Eur Biophys J (1992) 21, 185–191

European Biophysics Journal

Expression of a genomic clone encoding a brain potassium channel in mammalian cells using lipofection

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Received January 20, 1992/Accepted March 27, 1992

Abstract. A genomic clone encoding a mouse brain K^+ channel (MBK1) was isolated, characterized and expressed in COS cells using the lipofection technique. Transfected COS cells expressed voltage-dependent K^+ currents that activated within 20 ms at 0 mV and showed less than 10% inactivation during 250 ms depolarizing pulses at 60 mV. Expressed K^+ currents were reversibly blocked by 4-aminopyridine and tetraethylammonium, and were moderately sensitive to dendrotoxin, but insensitive to charybdotoxin. Thus MBK1, expressed transiently in a mammalian cell line, exhibits features characteristic of non-inactivating K^+ channels with a conspicuous insensitivity to charybdotoxin. Lipofection is, therefore, a valuable strategy for expression of channel proteins in mammalian cells.

Key words: K^+ channels – Channel clones – Lipofection/transfection – Patch clamp

Introduction

Voltage-activated K^+ channels are integral membrane proteins that control neuronal excitability through repolarization of the action potential and modulation of repetitive firing (for reviews see Hille 1991; Jan and Jan 1990). Electrophysiological measurements indicate that neurons express a heterogeneous population of K^+ channels with diverse voltage-dependence characteristics and pharmacological properties (Thompson 1977; Neher 1971; Moczydlowski et al. 1988). A molecular basis for this diversity was established by the isolation of multiple genes encoding K^+ channels that exhibit different biophysical properties after their expression in heterologous systems (Jan and Jan 1990; Baumann et al. 1988; Stühmer et al. 1988, 1989; MacKinnon and Miller 1989; Christie et al. 1989). The most common method for heterologous expression of channel proteins is the injection of mRNA into *Xenopus laevis* oocytes (Lester 1988). However, this method has some caveats, particularly with respect to post-translational modifications that might affect the gating characteristics or pharmacological specificity of the expressed channels. Accordingly, functional expression of channel proteins in mammalian cells, that do not normally express them, is a valuable way to pursue questions about the relation between protein structure and its function as an ion channel. Here, we describe the characterization of a clone encoding a mouse brain potassium channel (MBK1) isolated from a genomic library and its functional expression in COS cells using lipofectin-mediated transfection. The ease of this procedure and its efficiency establish lipofection as a valuable strategy for investigating structure-function relations of ion channels in a variety of mammalian cells. A preliminary account of this research was presented elsewhere (Ferroni et al. 1992).

Materials and methods

Materials

Special materials and sources were: Lipofectin reagent and opti-MEM I reduced serum medium (Gibco-BRL); the plasmid pSVK3 (Pharmacia); the plasmid pBluescript II (Stratagene); the mouse genomic library from Adult Balb/c liver (Clontech); COS 1 cells (ATCC, CRL 1650); charybdotoxin (CTX) (Calbiochem, Peptides Internationals, and a kind gift of M. L. Garcia, Merck Institute); dendrotoxin (DTX) (Calbiochem); tetraethylammonium (TEA) and 4-aminopyridine (4-AP) (Sigma).

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Abbreviations: 4-AP, 4 aminopyridine; TEA, tetraethylammonium; CTX, charybdotoxin; DTX, dendrotoxin, V, applied voltage; V_{rev}, reversal potential; I, current; G, conductance; MBK1, mouse brain potassium channel 1; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid

