ACTION OF HALOTHANE ON LARGE-CONDUCTANCE, CALCIUM-ACTIVATED POTASSIUM CHANNELS IN RAT CEREBROVASCULAR SMOOTH MUSCLE CELLS

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ABSTRACT

Halothane anesthesia is associated with increased cerebral blood flow and dilatation of cerebral vessels. The mechanisms which underlie this drug action are presently unclear. Cerebrovascular tone is probably regulated in part by the opening of large conductance, Ca^{2+}-activated potassium channels (BK channels) in cerebral artery smooth muscle cells. The present experiments utilized extracellular patch clamp techniques to investigate whether clinically relevant concentrations of halothane directly alter the biophysical properties of these channels.

Cerebrovascular smooth muscle cells (CVSMCs) were dispersed from the basilar, middle, posterior communicating, and posterior cerebral arteries of adult Wistar rats using collagenase and trypsin and maintained *in vitro* for 48 hrs prior to use. Recordings were made from isolated inside-out membrane patches at room temperature (21-23^{o}C) using a List EPC-5 patch clamp amplifier.

Under control conditions, amplitude distributions of single BK channel currents were well described by a single Gaussian term. This behavior was maintained during exposure to halothane. The mean conductance of single BK channels, which was 194 ± 6.1 pS in symmetrical 140 mM K^+ solutions, was unchanged by application of halothane at any of the concentrations tested (0.5, 1.6, 2.8 mM).

Halothane caused a dose-dependent, reversible decrease in the open probability (P_o) of BK channels. Halothane reduced P_o by 14 % and 55 % on application of 1.6 mM (n=11) and 2.8 mM (n=11) halothane respectively, while 0.5 mM halothane had no significant effect on the open probability (n=7).

Kinetic analysis of BK channel currents showed that halothane altered the gating of these channels. Halothane reduced the mean channel open time by 23 % and 56 %, and increased the mean channel closed time by factors of 2.1 and 9.3 when

applied at concentrations of 1.6 mM or of 2.8 mM, respectively.

The inhibitory effect of halothane on BK channel function is unlikely to result solely from the fluidization of membrane lipids by the anesthetic, since this would probably increase the channel opening probability. Rather, halothane appears to alter BK channel function by binding to hydrophobic domains within the channel protein, or by interfering with protein-lipid interactions in the membrane.

A halothane-induced decrease in the open probability of BK channels in CVSMCs might be expected to reduce outward potassium current, resulting in enhanced contraction of blood vessel walls. Hence, the direct inhibitory effects of halothane on BK channels obtained from cerebral artery cells cannot explain the marked cerebral vasodilation caused by the anesthetic. This vasodilation must therefore result from other drug actions on vascular smooth muscle cells, which include reduction in calcium influx through voltage-dependent calcium channels, decreased accumulation of intracellular free calcium, and lowered sensitivity of contractile proteins to calcium.

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INTRODUCTION

1. Cerebral Vasodilatory Action Of Halothane

Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is a fluorinated hydrocarbon widely used as a volatile surgery anesthetic at concentrations in the range of 0.5 to 3% (v/v) in air (Atkinson et al., 1987). Halothane not only suppresses consciousness by acting on the central nervous system, but also exerts a hypotensive effect, due to decreased cardiac contractility (Brown & Crout, 1971) and reduced vascular tone (Sprague et al., 1974; Longnecker & Harris, 1980; Spiss et al., 1985). The effects of halothane anesthesia on cerebral blood flow have been studied extensively in both animals (Wollman et al., 1964; Christensen et al., 1967; Chen et al., 1984; Drummond et al., 1984) and humans (Fleischer & Inni, 1989; Sato et al., 1988; Kitazawa et al., 1991). Increases in human cerebral blood flow have been reported with 0.5 to 2 % halothane (McDowall, 1967). Cerebral vascular resistance in the dog has been shown to decrease progressively on increasing the halothane dose from 0.5 to 4 % (Smith & Wollman, 1972). Thus, halothane appears to be a cerebral vasodilator at most clinically useful concentrations. This vasodilation may increase intracranial pressure and cerebral blood flow, reducing the usefulness of halothane for many neurosurgical procedures (Eintrei et al., 1985).

Despite these important clinical effects, the cellular mechanisms by which halothane dilates blood vessels remain largely unknown. However, studies carried out on a variety of smooth muscle and cardiac muscle preparations indicate that several mechanisms probably play roles in the actions of halothane on muscle cells (Muldoon et al., 1988). These include drug effects on intracellular Ca^{2+} mobilization (Wood & Wood, 1990; Su & Bell, 1986; Katsuoka et al., 1989), on the activation of contractile proteins by Ca^{2+} (Harder et al., 1985; Housmans, 1990) and

alteration of the properties of transmembrane ion channels in muscle cells (Haydon et al., 1988).

2. Effects Of Halothane On Intracellular Calcium Mobilization In Muscle Cells

Tension generation and maintenance in vascular smooth muscles depend upon processes that modulate intracellular levels of free Ca^{2+}, $[Ca^{2+}]_i$ (Fleischer & Inni, 1989; Sato et al., 1988; Kitazawa et al., 1991; Van Breemen & Saida, 1989; Somlyo et al., 1988). In the relaxed cell, $[Ca^{2+}]_i$ is in the range of 40-130 nM (Nabika et al., 1985; Takata et al., 1988; Kuriyama et al., 1982; Wang & Mathers, 1993), increasing during contraction up to 1 uM (DeFeo & Morgan, 1986). Although much of the Ca^{2+} utilized for contraction is of extracellular origin, Ca^{2+} release from the sarcoplasmic reticulum (SR) also plays an important role in smooth muscle cells (Fleischer & Inni, 1989).

Considerable evidence indicates that halothane inhibits intracellular Ca^{2+} mobilization. In isolated heart cells and in vascular smooth muscle cells (VSMCs), halothane decreases the uptake of Ca^{2+} by the SR. The drug also increases the release of Ca^{2+} from the SR by stimulating the process of Ca^{2+}-induced Ca^{2+} release (Bosnjak et al., 1992; Wheeler et al., 1988; Katsuoka et al., 1989; Su & Kerrick, 1978; 1979; Iaizzo, 1992; Su & Zhang, 1989). The calcium so released is probably extruded from the cell, thereby leaving a depleted store in the SR and reducing the Ca^{2+} available for subsequent release by agonists (Wheeler et al., 1988; Katsuoka et al., 1989; Sill et al., 1991; Szocik et al., 1989).

In VSMCs dispersed from coronary artery and thoracic aorta, halothane also attenuates the increases in $[Ca^{2+}]_i$ evoked by two vasoactive modulators, norepinephrine and vasopressin (Sill et al., 1991; Tsuchida et al., 1993). These agents

elevate $[Ca^{2+}]_i$ by stimulating the phospholipase C family of enzymes, which hydrolyze minor membrane phospholipids to form second messengers, including inositol 1,4,5-trisphosphate (IP_3). IP_3 is primarily responsible for discharging Ca^{2+} from intracellular stores through IP_3-activated channels (Berridge & Irvine, 1989; Hashimoto et al., 1986; Suematsu et al., 1984; Grillone et al., 1988). Therefore, inhibition of IP_3 formation by halothane may also contribute to the attenuation of Ca^{2+} mobilization in these vascular muscle cells (Sill et al., 1991; Tsuchida et al., 1993).

3. Effect Of Halothane On The Activation Of Contractile Proteins By Calcium In Smooth Muscle Cells

In smooth muscle, an increase in intracellular free calcium concentration activates myosin light chain kinase, which phosphorylates the myosin light chain and induces contraction (Hai & Murphy, 1989; Kamm & Stull, 1985). Modulatory processes which alter the sensitivity of the contractile filaments to Ca^{2+} also regulate the contraction and relaxation of smooth muscle. In smooth muscle cells of dog trachea, clinical concentrations of halothane suppress the increase in $[Ca^{2+}]_i$ and of muscle tension produced by application of carbachol. However, the depressant effect of the drug is more marked on muscle tension than on changes in $[Ca^{2+}]_i$ (Yamakage, 1992). These observations indicate that halothane probably suppresses the sensitivity of contractile elements to $[Ca^{2+}]_i$. Cyclic adenosine 3,5-monophosphate (cAMP), as well as cyclic guanosine 3,5-monophosphate (cGMP) are known to decrease both $[Ca^{2+}]_i$ and the sensitivity of contractile elements to Ca^{2+} (Itoh et al., 1982; Meisheri & Breeman, 1982). The latter effect is due to inhibition of myosin light chain kinase (Adelstein et al., 1978). Clinically relevant concentrations of halothane increase the level of cAMP and cGMP in smooth muscle cells of dog

trachea, rat aorta and dog cerebral artery (Yamakage, 1992; Nakamura et al., 1991; Eskinder et al., 1992; Sprague et al., 1974). It seems likely, therefore, that the halothane-induced relaxation of smooth muscle is partly mediated by an increase in cAMP and/or cGMP concentration.

4. Alteration Of The Properties Of Transmembrane Ionic Channels By Halothane

The contractile state of VSMCs is partially controlled by the electrical potential across the cell membrane. In general, membrane depolarization increases the degree of contraction, while membrane hyperpolarization produces relaxation. Alteration of trans-sarcolemmal ion fluxes offers a mechanism by which drugs might influence the contractile state of VSMCs.

