIH, the Cystic Fibrosis Foundation, and the Lucille P. Markey Charitable Trust. N.A.B. and T.J. are Research Fellows of the Cystic Fibrosis Foundation, K.L.K. is an Established Investigator of the American Heart Association, and E.J.S. is a Lucille P. Markey Scholar.

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Regulation of Arterial Tone by Activation of Calcium-Dependent Potassium Channels

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Blood pressure and tissue perfusion are controlled in part by the level of intrinsic (myogenic) vascular tone. However, many of the molecular determinants of this response are unknown. Evidence is now presented that the degree of myogenic tone is regulated in part by the activation of large-conductance calcium-activated potassium channels in arterial smooth muscle. Tetraethylammonium ion (TEA^+) and charybdotoxin (CTX), at concentrations that block calcium-activated potassium channels in smooth muscle cells isolated from cerebral arteries, depolarized and constricted pressurized cerebral arteries with myogenic tone. Both TEA^+ and CTX had little effect on arteries when intracellular calcium was reduced by lowering intravascular pressure or by blocking calcium channels. Elevation of intravascular pressure through membrane depolarization and an increase in intracellular calcium may activate calcium-activated potassium channels. Thus, these channels may serve as a negative feedback pathway to control the degree of membrane depolarization and vasoconstriction.

Smooth muscle cells in small arteries depolarize and contract in response to increased intravascular pressure. The resulting vasoconstriction is independent of regional innervation and circulating hormones and has been termed myogenic (*1*). Myogenic tone is abolished by the removal of external Ca^{2+}, by Ca^{2+} channel blockers (*2*), and by membrane hyperpolarization (*3*), which suggests that myogenic tone depends on Ca^{2+} influx through voltage-dependent Ca^{2+} channels (*4*). The ability of arteries to grade myogenic tone and to sustain it at steady levels suggests the ability to grade depolarization and Ca^{2+} entry. One way this could be achieved is if Ca^{2+} entry and depolarization are opposed by hyperpolarizing mechanisms linked to Ca^{2+} accumulation in cells. Thus, a negative feedback (vasodilating) pathway might exist that is activated by pressure, intracellular free Ca^{2+}, or membrane depolarization. Interruption of this pathway could lead to poorly controlled and perhaps highly constricted arteries. The large-conductance Ca^{2+}-activated K^+ (K_{Ca}) channel is an ideal candidate for such a pathway because it is activated by both membrane depolarization and intracellular Ca^{2+} (*5*). However, although K_{Ca} channels exist in a variety of smooth muscle types, there is little evidence for any function of these channels in

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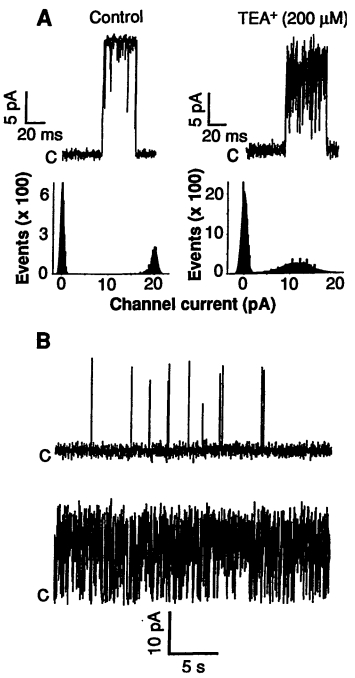

Fig. 1. Blockage of Ca^{2+}-activated (K_{Ca}) channels by external TEA^+. (**A**) Recordings and amplitude histograms from two inside-out patches from rabbit cerebral arteries held at +50 mV. According to the usual convention, membrane potentials are expressed inside relative to outside, and outward currents are given a positive sign. Pipette solutions contained no TEA^+ (left) and 200 μM TEA^+ (right). Gaussian curves were fitted to the peaks of the control amplitude histogram to yield a unitary current of 20.0 pA. The TEA^+ reduced the mean unitary current and broadened the open level peak. The mean amplitudes of unitary current before and after treatment with TEA^+ (200 μM) were 5.85 and 2.85 pA (n = 4) at 0 mV and 16.5 and 9.3 pA (n = 6) at +50 mV. (**B**) Ca^{2+}-activation of K_{Ca} channels. An inside-out patch was held at +50 mV in the presence of external TEA^+ (200 μM). Intracellular Ca^{2+} concentration was raised from approximately 1 nM (top) to 12.5 μM (bottom). The bath contained 136 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, and 15 mM Hepes (pH 7.2). The pipette solution contained 137 mM NaCl, 5.4 mM KCl, 0.55 mM KH_2PO_4, 2 mM $MgCl_2$, 4.17 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 0.05 mM EGTA, and 10 mM Hepes (pH 7.4). The closed level of the channel is indicated by C; filter, 2.5 kHz; sample rate, 10 kHz.

arterial smooth muscle (*4*, *6*). We now present evidence that K_{Ca} channels in small cerebral arteries participate in regulating the degree of myogenic tone.