Cloning, sequencing and expression

Standard molecular biological techniques were as described (Sambrook et al. 1989). 5×10^5 plaques from a mouse genomic library were screened with the mouse brain K^+ channel (MBK1) cDNA (Tempel et al. 1988) and washed to a final stringency of 0.1 X SSPE (17 mM NaCl, 1 mM NaH_2PO_4, 0.1 mM Na_2EDTA, 0.08 mM NaOH, pH 7.5), 0.1% SDS at 62 °C. Several positive clones were obtained. A clone corresponding to MBK1 was purified and characterized. A Hind III fragment generated from this clone, encompassing from nucleotide position −248 to 1641 in the cDNA (Tempel et al. 1988), was subcloned in pBluescript II SK+, and sequenced (Sanger et al. 1977). This construct was digested with Pst I (in the vector) and Nsi I (in nucleotide position 1605 in the 3′-untranslated region of cDNA). The fragment thus obtained was ligated with the expression vector pSVK3, previously digested with Pst I. The pSVK3 vector contains the SV40 early promoter, SV40 small T antigen splice site and the SV40 early polyadenylation site (Mongkolsuk 1988). COS cells were transfected with the resulting construct by lipofection (Felgner et al. 1987). COS cells were grown at 37 °C in Dulbecco's modified Eagle (DME) medium supplemented with 10% fetal calf serum. One day prior to lipofection, cells were split in a 35 mm dish at ~50% confluency. DNA (5 µg in 25 µl) was mixed with 25 µl of lipofectin reagent (25 µg in 25 µl) and incubated at 22 °C for 15 min. Cells were washed once with, and maintained in Opti-MEM I reduced serum medium. DNA-lipofectin reagent mixture (50 µl) was added to cells in Opti-Mem medium and were incubated overnight, thereafter. The cells were transferred to DME medium containing serum. Cells were plated for electrophysiological recordings 24 h later.

Electrical recordings and data analysis

Recordings were conducted from day 2 through day 5 after plating. Membrane currents were recorded in the whole-cell configuration as described in detail (Hamill et al. 1981). Voltage stimulation and data acquisition were performed with a microcomputer equipped with pClamp 5.5.1 software (Axon Instruments). Capacitance transients were minimized by analog compensation. Residual transients and leakage currents were corrected using a P/6 protocol (Armstrong and Bezanilla 1977). Cells were bathed in standard saline composed of (in mM): NaCl 140, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, Glucose 5, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) 5, pH 7.3. Osmolarity was adjusted at 330 mOsm with mannitol. For high K^+ solution, NaCl was substituted with equimolar concentrations of KCl; for Ca^{++}-free solution, 2 mM Ca^{++} was substituted with 6 mM Mg^{++}. The pipette solution was (in mM): KCl 140, $MgCl_2$ 1.5, EGTA 5, glucose 5, TES 5, pH 7.3. Drug application and changes of solutions were performed with a multibarreled perfusion system (Carbone and Lux 1987). Tabulated data points are mean ±SD; the minimum number of experiments per point, *N*, was three and the maximum was nine. Experiments were performed at 22 ± 2 °C.

Results

Isolation and sequence of a mouse genomic clone for a brain K^+ channel

A mouse genomic clone encoding the brain specific potassium chennel (MBK 1) was isolated and characterized (Fig. 1). This clone is approximately 14 Kb long. In addition to the coding sequence in one intronless region, it extends 3.5 Kb in the 5′-untranslated region and 8 Kb in the 3′-untranslated region. In the region that overlaps a previously described genomic clone (Chandy et al. 1990), the restriction enzyme patterns are identical. A Hind III fragment from *nt* position −248 to 1641 in cDNA, which covers the entire coding region was subcloned in pBluescript II and partially sequenced. The sequence is identical to the MBK1 cDNA (Tempel et al. 1988).

Transient expression in COS cells

The electrophysiological properties of the K^+ channel coded by the cloned DNA sequence were characterized after transfection in COS cells. COS cells were selected for expression because they are practically devoid of endogenous K^+ currents. Whole cell outward currents were ≤100 pA at depolarizations of 50 mV (Fig. 2B, •). In contrast, when cells were voltage clamped 2–5 days after transfection, large outward currents ($I \geq 1$ nA at 50 mV) were measured, as displayed in Fig. 2A. A family of outward currents in response to a set of test potentials (V_T) that ranged from −60 mV to 80 mV from a holding potential (V_H) of −80 mV is illustrated. These currents display a time course characteristic of non-inactivating K^+ channels (Fig. 2A–B, o): a fast increase (activation) and a slower and moderate decay (inactivation) during 250 ms depolarizing pulses. Outward currents were insensitive to external Ca^{++}. Cells that express this outward current (≤5% of the population) were round-shaped and grew more slowly than the non-transfected cells. These features have assisted in their identification: according to this selection, ~50% of the cells that exhibit these features and were patched expressed the outward K^+ current.