Action potentials in most VSMCs persist in the presence of tetrodotoxin (TTX), a Na^+-channel blocker, and are unaffected by removal of Na^+ ions from the extracellular fluid, suggesting that inward Na^+ movement is not responsible for the upstroke of the action potential (Itoh et al., 1981a; Kuriyama, 1971; Ito et al., 1977; Hirst et al., 1986). In contrast, these action potentials are abolished in Ca^{2+}-free medium and are blocked by Ca^{2+} channel antagonists, such as verapamil and nifedipine (Itoh et al., 1981; Hirst et al., 1986). These observations strongly suggest that inward Ca^{2+} current is responsible for generation of action potentials in VSMCs.

The predominant voltage-dependent Ca^{2+} channel in arterial smooth muscle cells is inhibited by the dihydropyridine Ca^{2+}-channel blockers and inactivates slowly during prolonged depolarization. It has been referred to as the "L-type" Ca^{2+} channel (Tsien et al., 1988). A dihydropyridine-insensitive, rapidly inactivating (T-type) form of Ca^{2+}-channel has also been reported in some VSMCs (Bean et al.,

1986; Benham et al., 1987), but appears to be absent in others (Aaronson et al., 1988; Nelson & Worley, 1989). L-type Ca^{2+} channels contribute strongly to the macroscopic calcium current during the action potential, since maintained arterial tone is strongly inhibited by dihydropyridines (Himpens & Somlyo, 1988).

4.1. Inward Calcium Currents

Volatile anesthetics have been shown to reduce the inward calcium current in isolated atrial and ventricular muscle cells (Lynch et al., 1981; Ikemoto et al., 1985; Bosnjak et al., 1991; Hirota et al., 1989; Puttick & Terrar, 1992). However, there have been relatively few reports on the effects of halothane on inward Ca^{2+} currents in VSMCs. Murray et al. (1989) have shown that halothane inhibits Ca^{2+} influx, as measured by $^{45}Ca^{2+}$, in pulmonary artery smooth muscle cells. Buljubasic et al. (1992) have reported, using whole-cell voltage clamp techniques, that in dog coronary artery cells, the inward Ca^{2+} current carried by nifedipine-sensitive Ca^{2+} channels is reduced by halothane. Since some VSMCs rely on Ca^{2+} entry for maintenance of contraction (Towar, 1981; Rush et al., 1985; Mullett et al., 1983; Bevan & Bevan, 1988), reduced Ca^{2+} influx by volatile anesthetics may represent an important vasodilatory mechanism.

4.2. Outward Potassium Currents

Outward K^+ currents are well known to play an important role in the modulation of vascular tone. Suppression of K^+ currents leads to depolarization and spontaneous action potential activity in many VSMCs (Bolton, 1979). Some vasodilators, such as cromakalim and pinacidil probably relax vessels by the hyperpolarizing effect of increased K^+ conductance (Nelson et al., 1990; Videbaek et

al., 1990).

In most isolated VSMCs, macroscopic outward K^+ current can be divided into two components, one gated by membrane voltage, and the other gated by both voltage and intracellular Ca^{2+}. Voltage-gated current is mainly carried by delayed rectifier K^+ channels (Rudy, 1988). Currents gated by Ca^{2+} and voltage appear to be predominantly carried by large conductance or BK channels (Benham et al., 1986; Ohya et al., 1987; Hume & Leblanc, 1989; Beech & Bolton, 1989a).

4.2.1. Delayed Rectifier Potassium Current

Delayed rectifier K^+ current has been studied at the whole-cell level in VSMCs isolated from pulmonary arteries ((Okabe et al., 1987; Beech & Bolton, 1989; 1989a), coronary arteries (Buljubasic et al., 1992; Volk et al., 1991) and human mesenteric artery (Smirnov & Aaronson, 1992). A few single channel studies have also been made (Beech & Bolton, 1989). This current is voltage-dependent, activating with depolarization at a threshold between -30 and -50 mV. Under a constant depolarizing stimulus, a slow exponential inactivation occurs, requiring several seconds for completion (Beech & Bolton, 1989; Smirnov & Aaronson, 1992; Buljubasic et al., 1992).

In VSMCs, delayed rectifier K^+ currents are blocked by 4-aminopyridine (K_d < 1.5 mM), but are quite insensitive to TEA^+ (Buljubasic et al., 1992; Smirnov & Aaronson, 1992). These characteristics are shared with delayed rectifier K^+ currents seen in other excitable tissues (Rudy, 1988). From single channel recordings in isolated membrane patches, single channels underlying delayed rectifier K^+ currents have a small unitary conductance (5 pS) in physiological solutions (Beech & Bolton, 1989).

Delayed rectifier K^+ currents are active during the repolarization phase of

action potential, thereby helping to terminate depolarization. In VSMCs from guinea-pig pulmonary artery and rat portal vein, 4-aminopyridine causes an increase in spike frequency, suggesting that delayed rectifier K^+ currents may also play a role in determining cell excitability (Hara et al., 1980).

Sugiyama et al. (1992) have reported that halothane hyperpolarizes guinea pig thalamic neurons by increasing a K^+ conductance of the delayed rectifier type. In contrast, in VSMCs obtained from dog coronary artery, halothane has been shown to suppress the amplitude of delayed rectifier K^+ currents (Buljubasic et al., 1992). However, halothane also decreased L-type Ca^{2+} current with a much higher potency in the same type of VSMCs. This dual effect on hyperpolarizing and depolarizing currents may explain why volatile anesthetics cause electromechanical uncoupling in cerebrovascular smooth muscle, i.e. membrane depolarization concurrent with vessel relaxation (Harder, et al., 1985).

4.2.2. Calcium-activated Potassium Currents

Potassium current carried by Ca^{2+}-activated potassium (K_{Ca}) channels is the predominant outward K^+ current in virtually every type of vascular smooth muscle cell so far investigated. Whole-cell patch clamp recordings have shown that I_{KCa} activates at a higher threshold than delayed rectifier K^+ currents (0 to -30 mV) and shows little tendency to inactivate during prolonged depolarization (Smirnov & Aaronson et al., 1992; Beech & Bolton, 1989). This current is sensitive to experimental manipulation of cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_i$, such that removal of external Ca^{2+} or addition of Ca^{2+}-channel blockers strongly decreases the amplitude of I_{KCa} (Weigel et al., 1979; Hirst et al., 1986; Benham et al., 1986; Smirnov & Aaronson, 1992; Akbarali et al., 1992). 4-aminopyridine, which shows some selectivity for purely voltage-gated over Ca^{2+}-activated K^+ currents

(Rudy, 1988), has little effect on I_{KCa} (Smirnov & Aaronson, 1992).

Using patch clamp recording techniques, two groups of K_{Ca} channels have been distinguished in VSMCs on the basis of their single-channel conductance and susceptibility to the blocking action of drugs. Ca^{2+}-activated K^+ channels of relatively modest conductance have been observed in VSMCs isolated from rabbit portal vein, human cystic and rat cerebral artery. These channels show single channel conductances in the range of 55 to 117 pS in symmetrical 140 mM K^+ solutions (Inoue et al., 1985; Akbarali et al., 1992; Wang & Mathers, 1991). These so called intermediate conductance, Ca^{2+}-activated K^+ (IK_{Ca}) channels show an increased open probability on increasing $[Ca^{2+}]_i$ or membrane depolarization, but open probability is low at normal resting potentials. In neurons, IK_{Ca} channels are relatively insensitive to the blocking action of charybdotoxin, a peptide produced by the scorpion *Leirus quinquestriatus* (K_d = 30-100 nM) (Castle et al., 1989).

Large conductance, Ca^{2+}-activated K^+ (BK) channels have been identified in a wide variety of VSMCs (Inoue et al., 1985; 1986; Benham et al., 1986; Bolton et al., 1985; Akbarali et al., 1992; Wang & Mathers, 1993). The channels exhibit a high conductance (150-300 pS) in 140 mM symmetrical K^+ solutions. Charybdotoxin is a relatively selective and potent blocker of BK channels in VSMCs (K_d < 10 nM) (Miller et al., 1985; Kovacs & Nelson, 1991; Blatz & Magleby, 1987).

In most neuronal and muscle preparations, low concentrations (0.1 - 1 mM) of TEA^+ applied to the external membrane face reversibly block current flow in BK channels with an apparent dissociation constant, K_d of about 0.3 mM. In these preparations, TEA^+ also blocks current flow in BK channels when applied to the internal membrane face, but with a much higher K_d (30 - 50 mM) (Yellen, 1984a; 1984b; Vergara & Latorre, 1983; Blatz & Magleby, 1984). However, in clonal pituitary cells (Wong & Adler, 1986) and in brain synaptosomal membranes (Farley & Rudy, 1988), BK channels display a high sensitivity to internal TEA^+, with K_ds of

0.08 mM and 0.8 mM, respectively. BK channels of rat cerebrovascular smooth muscle cells also show a high sensitivity to internally applied TEA^+ (K_d=0.8 mM) (Wang & Mathers, 1993).

K_{Ca} channels are frequent targets of modulation by neurotransmitters and second messengers (Rudy, 1988). cAMP has been shown to activate BK channels in rat aorta VSMCs (Sadoshima et al., 1988). In porcine coronary artery, the newly discovered vasoactive peptide endothelin has been found to enhance the open probability of BK channels (Hu et al., 1991).