Although K_{Ca} channels are present in vascular smooth muscle (*7*), the existence of K_{Ca} channels in smooth muscle cells isolated from cerebral arteries has not been documented. We recorded unitary currents through single K_{Ca} channels in membrane patches from smooth muscle cells isolated from rabbit cerebral arteries (*8*) (Figs. 1 and 2). Channel activity in inside-out excised patches was increased by depolarization (*9*) and by an increase in the concentration of intracellular Ca^{2+} (Fig. 1B). The mean unitary current at 0 mV with a physiological K^+ gradient was 5.85 pA (n = 4), which is comparable to the value (5.38 pA) measured at the same potential in systemic arteries (*10*). The slope conductance was 213 pS between 0 and +50 mV; in systemic arteries, slope conductance is 186 pS (*10*). In cerebral arteries, external TEA^+ decreased the mean single-channel current and increased the open-channel current noise of single K_{Ca} channels. This type of "flickery block" is characteristic of a blocking process with relatively rapid kinetics, so that blocking and unblocking events are not fully resolved at the recording frequency used (2.5 kHz). The concentration of TEA^+ required to decrease the current by 50% (apparent K_d) was 190 μM at 0 mV and 251 μM at +50 mV (Fig. 1) (*10*). Blocking and unblocking rate constants for TEA^+ at +50 mV were estimated to be 607 mM^{-1} ms^{-1} and 122 ms^{-1}, respectively, from the excess current variance that can be attributed to blockage of the channel (*10*, *11*). By comparison, external TEA^+ also induces a flickery block of K_{Ca} channels in systemic arteries with a K_d of 196 μM at 0 mV and 210 μM at +50 mV (*10*) and with rate constants that are similar to those estimated for cerebral arteries.

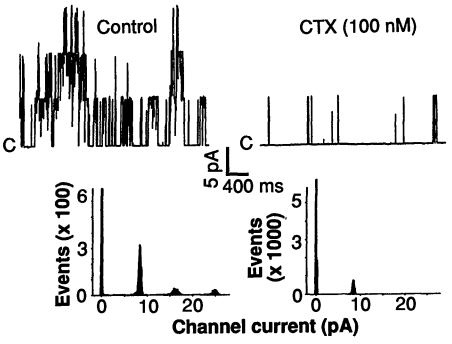

Fig. 2. Blockage of single K_{Ca} channels by charybdotoxin in outside-out membrane patches from rabbit cerebral arteries held at +20 mV. The bath solution contained 140 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM Hepes (pH 7.4, adjusted with KOH). The pipette contained 120 mM KCl, 10 mM EGTA, 8.8 mM $CaCl_2$ (0.5 μM free Ca^{2+}), 2 mM $MgCl_2$, 1 mM guanosine 5′-triphosphate, and 10 mM Hepes (pH 7.3, adjusted with KOH). Control is on the left; 100 nM CTX is on the right. Amplitude histograms for longer recordings are shown below the records. Filter, 0.5 kHz; sample rate, 3 kHz.

Charybdotoxin (CTX), a peptide produced by the scorpion *Leirus quinquestriatus*, is a relatively selective and potent blocker of K_{Ca} channels in smooth muscle and in other preparations, with a K_d between 1 and 20 nM (*12*). We found that CTX is an effective blocker of single K_{Ca} channels in outside-out patches from smooth muscle cells of cerebral arteries. At a concentration of 100 nM, CTX reduced the fractional open time (NP_{open}) by 86% (Fig. 2) (mean reduction 80.3%; n = 3), which, with a single-site interaction, corresponds to a K_d of 16.3 nM. We conclude, therefore, that sensitivity to TEA^+ and CTX is conserved in K_{Ca} channels and that external TEA^+ and CTX are useful probes for exploring the function of K_{Ca} channels in cerebral arteries. Furthermore, the K_{Ca} channel is the only known K^+ channel in smooth muscle that is blocked either by low concentrations of TEA^+ or by CTX (*4*).