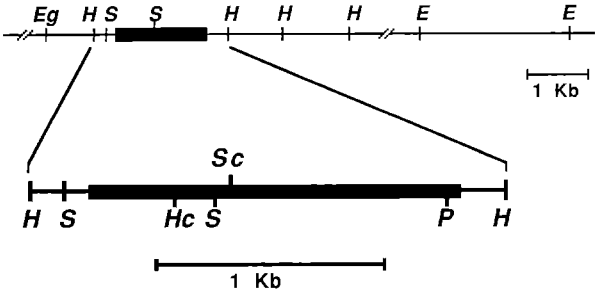

Fig. 1. Restriction map of the mouse genomic clone encoding MBK1: original clone of ~14 Kb (upper) and Hind III fragment used for its expression (lower). The thick band represents the coding region. E, Eco RI; Eg, Eag I; H, Hind III; Hc, Hinc II; Pvu II; S, Sma I; Sc, Sac I. Not all sites for Eco RI are given

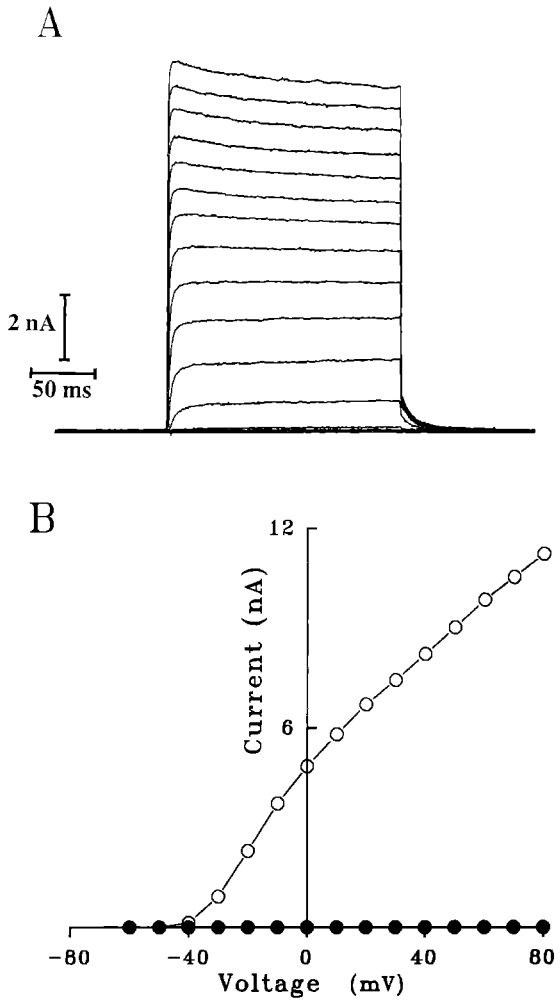

Fig. 2 A, B. Delayed outward currents in COS cells transfected with genomic MBK1 DNA. **A** Outward currents evoked from a holding potential of −80 mV in response to depolarizing voltage steps ranging from −60 to 80 mV in 10 mV increments. Linear leak current was subtracted. **B** Steady-state current from traces in A plotted as a function of membrane potential (o). Endogenous outward currents (•) never exceeded 100 pA

Ionic selectivity

The ionic selectivity of the expressed channels was determined from reversal potential (V_{rev}) measurements. Tail currents elicited by repolarizations to various voltages after stepping to 50 mV from a holding potential of −80 mV were recorded in different bath K^+ concentrations. Tail currents in 3 mM K^+ (Fig. 3 A) and 30 mM K^+ (Fig. 3 B) reversed polarity at −85 mV and −35 mV, respectively. In standard internal and external solutions, $V_{rev} = -83 \pm 3$ mV ($N = 6$) and shifted by 50 ± 2 mV ($N = 6$) with a 10-fold increase in external K^+ concentration (Fig. 3 C). This indicates that the cloned channel is highly selective for K^+ over Na^+ and Cl^-.