In VSMCs of guinea-pig mesenteric artery, BK channels were inactive at the resting membrane potential unless $[Ca^{2+}]_i$ was raised to micromolar levels (Benham et al., 1986). Since $[Ca^{2+}]_i$ in unstimulated vascular smooth muscle cells has been estimated at 40 - 130 nM (Nabika et al., 1985; Takata et al., 1988; Kuriyama et al., 1982; Wang & Mathers, 1993), BK channels probably contribute little to resting potassium conductance in these cells. However, it seems likely that the function of Ca^{2+}-activated K^+ channels in VSMCs is to open when $[Ca^{2+}]_i$ rises and so to serve to repolarize the membrane towards the resting potential, terminating voltage-dependent calcium entry (Bolton et al., 1985). It has also been proposed that BK channels may play a role in the after-hyperpolarization phase of the action potential seen in cerebral artery and bladder smooth muscle cells (Hirst et al., 1986; Fujii, 1987).

Clinical concentrations of halothane are known to alter the activity of K_{Ca} channels in excitable cells. In human red blood cells, halothane produces biphasic effects on the calcium-dependent efflux of $^{86}Rb^+$, having a stimulatory action at low anesthetic concentrations (< 1 mM) and an inhibitory action at higher drug concentrations (Scharff & Foder, 1989; Caldwell & Harris, 1985). In isolated hippocampal neurons, the amplitude of the fast after-hyperpolarization (AHP), which is due to activation of BK channels (Lancaster et al., 1991), is readily depressed by

halothane at clinically relevant concentrations (Fujiwara et al., 1988; Southan & Wann, 1989). Halothane (0.5-2 %) also reduces ion flux through charybdotoxin-sensitive K_{Ca} channels of BK channel type in the rat glioma C6 cell line (Tas et al., 1989). In all of these studies, halothane did not measurably interfere with the entry of calcium into cells. This suggests that the suppressive effect of halothane on K_{Ca} channel function was direct rather than mediated via changes in the Ca^{2+} flux into the cells.

Experimental Rationale

BK channels occur widely in vascular smooth muscle cells and may play an important role in controlling action potential duration and Ca^{2+} entry into these cells, thereby controlling the contractile state of muscle cells. In addition, there is considerable evidence that clinically relevant doses of halothane affect the activity of BK channels in a variety of preparations. The purpose of this study was to examine the direct effects of halothane on the biophysical properties of BK channels in VSMCs obtained from cerebral arteries of the adult rat. This approach should allow a clear appraisal of how volatile anesthetics interact with BK channels in vascular smooth muscle, and of the role of this action in the cerebral vasodilation caused by the volatile anesthetic.

The opening of BK channels is strongly promoted by raising the intracellular concentration of free Ca^{2+} ions (Latorre et al., 1989; Wang & Mathers, 1993). It was necessary, therefore, to minimize the possible action of halothane on calcium fluxes during these experiments. This was achieved by using isolated inside-out membrane patches, in which the value of $[Ca^{2+}]_i$ could be kept constant (Hamill et al., 1981). A low extracellular concentration of free calcium ions was also employed, in order to suppress inward Ca^{2+} current on depolarization of these membrane patches.

METHODS

1. Preparation Of Cerebrovascular Smooth Muscle Cells

Experiments were performed on cerebrovascular smooth muscle cells (CVSMCs) isolated from the middle, basilar, posterior communication and posterior cerebral arteries of adult male Wistar rats (200-250 g, Charles River, Montreal). Rats were exposed to CO_2 until unconscious, then decapitated. The brain was carefully removed under aseptic conditions following removal of the parietal bone, and placed in a 60 mm culture dish filled with brain dissecting buffer solution containing Ca^{2+} and Mg^{2+} free Hank's Balanced Salt Solution (HBSS, Gibco Laboratories, Grand Island, NY) of the following composition (in mM): 138 NaCl, 5 KCl, 0.3 KH_2PO_4, 0.3 $Na_2HPO_4 \cdot 7H_2O$, 18 Dextrose, 4 $NaHCO_3$, 15.7 HEPES with penicillin 100 U/mL and streptomycin 100 μg/mL (Sigma Chemical Company, St. Louis, MO), pH 7.4. Under the low power of a dissection light microscope, the basilar, middle, posterior communicating and posterior cerebral arteries and their first order and second order branches were collected using iridectomy scissors and fine forceps and placed in a 65mm culture dish filled with potassium glutamate (KG) buffer solution containing (in mM): 140 glutamic acid monopotassium, 16 $NaHCO_3$, 0.5 NaH_2PO_4, 16.5 Dextrose and 25 HEPES, pH 7.4.

After incubation in KG solution for 10 minutes at 37^{o}C, the vessels were minced with iridectomy scissors into 0.5 mm fragments. The fragments were then transferred into a 15 mL centrifuge tube containing 3 mL of 0.1 % trypsin and incubated at 37^{o}C for 8 minutes (Type C, Sigma, dissolved in KG solution). The tissue suspension was then incubated in 3 mL of 0.3 % collagenase (Type 1A, Sigma, dissolved in KG solution) and 0.2 mL of 0.5 % trypsin inhibitor (Sigma, dissolved in KG solution) at 37^{o}C for 15 minutes. The cell suspension was centrifuged and the

supernatant was removed. Isolated cells were resuspended in 3 mL of horse serum (heat-inactivated, Gibco) at 4^{o}C in order to inhibit the activity of enzymes. Then this cell suspension was washed three times in 6 mL of KG solution. A final cell suspension in 1 mL of KG solution was prepared.

A 0.2 mL volume of this cell suspension was pipetted onto a glass coverslip precoated with poly-D-lysine and laminin (Sigma). This coverslip was placed in a 35 mm culture dish filled with 2 mL of maintenance solution containing (in mM): 133 NaCl, 5 KCl, 0.8 $CaCl_2$, 1.3 $MgCl_2$, 5 Glucose and 10 HEPES with penicillin (100 U/mL, Sigma) and streptomycin (100 μg/mL, Sigma) (Zhang et al. 1991). Cells were kept at 4^{o}C in a refrigerator for 48 hours prior to use, in order to allow firm attachment to the substrate.

2. Identification Of Isolated CVSMCs

Cerebrovascular smooth muscle cells were identified using the Masson trichrome stain (Masson, 1929; Spatz et al. 1983). In this method, the cells on the coverslip were first fixed with 2.5 % formalin in phosphate buffer solution (PBS) containing (in mM): 149 NaCl, 2 KH_2PO_4, 4.2 Na_2HPO_4, pH 7.4 for 10 minutes. Nuclei were stained with 50 % iron hematoxylin (1:1 in H_2O) for 5 minutes, followed by differentiation with 1 % acid alcohol for several seconds. After washing with distilled water, the cells were treated with 2 % Ponceau acid solution (dissolved in 1 % acetic acid) for 2.5 minutes. This dye stained the cytoplasm of smooth muscle cells. The cells were washed with distilled water and finally differentiated with 1 % phosphotungstic acid for 5 minutes. The nuclei of CVSMCs and background cells (endothelia and fibroblasts) were stained black. The cytoplasm of CVSMCs was stained red, while the cytoplasm of connective tissue cells was stained blue (Masson, 1929).

3. Electrophysiology

Patch clamp recordings were carried out at room temperature (21-23^{O}C). At the time of recordings, one culture dish containing cells was taken out of the refrigerator. The maintenance solution was drawn off and replaced with 2 mL of a saline appropriate to the experimental design. The culture dish was then mounted on the stage of an inverted, phase-contrast microscope (Olympus CK, Tokyo, X300 magnification).

Single BK channel currents were recorded using standard patch clamp techniques. Since halothane is applied to the external face of cell membranes during surgical anesthesia, the outside-out patch configuration may be considered the optimal recording mode in studies of halothane action. However, in the present study, the inside-out patch mode was utilized, since it proved difficult to routinely obtain outside-out patches from CVSMCs. This difficulty reflected problems in forming the intermediate, whole-cell recording state, probably due to the use of relatively high resistance patch electrodes. Halothane is a highly lipid soluble agent, which can readily pass through the lipid bilayer. It was assumed that the agent equilibrated rapidly with its sites of action by dissolving in the lipid bilayer.

To get inside-out patches, a low resistance seal was produced upon mechanical contact between the patch electrode and the cell membrane. With gentle suction, a cell-attached gigaohm seal was formed. To form an inside-out patch, the electrode was withdrawn from the cell surface, creating a membrane vesicle at the electrode tip. The outer membrane of this vesicle was ruptured by rapid passage through the solution/air interface (Hamill et al. 1981).

Patch electrodes were fabricated from borosilicate glass (1.5 mm OD X 0.75 mm ID, Frederik Haer Corp, Brunswick, ME) using a two-stage vertical puller (David Kopf 700). The electrode tip outer diameters ranged from 2-4 μm prior to fire

polishing. Patch electrodes were coated to near the tip with 3140 RTV sealant (Dow Corning Corporation, Midland, Michigan) and were fire-polished just before use to produce a clean and smooth tip rim. This facilitated the formation of a large resistance seal between the electrode and the cell membrane (Hamill et al. 1981). Patch electrode resistance was 10-20 Mohm when filled with experimental salines.

4. Data Acquisition And Analysis

A List EPC-5 amplifier (Medical Systems Corp., New Jersey) was used to measure single channel currents. Amplified currents were displayed on a rectilinear pen recorder (Model 220, DC-100 Hz, Gould, Ohio) and stored on FM wideband tape at a bandwidth of DC-3 kHz (-3dB, Bessel) using an instrumentation recorder (Store 4DS, Racal-Decca, England).