Elevation of transmural pressure depolarizes and constricts isolated cerebral arteries (*3*, *13*). Ten to 15 min after the pressure was raised from 10 mmHg (resting) to 75 mmHg, the internal diameter of these arteries decreased to about 70% of the fully relaxed diameter, which was determined in the presence of 0.1 μM nimodipine or by the removal of external Ca^{2+} (*14*). After myogenic tone developed, addition of K_{Ca} channel blockers induced vasoconstriction. At concentrations of 0.05 to 1.00 mM, TEA^+ decreased the diameter of arteries by as much as 30% (Fig. 3A). At concentrations below 1 mM, TEA^+ preferentially

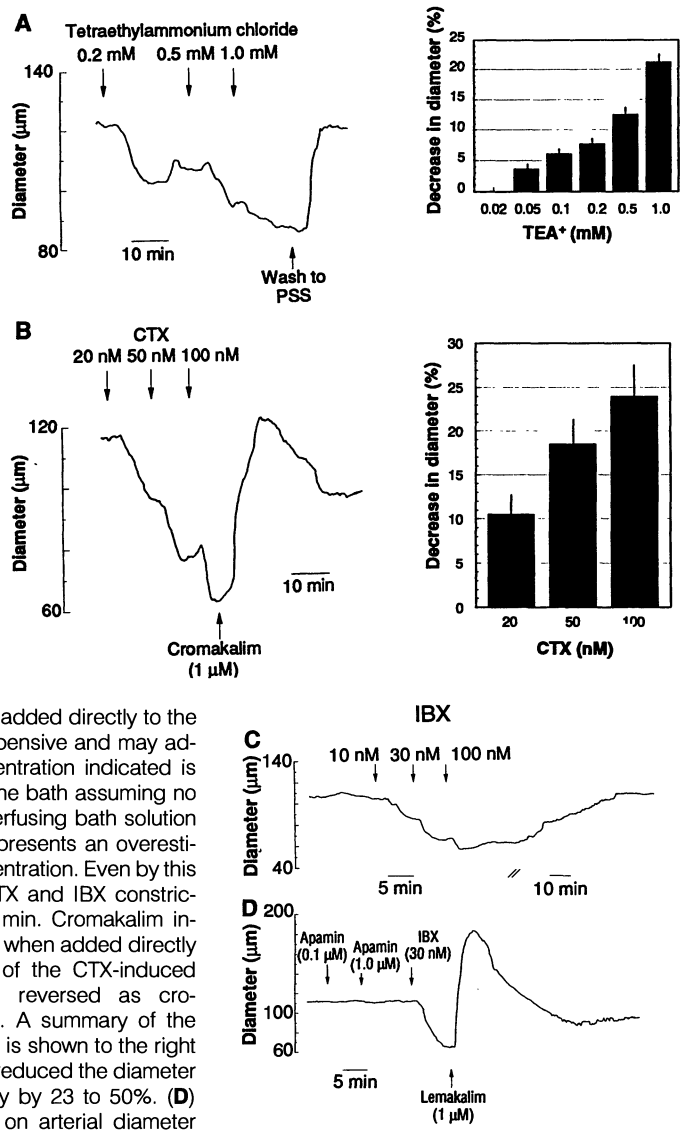

Fig. 3. Constriction of cerebral arteries in response to TEA^+, CTX, and IBX but not apamin. Arteries were pressurized to 75 mmHg. After myogenic tone developed, the arteries were exposed to K^+ channel blockers. (**A**) Various concentrations of TEA^+ were included in the physiological salt solution (PSS) [composition (in mM): NaCl, 118.5; KCl, 4.7; $NaHCO_3$, 24.0; KH_2PO_4, 1.2; EDTA, 0.023; $CaCl_2$, 1.6; $MgSO_4$, 1.2; and glucose, 11.0] that superfused the artery at 3 ml/min (bath volume = 6 ml). A summary of the effects of TEA^+ on diameter is shown to the right of the trace. Data are mean ± SEM (n = 12 to 20). Concentrated CTX (**B**) and IBX (**C**) were added directly to the bath because they are expensive and may adhere to tubing. The concentration indicated is the final concentration in the bath assuming no dilution by the slowly superfusing bath solution and therefore probably represents an overestimate of the true bath concentration. Even by this route of administration, CTX and IBX constrictions lasted from 5 to 40 min. Cromakalim induced a large vasodilation when added directly to the bath at the peak of the CTX-induced constriction. The dilation reversed as cromakalim was washed out. A summary of the effects of CTX on diameter is shown to the right (n = 12 to 15). Iberiotoxin reduced the diameter of a middle cerebral artery by 23 to 50%. (**D**) Lack of effect of apamin on arterial diameter followed by a substantial IBX-induced vasoconstriction. In the presence of IBX, lemakalim caused a large dilation which then reversed as lemakalim washed out.