Gating properties

The steady-state voltage-dependence of inactivation (Fig. 4 A) of MBK1 channels was studied by using a pulse

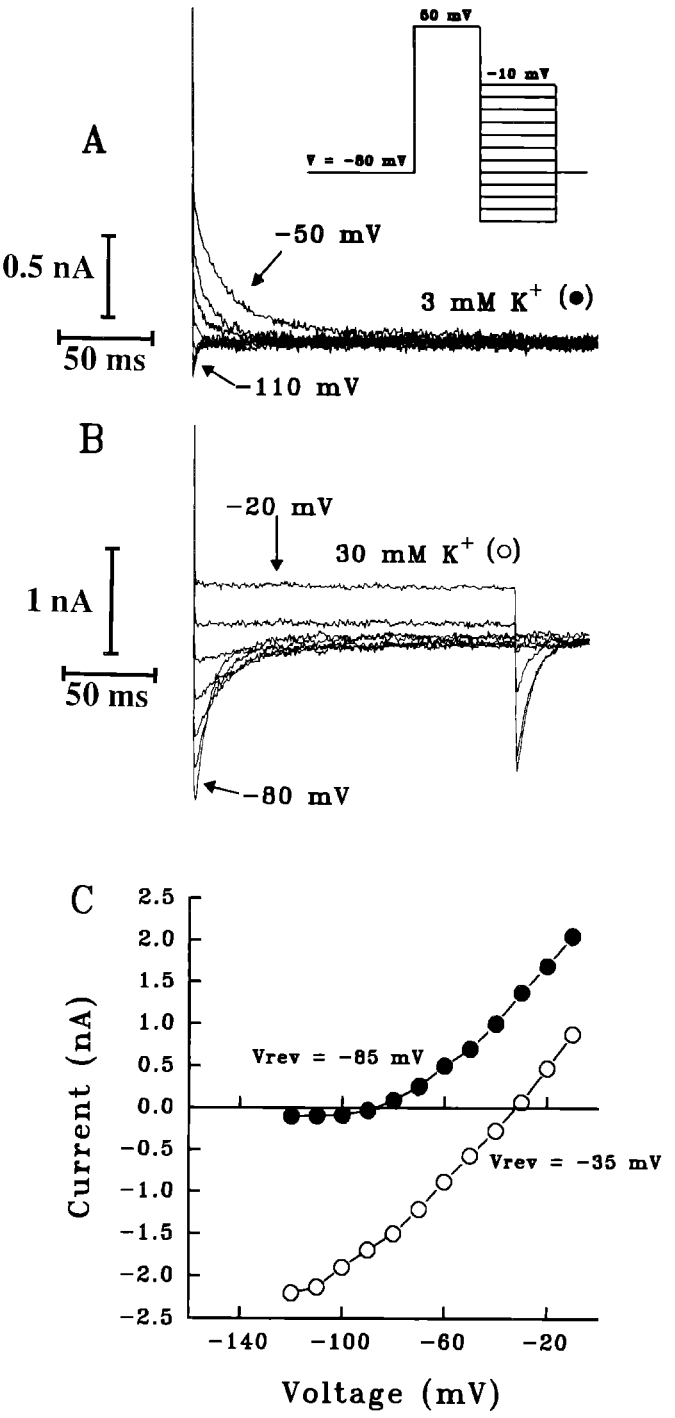

Fig. 3 A–C. K^+ carries the expressed currents. **A** Tail currents recorded in 3 mM K^+ in the external bath, were evoked by a depolarization to 50 mV followed by repolarizing steps to different test potentials. **B** Tail currents from the same cell shown in **A** recorded in 30 mM K^+. **C** Tail currents from **A** (•) and **B** (o) plotted as function of test potential. $V_{rev} = -85$ mV and −35 mV, respectively

protocol in which the test voltage step to 50 mV was preceded by 1 min depolarizing pulses at different potentials. As shown in Fig. 4 A and C, inactivation of the K^+ current is a voltage-dependent process. The current inactivates by 50% following a prepulse at −45 mV and by 90% at −20 mV (Fig. 4 A). The midpoint of the steady-state inactivation ($I_{1/2}$), extracted from a fit of the data