Analysis was performed offline on an Atari Mega 4 computer (Atari, Sunnyvale, California) using the RECORD and TAC software programs devised by Instrutech Corporation, New York. The current signal was sampled at 8 kHz and subjected to a digital filter with Gaussian characteristics (f_c = 2 kHz). A threshold for event detection was set at 50 % of the mean open channel current amplitude for BK channels recorded in the patch under study. Frequency distributions for channel open times, closed times and amplitudes were calculated by analyzing 1000 - 1500 events in each data set. Use of a high intracellular free calcium concentration (100 uM) suppressed the non-stationary kinetics which BK channels exhibit when the cytoplasmic free calcium is low.

The probability, P_o of a single BK channel being open during a recording of duration T_{tot} was calculated from the expression:

$$P_o = (T_1 + T_2 + ... + T_N)/NT_{tot}.$$

Here N is the number of functional BK channels in the patch, and T_1, T_2...T_N are the

times for which at least 1,2...N channels are open (Mayer et al., 1990). Mean single channel current amplitude was obtained as the midpoint of a Gaussian curve fitted by eye to the frequency distribution of amplitudes.

Frequency distributions of channel open and closed times were plotted on a logarithmic time axis. This transforms the exponential function $y = N.e^{-t/TAU}$ into a curve with peak amplitude at the time constant, TAU, and an area proportional to the number of events in that component (Sigworth & Sine, 1987). These distributions were fitted by sums of two or three exponential terms using the method of maximum likelihood (Colquhoun & Sigworth, 1983). Reversal potentials for single channel currents were determined as the zero current intercepts of theoretical curves fitted to the data points by linear regression. Currents and voltages were denoted with respect to the cytoplasmic face of the membrane in all recordings.

5. Experimental Solutions

Recording pipettes were filled with a saline of composition (in mM): 140 KCl, 1.48 $CaCl_2$, 3 EGTA, 10 HEPES, pH 7.4. The free calcium concentration in this saline was calculated at 50 nM, using the programme Max Chelator, obtained from Standford University, California. The cytoplasmic face of the membrane patches was normally bathed in a control saline of composition (in mM): 140 KCl, 0.1 $CaCl_2$, 10 HEPES, pH 7.4. To demonstrate the Ca^{2+} sensitivity of BK channels, a low Ca^{2+} internal saline was also used, having a composition (in mM): 140 KCl, 10 HEPES, 3 EGTA, 0.27 mM $CaCl_2$, pH 7.4, free $[Ca^{2+}]_i$ = 5 nM. The effect of the potassium channel blocker tetraethylammonium (TEA^+) on BK channels was determined by dissolving the chloride salt of the drug (Eastman Kodak, Rochester, New York) in the saline bathing the cytoplasmic face of isolated membrane patches.

6. Gravity Perfusion System For Application Of Experimental Solutions

The saline bathing the cytoplasmic face of the patch could be exchanged using a gravity-fed perfusion system. The system consisted of the 60 mL reservoir mounted above the stage of the microscope. A short length of plastic tubing ran from the end of the reservoir to an L-shaped glass tube held in a micromanipulator. To facilitate rapid exchange of saline at the membrane patch, the tip of the patch electrode was positioned within 200 μm of the outlet of the perfusion system. During recording, patches were continuously perfused with experimental salines at a flow rate of 5 mL/min. Under these conditions, saline exchanges were effectively complete within 30 seconds of switching to the new solution.

7. Administration of Halothane

Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane, Ayerst, Montreal) was delivered to the 60 mL reservoir of perfusion saline in a compressed air carrier gas at a flow rate of 1 L/min using a Fluotec-3 vaporizer (Cyprane, Keighley, Yorkshire). Prior to recording, the experimental saline in the reservoir was bubbled with the gaseous mixture for a minimum of 45 minutes, in order to ensure complete equilibration with the anesthetic. During the recording session, the saline in the reservoir was continuously bubbled with the gaseous mixture to avoid loss of halothane to the atmosphere. Control experiments were performed using the saline bubbled with carrier gas only (0 % halothane). The pH of experimental salines was unaltered by equilibration with the anesthetic.

8. Determination Of Halothane Concentrations

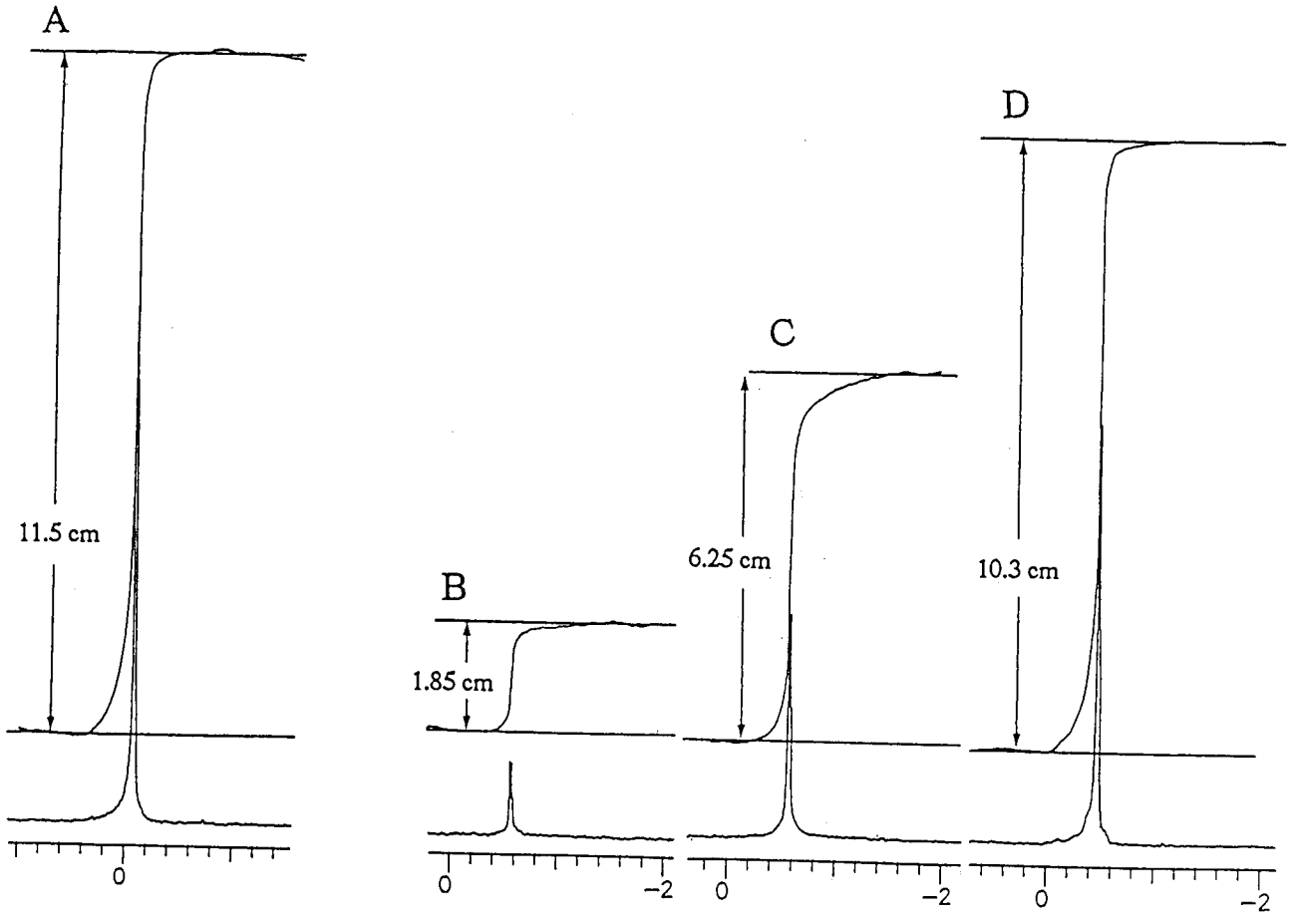
The concentration of halothane present in experimental salines was

determined at 21-23^{o}C using ^{19}fluorine nuclear magnetic resonance (^{19}F-NMR) techniques (Hausser & Kalbitzer, 1991) using a VARIAN XL-300 magnetic resonance machine (Varian Associates Inc., Palo Alto, CA). Aliquots (2.4 mL) of pre-equilibrated salines were collected from the reservoir and injected into NMR sampling tubes with an air-tight syringe. Trifluoroacetic acid (3 mM) was employed as an external standard solution (ESS) and was prepared fresh by dissolving the liquid stock solution in control saline. Halothane concentrations in salines were determined using the relation: [Halothane] = (I_{saline}/I_{ESS}).[ESS]. Here I_{saline} and I_{ESS} are the integrals for the resonance lines of halothane-containing salines and of ESS, respectively (Hausser & Kalbitzer, 1991; Miu & Puil, 1989). Vaporizer settings of 1, 3, and 5 % halothane in carrier gas were found to yield measured solution concentrations of 0.5, 1.6, and 2.8 mM, respectively (Fig. 1). These values were in good agreement with concentrations predicted by Avogadro's theory, assuming a saline/gas partition coefficient for halothane of 1.22 at 23^{o}C (Stoelting & Longshore, 1972). The aqueous EC_{50} for halothane anesthesia is 0.21 mM for humans, and 0.29 mM for rats (Steward et al., 1973). 1 - 2.5 EC_{50} is typically used to maintain surgical anesthesia (Atkinson et al., 1987). Therefore, the 0.5 mM concentration of halothane used in the present study is within the range routinely employed in clinical anesthesia.