blocks K_{Ca} channels, although at much higher concentrations (>5 mM), it blocks other types of K^+ channels in smooth muscle (*4*, *6*). Therefore, we tested two additional toxins, CTX (*12*) and iberiotoxin (IBX) (*15*), which are more selective than TEA^+ as blockers of K_{Ca} channels. At concentrations (10 to 100 nM) that block K_{Ca} channels (Fig. 2) (*12*), CTX caused dose-dependent vasoconstriction (Fig. 3B). In some cases, CTX decreased arterial diameter by more than 40% (Fig. 3). At a concentration of 100 nM, CTX had a maximal effect on diameter; higher concentrations (300 nM) had no additional effect (n = 3). Iberiotoxin, a peptide purified from the venom of the scorpion *Buthus tamulus*, is a highly selective blocker of K_{Ca} channels and blocks them by binding to a site distinct from the CTX binding site (*15*). Iberiotoxin also constricted pressurized arteries (Fig. 3C), which further supports a specific role for K_{Ca} channels in regulating myogenic tone. At a concentration of 100 nM, IBX decreased arterial diameter by 41 ± 9% (n = 6).

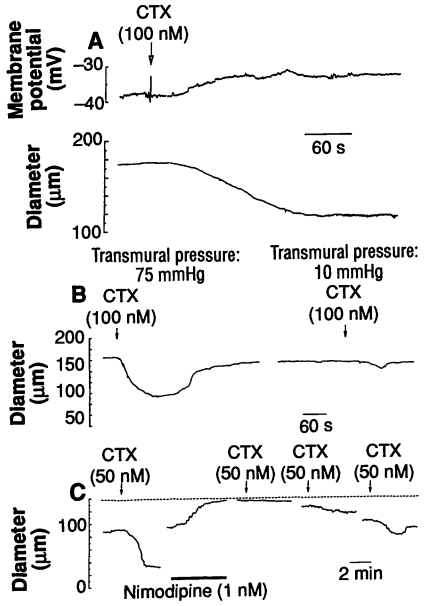

Fig. 4. Effect of CTX on membrane potential and arterial diameter. (**A**) Charybdotoxin was added to the myograph bath at the time indicated by the arrow. The artery was pressurized to 75 mmHg. (**B**) Charybdotoxin was first applied in the presence (pressure = 75 mmHg) and then in the absence (pressure = 10 mmHg) of myogenic tone in the same artery. The gap in the trace represents 140 min. By coincidence, the initial diameters are nearly the same in the two cases. The diameter is small at low pressure as a result of a diminished passive (pressure-induced) force on the artery. (**C**) Effects of nimodipine on diameter changes induced by CTX in an artery pressurized to 75 mmHg. Gaps in the trace represent 60, 20, 60, and 10 min, respectively, from left to right. Nimodipine was applied in the superfusate and was present for the time indicated by the bar. The dashed line indicates the fully relaxed diameter in the absence of Ca^{2+} in the bathing medium.

Small-conductance K_{Ca} channels (*16*) might be activated by the Ca^{2+} influx associated with myogenic tone. However, apamin, a specific inhibitor of small conductance K_{Ca} channels, had no effect on arterial diameter at concentrations as high as 1 μM (n = 4) in arteries that constricted in response to IBX (Fig. 3D). Glibenclamide (3 μM), a selective inhibitor of adenosine triphosphate (ATP)–sensitive K^+ (K_{ATP}) channels, also had no effect on myogenic tone (n = 4). Thus, K_{ATP} channels do not appear to be activated in association with myogenic tone in cerebral arteries (*17*). Cromakalim and lemakalim (a more active isomer of cromakalim) activate K_{ATP} channels (*4*, *6*, *18*) and, as would be predicted, dilated myogenic cerebral arteries in the presence of inhibitors of K_{Ca} channels (Fig. 3, B and D).