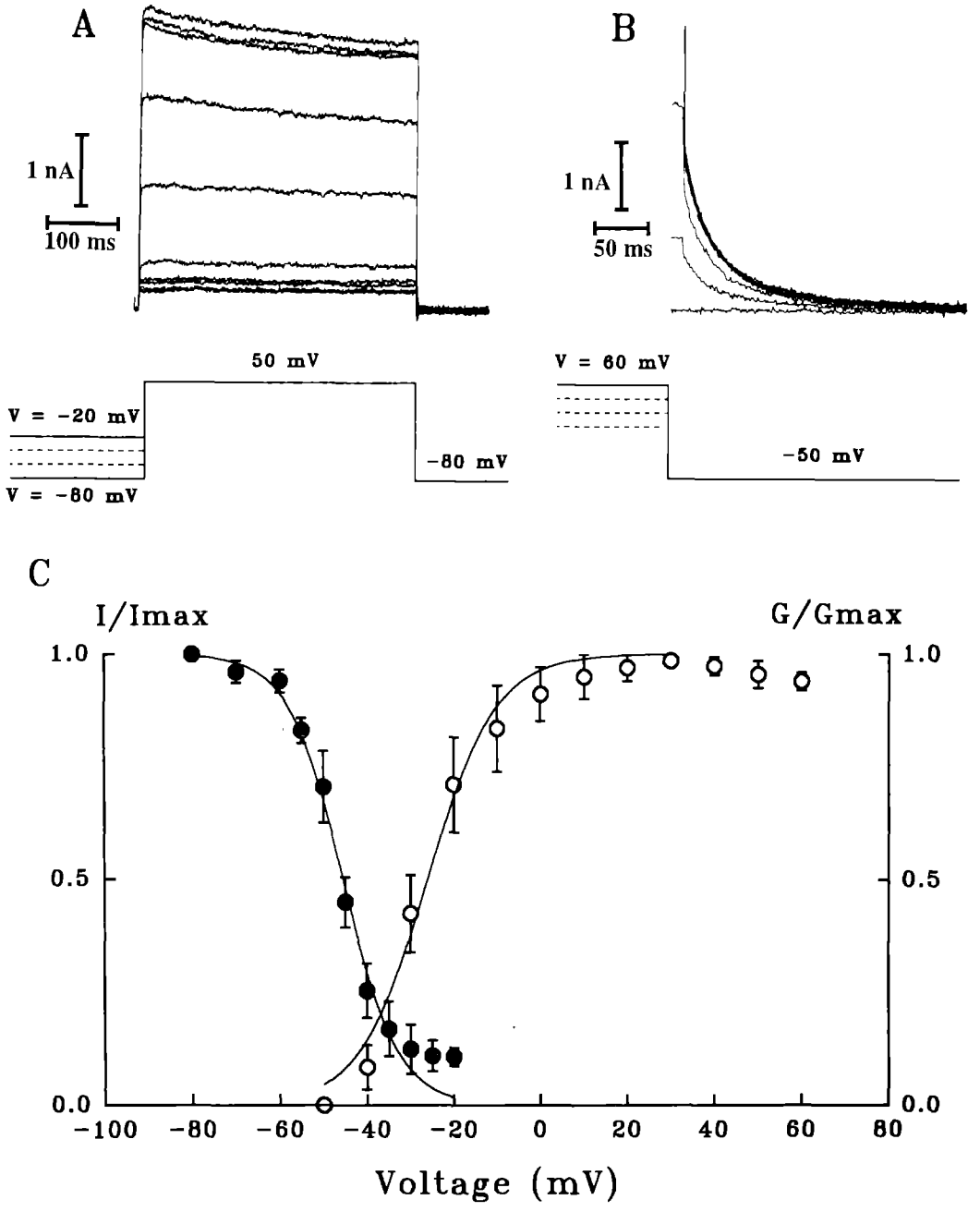

Fig. 4A–C. Steady-state inactivation and conductance-voltage characteristics of MBK1. **A** Steady state inactivation. Delayed outward MBK1 currents evoked from a holding potential $V = -80$ mV; one minute depolarizing prepulses (V_{pp}) to potentials ranging from -70 to -25 mV in 5 mV increments preceded the test potential to 50 mV. **B** Conductance-voltage relationship. Tail currents were recorded at the test potential of -50 mV, stepping back from different depolarizing potentials. **C** Data points of the steady-state inactivation (•) plotted as the ratio between steady-state currents following different prepulses and plotted as function of prepulse potential. Solid line depicts the best fit to data points and is given by $I/I_{max} = 1/[1+\exp(V_{pp}-V_{1/2})/a_h]$, were I denotes current, I_{max}, the maximum I, V_{pp}, prepulse V and a_h the inactivation slope. Relative conductance values (o) were normalized according to the relationship $g = I_K/I_{K\,max}$, where $I_{K\,max}$ is the maximum amplitude of the instantaneous K^+ tail currents and I_K are the values relative to $I_{K\,max}$. The *solid line* depicts the best fit given by a Boltzmann distribution $G/G_{max} = 1/[1+\exp[(V_{1/2}-V)/a_n]$, where G denotes conductance, $V_{1/2}$, the half-activation V, and a_n, the slope conductance