9. Statistics

All data are presented as mean ± standard error of the mean (S.E.M.). Analysis of effects of halothane on BK channel properties was normally carried out using the Paired *t*-test. In some data sets, it was noted that the variances of control and halothane data were not equal. In these cases, a non-parametric test (*Sign* test) was applied to the data, as indicated in the result section. Data sets for which $P < 0.05$ were considered significantly different.

Figure 1. ^{19}F-NMR spectra (peaked curves) of 1 % (B), 3 % (C) and 5 % (D) halothane equilibrated in standard experimental saline at room temperature (21 - 23^{o}C). Panel A shows the corresponding spectrum for 3 mM trifluoroacetic acid, dissolved in experimental saline and employed as the external standard. The S-shaped curves above each spectrum indicate the integral of that spectrum, with amplitudes as shown by the vertical arrows. These integrals were used to calculate the concentration of halothane in each of the samples, as described in the text. The values obtained were (B) 0.5 mM, (C) 1.6 mM and (D) 2.8 mM. The theoretical concentration of halothane in each saline was calculated assuming a saline/gas partition coefficient of 1.22 at 23^{o}C. This yielded theoretical values of (B) 0.5 mM, (C) 1.5 mM and (D) 2.5 mM, in good agreement with the measured concentrations.


A
11.5 cm
0
B
1.85 cm
0
−2
C
6.25 cm
0
−2
D
10.3 cm
0
−2

RESULTS

SECTION I: IDENTIFICATION OF THE BK CHANNEL

Isolated inside-out membrane patches excised from CVSMCs displayed a variety of single channel currents when exposed to 140 mM KCl solutions at both membrane faces. In some cases, small currents corresponding to single channel conductances in the range 40 - 60 pS were observed. These events could readily be distinguished from the activity of BK channels by their smaller conductances and insensitivity to changes in calcium ion concentration at the cytoplasmic membrane face. Large conductance, Ca^{2+}-activated K^+ (BK) channel currents were observed in 73 % of inside-out patches studied, as expected from previous studies on these cells (Wang & Mathers, 1993). BK channels were identified on the basis of a large single channel conductance (> 150 pS), dependence of open probability on $[Ca^{2+}]_i$ and sensitivity to block by tetraethylammonium ions. No other calcium-dependent channels of comparably large conductance were present in these patches.

1. Conductance And Reversal Potential.

Single BK channel currents were observed in 29/40 inside-out patches studied. Fig. 2 shows currents flowing through a single BK channel in an inside-out patch voltage-clamped at a variety of membrane potentials, V. This patch was exposed to symmetrical 140 mM K^+ solutions and $[Ca^{2+}]_i$ was 100 μM.

Fig. 3a shows the amplitude histogram for single BK channel currents recorded from an isolated patch voltage-clamped at V = -60 mV. In all patches studied, these amplitude histograms were well fit by single Gaussian terms, indicating that no significant contribution from sub-state conductance levels was evident.

Figure 2. Single BK channel currents recorded in an inside-out patch of CVSMC membrane voltage-clamped at the indicated membrane potentials, V. Channel closed current level is denoted by 0, while 1 indicates channel open current level. Inward current is represented by downward deflection from baseline. Currents reversed polarity at V = 0 mV. The patch was exposed to symmetrical 140 mM K^+ solutions, $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$ were 100 μM and 50 nM, respectively. Bandwidth of recording DC-2 kHz. Temperature, 23^oC.

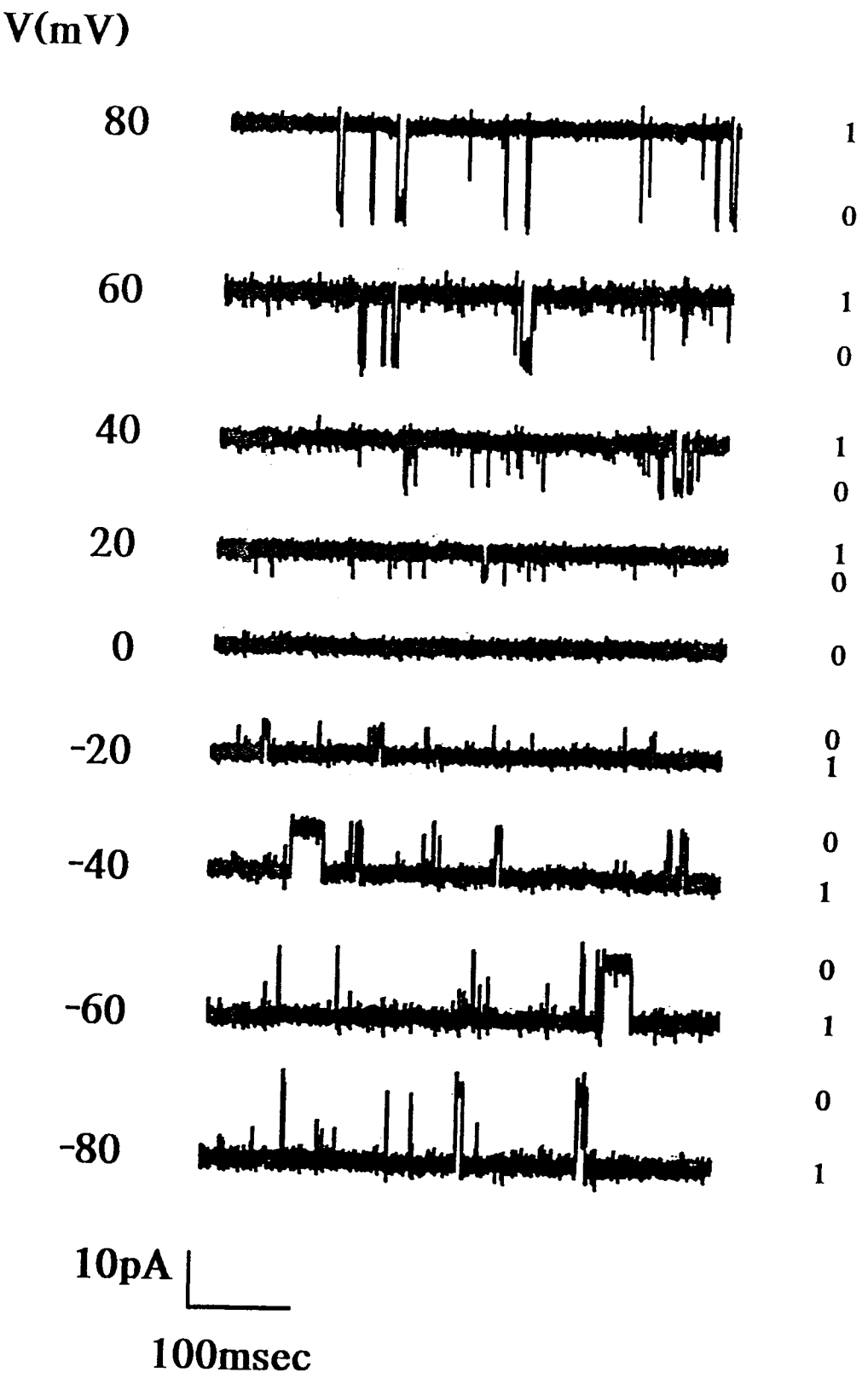
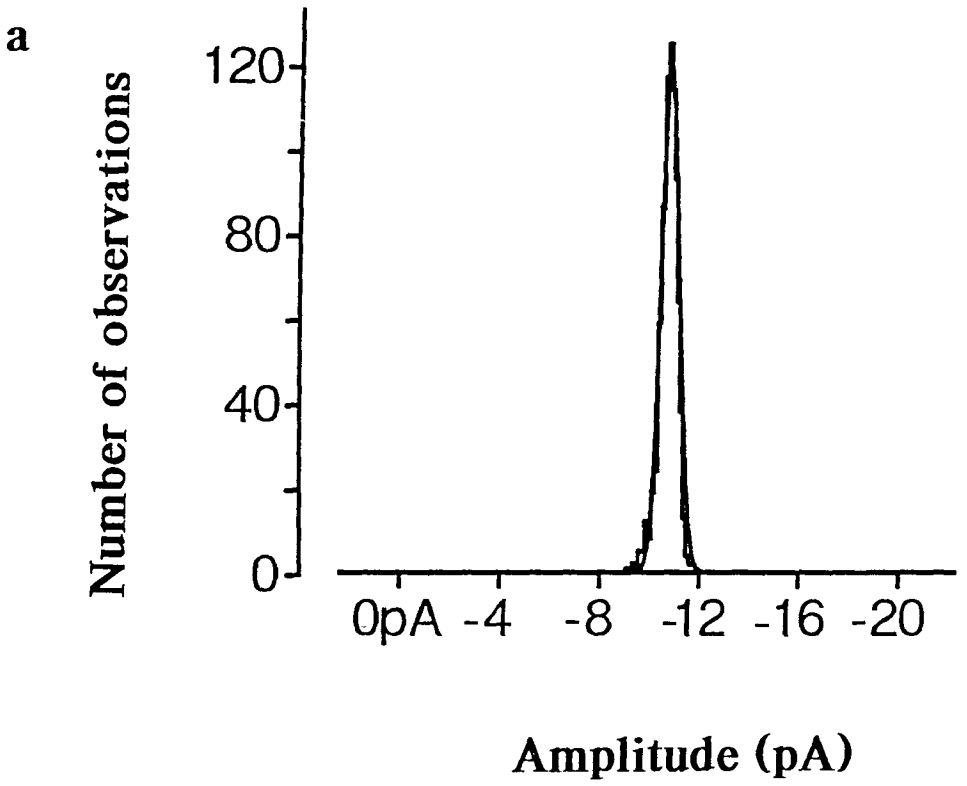
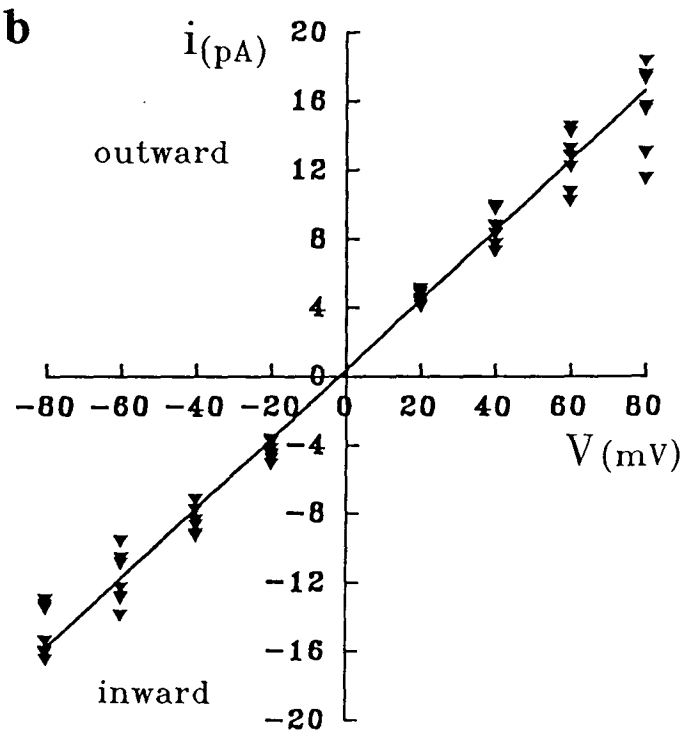