Myogenic tone can also be altered by factors released from the endothelium (*19*). K_{Ca} channel blockers could constrict arteries by modulating release of endothelial factors, such as endothelium-derived relaxing factor or endothelium-derived hyperpolarizing factor. We found, however, that removal of the endothelium (*20*) affected neither the degree of pressure-induced tone nor the vasoconstrictor response to IBX. [Iberiotoxin (100 nM) decreased vascular diameter by 45 ± 8% in the absence of the endothelium (n = 3).]

If K_{Ca} channels regulate myogenic tone, then K_{Ca} channel blockers should depolarize the smooth muscle cells within the blood vessel wall in parallel with their constrictive effect. The membrane potential of vascular smooth muscle cells in pressurized cerebral arteries was in the range of −35 to −40 mV (*3*, *13*). Charybdotoxin induced a depolarization of as much as 12 mV (mean = 7 ± 1 mV; n = 11), and this change in membrane potential was associated with a large vasoconstriction (Fig. 4A) (*21*). Similarly, TEA^+ (1 mM) depolarized the vascular smooth muscle cells of pressurized arteries by 4 ± 1 mV (n = 5).

If the membrane depolarization and the subsequent elevation of intracellular Ca^{2+} associated with myogenic tone activates K_{Ca} channels, then a decrease in Ca^{2+} concentration or hyperpolarization of the membrane should diminish the contribution of K_{Ca} channels to the arterial smooth muscle membrane potential, and, likewise, the effects of K_{Ca} channel blockers on tone should be reduced. We therefore studied the arterial response to K_{Ca} channel blockers under two conditions where Ca^{2+} influx is likely to be diminished. At low transmural pressure (10 mmHg), myogenic tone and, presumably, concentrations of intracellular Ca^{2+} are low, and the membrane potential is more polarized (−60 mV). Under these conditions, CTX (100 nM) (Fig. 4B) and TEA^+ (1 mM) decreased diameter by only 3 ± 2% (n = 6) and 2 ± 1% (n = 5), respectively (*22*). Correspondingly, at low transmural pressure, CTX changed membrane potential by less than 1 mV (n = 2) (*23*). In the presence of dihydropyridine Ca^{2+} channel blockers such as nimodipine, which are potent dilators of myogenic cerebral arteries (*4*, *24*), constrictions in response to K_{Ca} channel blockers were also reduced. Before addition of nimodipine, CTX reduced the diameter of an artery from about 90 to 30 μm (Fig. 4C). Nimodipine (1 nM) relaxed the artery to a diameter similar to that observed in the absence of Ca^{2+}. Under these conditions, CTX was essentially without effect on vascular diameter (Fig. 4C). In the presence of nimodipine at concentrations that cause substantial dilation (>1 nM), CTX (100 nM) and TEA^+ (1 mM) decreased diameter by only 2 ± 1% (n = 5) and 1 ± 1% (n = 6), respectively, and CTX (100 nM) depolarized the smooth muscle membrane by less than 1 mV (n = 4). As the concentration of intracellular Ca^{2+} increased and tone slowly returned during the washout of nimodipine, the effect of CTX on diameter progressively increased (Fig. 4C). These observations suggest a direct link between Ca^{2+} influx via voltage-dependent Ca^{2+} channels and activation of K_{Ca} channels.

Myogenic tone has been observed in the cerebral circulation of many species and in resistance arteries of other vascular beds (*25*) and probably participates in the autoregulatory response that is prevalent in the cerebral and coronary circulations in particular. The mechanism of initiation of the myogenic response to increased transmural pressure is not known. However, we propose that activation of K_{Ca} channels, caused by the depolarization and Ca^{2+} influx associated with myogenic tone (*26*), is an important negative feedback mechanism that regulates the level of vascular tone (*27*). This regulatory pathway is likely to influence arterial tone in many vascular beds, including coronary circulation (*28*). Activation of K_{Ca} channels by any means could lead to vasodilation of myogenic arteries. In contrast, a number of constrictor substances can close K_{Ca} channels from arterial, airway, and colonic smooth muscle (*29*), implying that modulation of this K^+ channel may be a common control mechanism in smooth muscle. Alterations in K_{Ca} channel activity might also initiate or aggravate pathophysiological states such as vasospasm and ischemia.