points to a Boltzmann isotherm, was -45.4 ± 0.6 mV with an e-fold decrease of the steady-state current (a_h) per 6.3 ± 0.5 mV ($N = 7$) (Fig. 4C). The conductance-voltage relationship was determined from tail currents measured at -50 mV after depolarizing steps ranging from -50 to 60 mV (Fig. 4B). Instantaneous currents were determined by extrapolation from single exponential fits of tail currents and plotted as a function of the depolarizing pulses. The voltage at which the conductance was half-maximal ($G_{1/2}$), obtained from a Boltzmann fit to the data points, was -26 ± 1 mV with a change of 7 ± 1 mV ($N = 6$) for an e-fold increase in conductance (a_n).

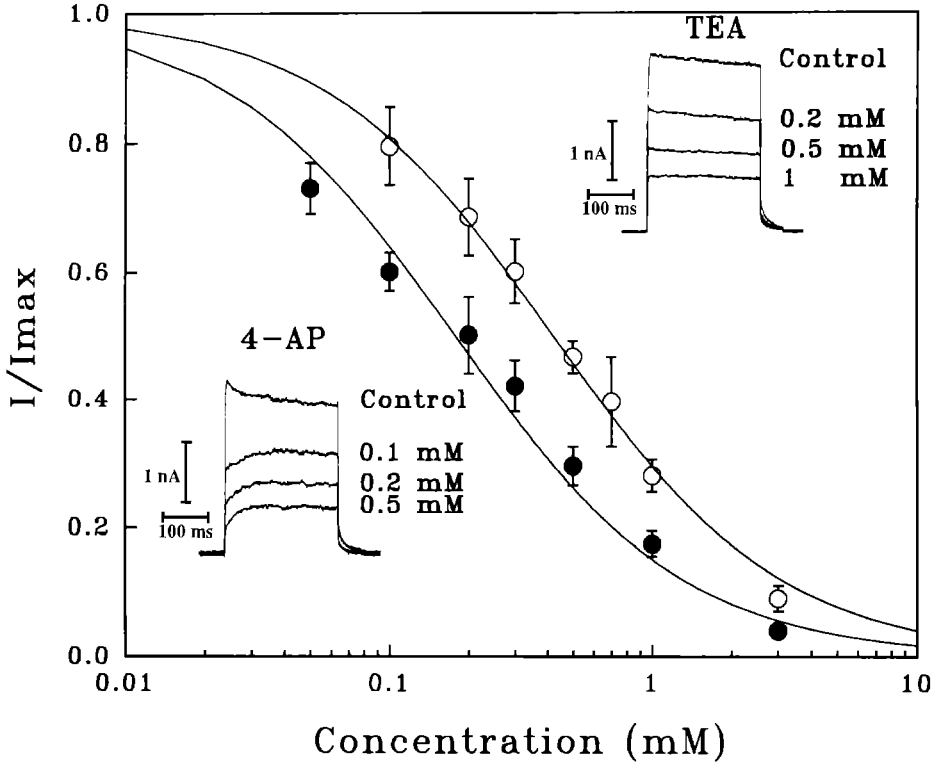

Fig. 5. Pharmacology of MBK1. Outward K^+ currents recorded at $V = 50$ mV in absence and presence of different concentrations of TEA (right inset) and 4-AP (left inset). Relative inhibition was plotted as a function of blocker concentration. Solid lines are best fits to inhibition isotherms. $K_{1/2}$ for TEA (o) and 4-AP (•) were 0.4 mM and 0.2 mM