V(mV)
80
60
40
20
0
-20
-40
-60
-80
1
0
1
0
1
0
1
0
0
0
1
0
1
0
1
0
1
10pA
100msec

Figure 3.

a. Amplitude distribution for BK channel currents recorded from an inside-out patch voltage-clamped at V = - 60 mV. This distribution was fitted by a single Gaussian term (smooth curve) with modal value at - 10.8 pA.

b. Current-voltage relationship of BK channels recorded from 8 inside-out patches. The straight line was fitted to these data by least squares regression and corresponded to a mean conductance of 205 ± 5.5 pS. The interpolated reversal potential for BK channel currents was - 2 mV. Recording conditions as in Fig. 2.


a
Number of observations
120
80
40
0
0pA -4 -8 -12 -16 -20
Amplitude (pA)


b
i(pA)
20
16
12
8
4
0
outward
-80 -60 -40 -20 0 20 40 60 80
V (mV)
-4
-8
-12
-16
-20
inward

As shown in Fig. 3b, the current-voltage relationship of BK channels was linear over a voltage range of - 80 mV to + 80 mV, showed a reversal potential of -2 mV and a mean slope conductance of 205 ± 5.5 pS.

2. Effect Of Varying $[Ca^{2+}]_i$ On The Open Probability Of BK Channels.

To demonstrate the sensitivity of BK channels to internal free calcium concentrations ($[Ca^{2+}]_i$), single channel recordings were made from isolated inside-out patches (n=9) exposed to symmetrical 140 mM K^+ solutions, while the membrane potential of the patch was voltage-clamped to V = -60 mV and $[Ca^{2+}]_i$ was decreased from 100 μM to 5 nM. Fig. 4 shows single BK channel currents recorded from a membrane patch containing one active BK channel. When $[Ca^{2+}]_i$ was 100 μM, this channel remained open most of the time (P_O = 0.91). On decreasing $[Ca^{2+}]_i$ to 5 nM, very few channel openings were seen (P_O = 0.0004). This effect reversed on return to saline containing 100 μM free calcium ions. These observations indicate that the open probability of this channel was sensitive to internal free Ca^{2+} concentration, and that the open probability was strongly promoted by an increase in $[Ca^{2+}]_i$.

3. Blocking Effect Of Internal TEA^+ On Current Flow In Single BK Channels

Internal application of tetraethylammonium ions, TEA^+ is known to block BK channels of CVSMCs (Wang & Mathers, 1993). The effect of internal TEA^+ on the BK channel was studied in isolated inside-out membrane patches voltage-clamped at V = + 40 mV with $[Ca^{2+}]_i$ = 100 μM. Fig. 5 shows the effect of this

Figure 4. Effect of changing $[Ca^{2+}]_i$ from 100 μM to 5 nM on the opening of BK channels. Recordings show single channel currents flowing through a BK channel in an inside-out patch bathed in symmetrical 140 mM K^+ solutions. The recording pipette contained 50 nM free Ca^{2+}. The membrane patch was voltage-clamped at a potential of -60 mV throughout. O indicates channel closed and 1 indicates channel open. Bandwidth DC-2 KHz.

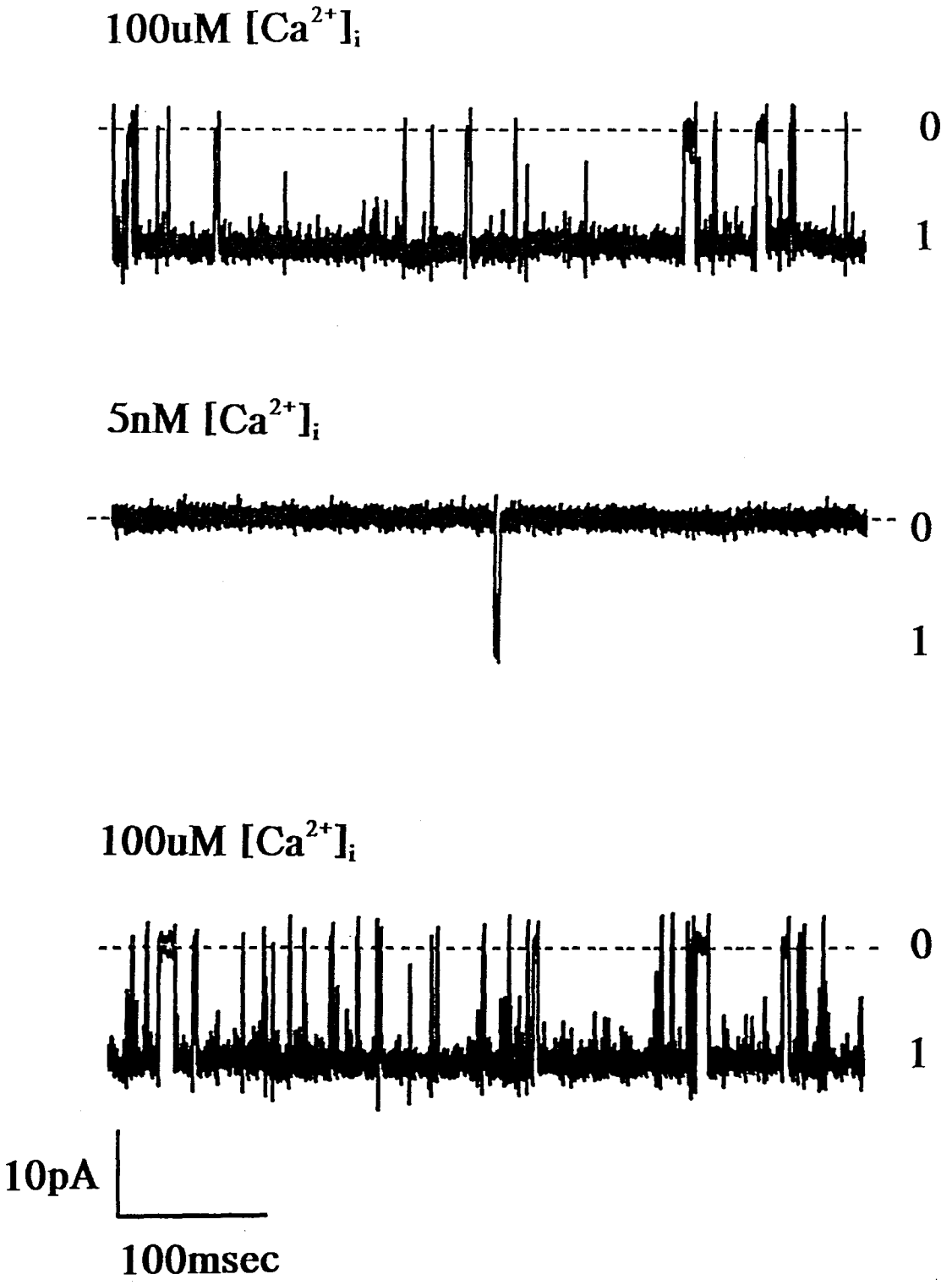
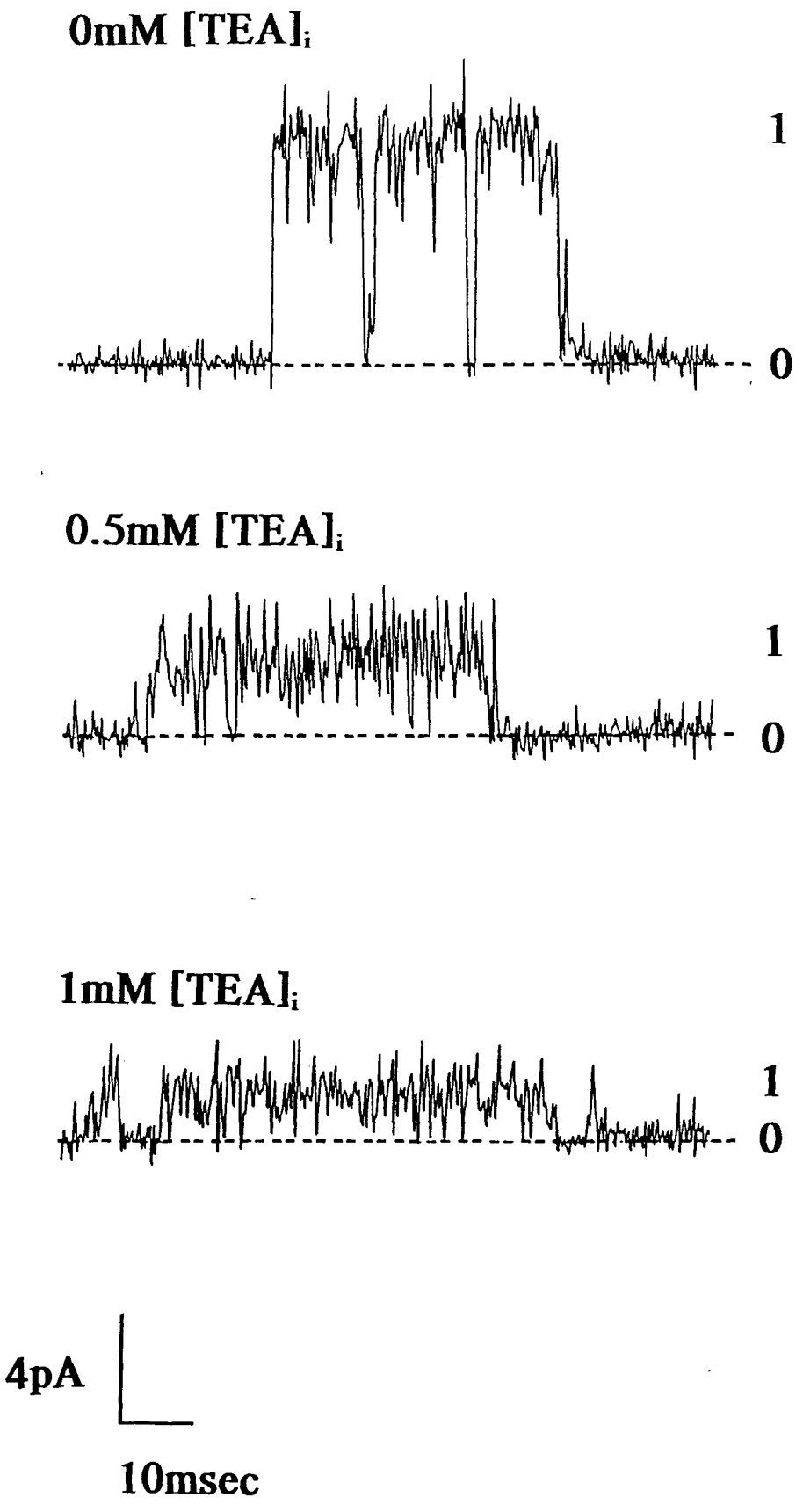