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9. Membrane depolarization from 0 to +50 mV increased the open state probability of these K_{Ca} channels by 67-fold (mean of three experiments) when intracellular Ca^{2+} was low (<100 nM). This voltage dependence is very similar to that reported for K_{Ca} channels in other tissues (*5, 7, 10*).
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20. We disrupted the endothelium by placing an air bubble in the lumen for 1 min and then perfusing the lumen with distilled water for 30 s. Damage to the endothelium was verified by the absence of a dilator response to acetylcholine (1 μM) after myogenic tone developed.
21. Tone in isolated mesenteric and cerebral arteries is extremely sensitive to small changes in membrane potential [M. T. Nelson, N. B. Standen, J. E. Brayden, J. F. Worley III, *Nature* **336**, 382 (1988); J. E. Brayden, *Circulation* **84** (suppl. III), II-406 (1991) (*3, 4*). Thus, a change in membrane potential of only a few millivolts would be expected to cause a substantial change in diameter.
22. At intermediate levels of myogenic tone (pressure: 40 mmHg; diameter: 86 ± 4% of maximum; $n = 3$) the vasoconstrictor response to IBX (100 nM) was reduced (16 ± 6% decrease in diameter) compared to responses in arteries pressurized to 75 mmHg, which further supports a direct relation between the degree of myogenic tone and the extent of activation of K_{Ca} channels.
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Isolation of a Complementary DNA That Encodes the Mammalian Splicing Factor SC35

Xiang-Dong Fu and Tom Maniatis

The mammalian splicing factor SC35 is required for the first step in the splicing reaction and for spliceosome assembly. The cloning and characterization of a complementary DNA encoding this protein revealed that it is a member of a family of splicing factors that includes mammalian SF2/ASF. This family of proteins is characterized by the presence of a ribonucleoprotein (RNP)-type RNA binding motif and a carboxyl-terminal serine-arginine–rich (SR) domain. A search of the DNA sequence database revealed that the thymus-specific exon (E_T) of the c-*myb* proto-oncogene is encoded on the antisense strand of the SC35 gene.

Splicing of nuclear pre-messenger RNA (pre-mRNA) takes place in complex ribonucleoprotein particles called spliceosomes, which contain pre-mRNA, the small nuclear ribonucleoproteins (snRNPs) U1, U2, U4/6, and U5, and non-snRNP proteins (*1*). Three non-snRNP mammalian splicing factors have been purified to homogeneity and characterized, namely SF2/ASF (*2*), U2AF (*3*) and SC35 (*4, 5*). The SC35 protein is required for formation of the earliest adenosine triphosphate (ATP)–dependent splicing complex (A complex) (*4*) and interacts with spliceosomal components bound to both the 5′ and 3′ splice sites during spliceosome assembly (*5*). In addition, SC35 is required for the ATP-dependent interactions of both U1 and U2 snRNPs with pre-mRNA.

We purified SC35 using standard chromatographic procedures (*6*) and generated tryptic peptides from the purified protein. These tryptic peptides were purified, and two were sequenced. The sequence of the first five NH_2-terminal amino acid residues of peptide 1 (Fig. 1) was identical to a sequence present in the splicing factor SF2/ASF (*2*), while the remaining 12 residues of the peptide were not present in this protein. The amino acid sequence of peptide 2 was identical to another SF2/ASF peptide (Fig. 1). Therefore, the purified SC35 preparation used to obtain the tryptic peptides was either contaminated with SF2/ASF, or the two proteins share common sequences.

We used the peptide 1 sequence to design oligonucleotide probes. One (probe A) corresponded to the SF2/ASF DNA sequence at the NH_2-terminus of peptide 1, and the other (probe B) corresponded to the unique region in the middle of peptide 1 (Fig. 1). We then used both probes simultaneously to screen a human primary cDNA library (*7, 8*). Of the five cDNA clones obtained, four hybridized to probe A and one to probe B. None of the clones hybridized to both probes. DNA sequence analysis of the two classes of cDNA clones revealed that the four clones that hybridized to probe A were SF2/ASF, while the sequence of the clone that hybridized to probe B (p1B) did not correspond to any known gene (Fig. 2A). The nucleotide sequence of the p1B cDNA clone encoded peptide 1, but did not contain the nucleotide sequence that corresponded to probe A or to the peptide 2 sequence found in SF2/ASF. Thus, the pooled SC35-containing MonoQ fractions were indeed contaminated by SF2/ASF, and

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