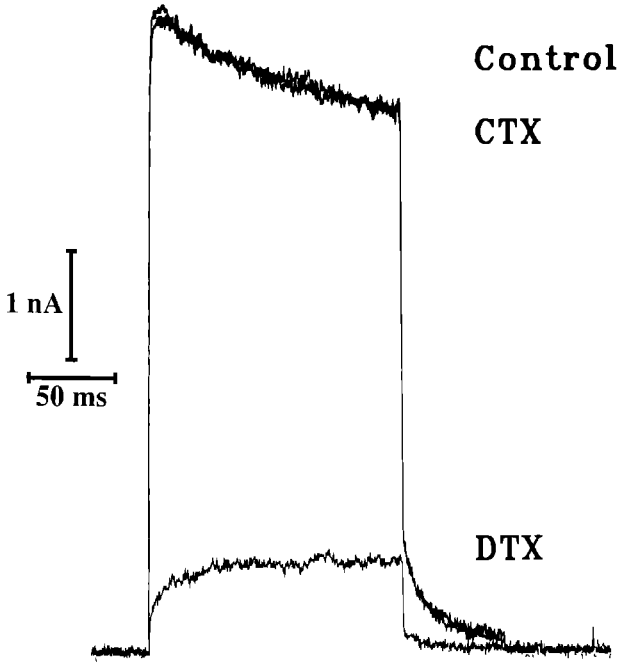

Fig. 6. MBK1 currents are blocked by dendrotoxin but not by charybdotoxin. Test potential was 50 mV; toxin concentration was 500 nM. Other conditions were as for Fig. 5

Pharmacology

The sensitivity of MBK1 to the K^+ channel blockers TEA and 4-AP (Fig. 5) was measured. Currents were recorded at 50 mV stepping from a holding potential of −80 mV. Cells were perfused for 30 s at different concentrations. The concentrations at which the current amplitudes were reduced by 50% ($K_{1/2}$) were 0.4 mM for TEA and 0.2 mM for 4-AP (Fig. 5). Blockade by TEA was fully reversible at concentrations ≤ 0.4 mM. Inhibition by 4-AP was voltage-dependent and use-dependent, increasing with more negative potentials and after the first pulse. At concentrations > 0.1 mM, inhibition was only partially reversible, the currents recovering up to 70% of control.

MBK1 channels are blocked by the snake toxin dendrotoxin but not by the scorpion toxin charybdotoxin (Fig. 6). Application of 150 nM DTX inhibited the K^+ currents evoked at $V = 50$ mV by $65 \pm 10\%$ ($N = 6$). Blockade was reversible up to ~80% of the initial current amplitudes. In contrast, CTX from three different sources and at concentrations up to 500 nM did not affect the expressed K^+ current ($N = 15$).

Discussion

Lipofection has successfully been used to introduce foreign DNA into mammalian cells (Felgner et al. 1987) and this is the first report demonstrating its use for the expression of channel proteins. Lipofection is effective, convenient and gentler than the calcium-phosphate-mediated transfection method (Graham and Van der Eb 1973) as reflected by the high quality of the electrical recordings. Viral expression vectors, such as vaccinia virus (Leonard et al. 1990) and baculovirus (Klaiber et al. 1990) have been used for the expression of Shaker K^+ channels. Use of these viral vectors requires specialized handling or is confined to the range of host cells permissive to viral infection. In contrast, lipofection is technically simple and ap-

plicable to a broad range of cell lines. Lipofection is, therefore, a valuable tool for the expression of channel proteins in mammalian cells.

We describe the isolation of a genomic MBK1 clone that encodes a functional K^+ channel and the characterization of the K^+ channel coded by this gene in COS cells transfected by the lipofection technique. A cDNA clone (Tempel et al. 1988) and a genomic clone (Chandy et al. 1990) for this protein were previously reported, but not expressed. MBK1a, a homolog with a coding sequence identical to MBK1 except that the nucleotide sequence around the initiation codon was derived from a PCR product, was expressed in *Xenopus* oocytes (Hoger et al. 1991). The kinetics of the MBK1 currents expressed in COS cells match those described for MBK1a expressed in oocytes (Hoger et al. 1991) and those of non-inactivating K^+ currents in nerve (Hodgkin and Huxley 1952).