100uM [Ca2+]i
0
1
5nM [Ca2+]i
0
1
100uM [Ca2+]i
0
1
10pA
100msec

Figure 5. Blockade of the BK channel by internal application of TEA^+. Single BK channel currents were recorded from an isolated inside-out patch voltage-clamped at V = + 40 mV and exposed to symmetrical 140 mM K^+ solutions. TEA^+ was applied to the cytoplasmic membrane face of the patch at the concentrations denoted by $[TEA]_i$. $[Ca^{2+}]_i$ = 100 μM. Channel closed current level is denoted by 0, while 1 indicates channel open level. Bandwidth DC-2 kHz.


0mM [TEA]i
1
0
0.5mM [TEA]i
1
0
1mM [TEA]i
1
0
4pA
10msec

drug on BK channel activity, examined in an inside-out membrane patch voltage-clamped at V = + 40 mV. This patch was exposed to symmetrical 140 mM K^+ solutions. TEA^+ was applied by bath perfusion to the cytoplasmic membrane face. Under these conditions, TEA^+ caused a dose-dependent and reversible reduction in the amplitude of single BK channel currents, as expected from previous studies (Wang & Mathers, 1993).

SECTION II: EFFECTS OF HALOTHANE ON BK CHANNEL CURRENTS

1. Effect Of Halothane On The Conductance Of Single BK Channels.

The effect of halothane on the conductance of the BK channel was studied in 29 inside-out membrane patches. Figures 6, 7 and 8 show currents flowing through single BK channels recorded in inside-out patches before, during and after exposure to 0.5, 1.6 and 2.8 mM halothane, respectively. Inspection of these traces suggested that halothane had no discernable effect on the amplitude of currents flowing in open BK channels, while the two higher doses of the anesthetic apparently reduced the open probability of these channels in a reversible manner.

Fig. 9 shows histograms of BK current amplitudes measured before, during and after application of 2.8 mM halothane to a single membrane patch voltage-clamped to V = -60 mV. As in all the membrane patches examined in this study, these amplitude histograms were well fit by single Gaussian terms, indicating that no sub-state conductance levels were present, either in the absence or presence of halothane. It may be seen that halothane had no effect on the mean amplitude of BK channel currents measured in this patch.

Figure 6. Single BK channel currents recorded before, during, and after exposure to 0.5 mM halothane. Currents were obtained from an inside-out patch bathed in symmetrical 140 mM K^+ solutions. Halothane was applied to the cytoplasmic face of the patch. Channel closed current level is denoted by 0, while 1 shows open channel current level. Membrane potential was V = -60 mV. $[Ca^{2+}]_i$ = 100 μM. Bandwidth DC-2 kHz.

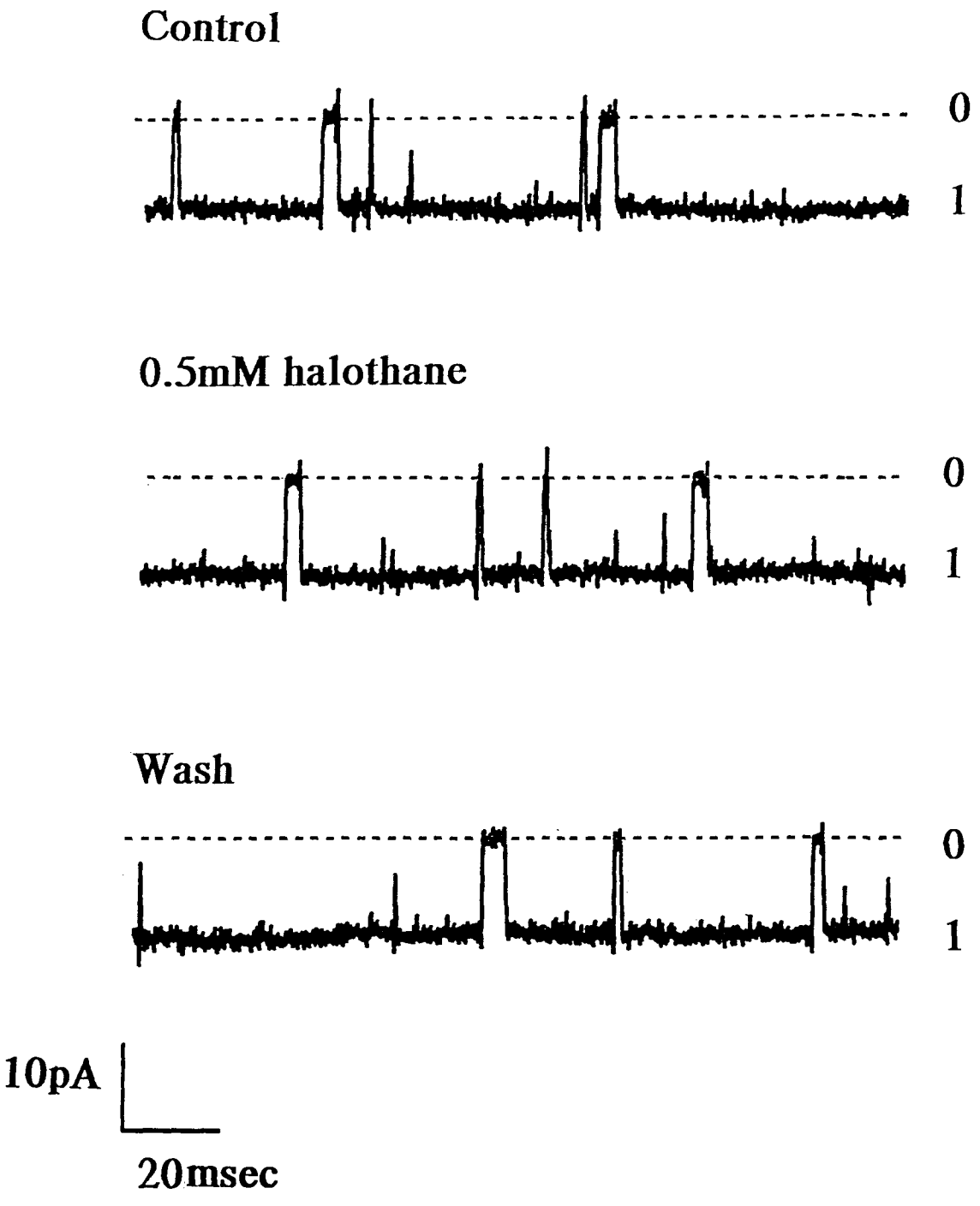

Control
0
1
0.5mM halothane
0
1
Wash
0
1
10pA
20msec

Figure 7. Single BK channel currents recorded before, during, and after exposure to 1.6 mM halothane. Currents were obtained from an inside-out patch bathed in symmetrical 140 mM K^+ solutions. Halothane was applied to the cytoplasmic face of the patch. Channel closed current level is denoted by 0, and 1 shows open channel current level. Membrane potential was V = -60 mV. $[Ca^{2+}]_i$ = 100 μM. Bandwidth DC-2 kHz.