DNA sequence alignment shows that MBK1 belongs to the gene family of *Drosophila Shaker* K^+ channels (Tempel et al. 1988) that mediate the fast inactivating K^+ current I_A (Schwarz et al. 1988; Pongs et al. 1988; Kamb et al. 1988). However, a notable distinction from *Shaker* proteins is in the inactivation kinetics: *Shaker* channels inactivate in the millisecond time range whereas MBK1 channels inactivate in seconds (Fig. 2). This disparity appears to arise from the diversity in length of the amino-terminal domain (Stühmer et al. 1989; Hoshi et al. 1990). The gating properties of MBK1 are, however, comparable to those of its rat brain homologs, RCK1 and RBK1 (Table 1) that, in accord, have a similar cytoplasmic amino-terminal stretch (Baumann et al. 1988; Stühmer et al. 1988, 1989; Christie et al. 1989).

CTX and DTX show differences in their inhibitory action (Table 1) in spite of the extensive sequence similarity of these brain K^+ channel clones. The CTX insensitivity of MBK1 expressed in COS cells and in oocytes (Ferrer-Montiel A, Planells-Cases R, Montal M, unpublished results) is noteworthy because the aspartic acid residue, corresponding by alignment to position 431 of *Shaker* and proposed to contribute to the CTX binding site (MacKinnon 1991), is absolutely conserved in RCK1, RBK1, and MBK1. RCK1 was expressed in a myoblast cell line but blockade by CTX was not reported (Koren et al. 1990), and RBK1 has only been expressed in oocytes (Christie et al. 1989). CTX blocking of K^+ channels is notoriously dependent on the purity of the toxin used, a consideration that may account for the different affinities reported (Oliva et al. 1991; McKinnon 1991) and summarized in Table 1.

The question, however, arises as to whether the diversity of K^+ channel phenotypes is intrinsic to the expressed protein or derived, in addition, from differences in processing of the transcript by the host cell. A notable case in point is that of *Shaker*:*Shaker* genes expressed in *Shaker* mutant *Drosophila* myotubes are insensitive to CTX (Zagotta et al 1989) but sensitive when expressed in *Xenopus* oocytes (MacKinnon and Miller 1989) or in rat basophilic leukocytes (Leonard et al. 1989).

Most studies on the expression of ion channels and neurotransmitter receptors rely heavily on introduction of genetic material in *Xenopus* oocytes. Expression in mammalian cells offers the notable advantage of characterizing channel proteins in cell lines in which natural synthetic modifications occur and intracellular messengers act. Furthermore, the notion that properties of channel proteins are tissue specific may be resolved by expression in cells of different lineages.

Table 1. Electrical and pharmacological properties of MBK1, and the rat brain homologs RBK1 (Christie et al. 1989) and RCK1 (Stühmer et al. 1988, 1989). Values for half-inactivation potential, inactivation slope, half-activation potential and slope conductance were obtained from Fig. 4C. Selectivity values determined from changes of V_{rev} upon imposing a 10-fold increase in external K^+ concentration. $K_{1/2}$, blocker concentration at which current amplitude was reduced by 50%, for TEA, 4-AP, DTX and CTX. ND, not determined. NB, no block by ≤ 500 nM CTX. Other conditions were as for Figs. 4 and 5

	MBK1	RCK1	RBK1
Activation threshold (mV)	−40	−30	−50
$V_{1/2}$ (mV) [half-inactivation potential]	−45	−47	−30
Inactivation slope (mV/*e*-fold)	6	4	ND
$V_{1/2}$ (mV) [half-activation potential]	−26	−30	−30
Slope conductance (mV/*e*-fold)	7	7	13
Selectivity (mV/$\log_{10}$ mM)	50	55	55
Blockers			
TEA: $K_{1/2}$ (mM)	0.4	0.6	0.8
4-AP: $K_{1/2}$ (mM)	0.2	1	0.16
DTX: $K_{1/2}$ (nM)	100	12	ND
CTX: $K_{1/2}$ (nM)	NB	22	5

Acknowledgements. We thank L. Y. Jan for providing the cDNA probe of MBK1, M. L. García for supplying charybdotoxin, and the members of our group for their perceptive comments. This work was supported by grants from the National Institute of Mental Health (MH-44638), the Department of the Army Medical Research (DAMD 17-89-C-9032) and a Research Scientist Award from the Alcohol, Drug Abuse and Mental Health Administration (MH-00778) to M. M.

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