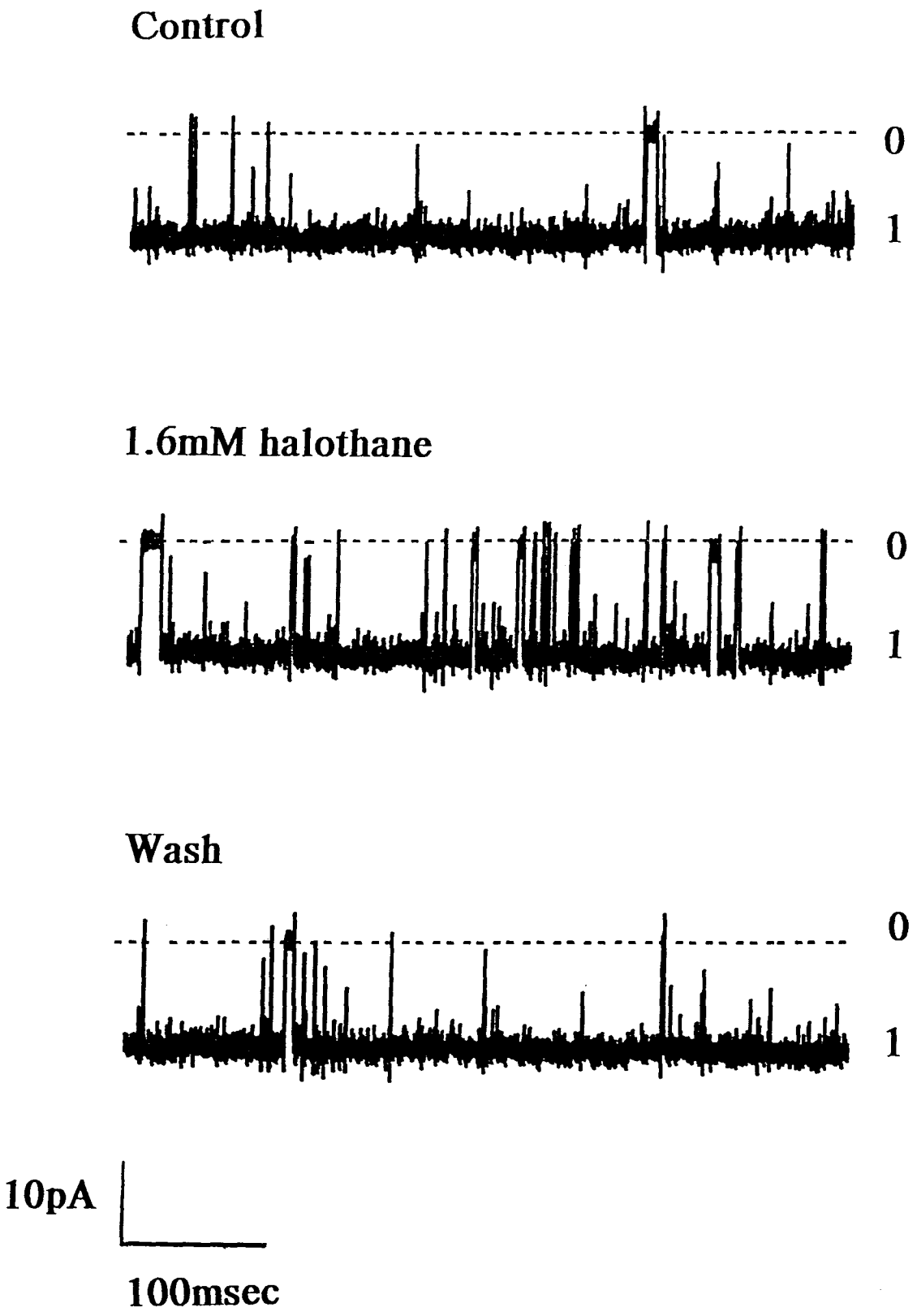

Control
0
1
1.6mM halothane
0
1
Wash
0
1
10pA
100msec

Figure 8. Single BK channel currents recorded before, during, and after exposure to 2.8 mM halothane. Currents were obtained from an inside-out patch bathed in symmetrical 140 mM K^+ solutions. Halothane was applied to the cytoplasmic face of the patch. Channel closed current level is denoted by 0, while 1 shows open channel current level. Membrane potential was V = -60 mV. $[Ca^{2+}]_i$ = 100 μM. Bandwidth DC-2 kHz.

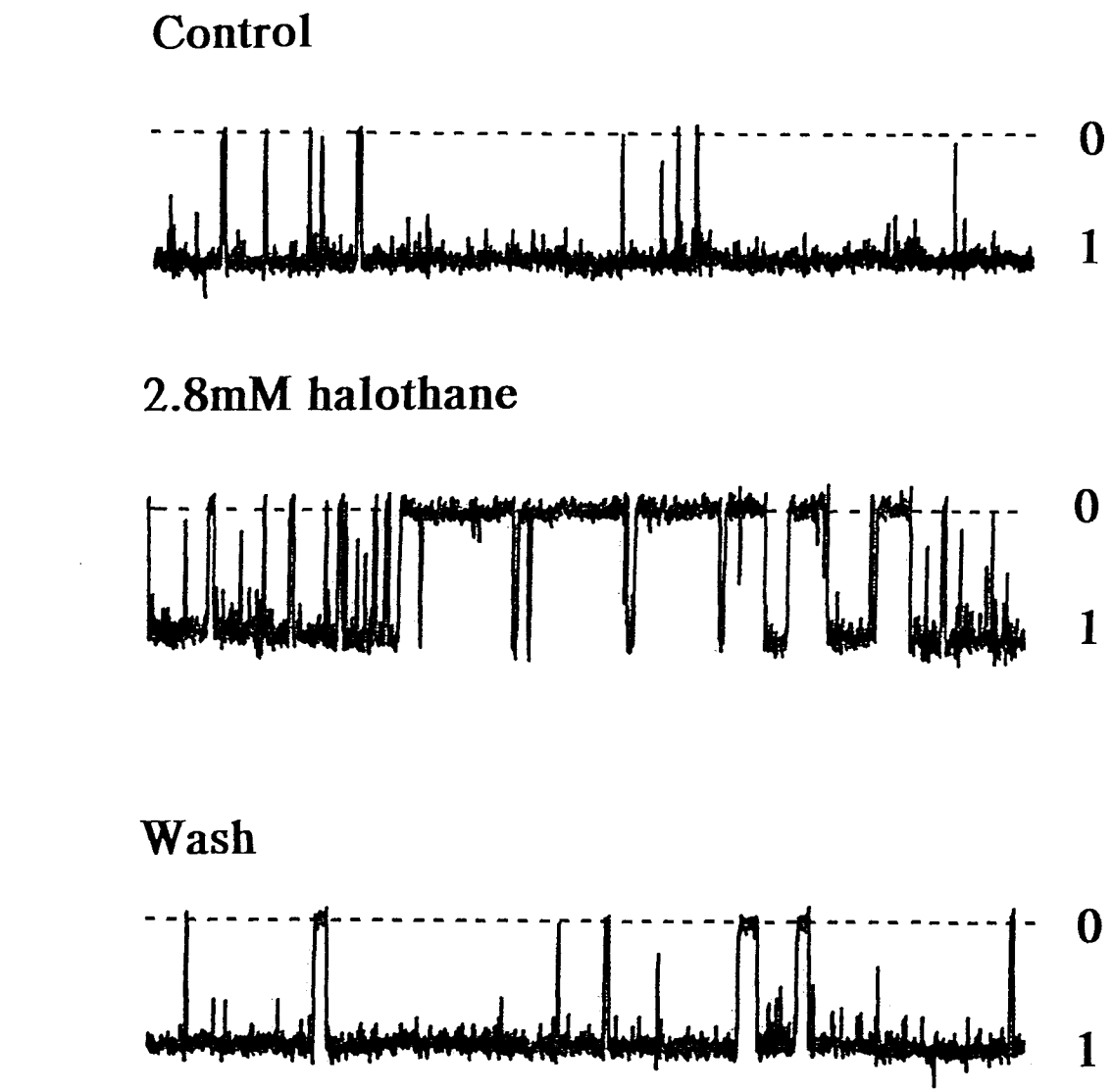

Control
0
1
2.8mM halothane
0
1
Wash
0
1
10pA
100msec

Figure 9. Effect of 2.8 mM halothane on the amplitude histogram of BK channel currents recorded from a single inside-out membrane patch. The patch was exposed to symmetrical 140 mM K^+ solutions and voltage-clamped to V = -60 mV. Halothane was applied to the cytoplasmic face of the patch. All three amplitude distributions were well fitted by single Gaussian functions (smooth curves), with modal values at -12.9 pA, -13.0 pA and -13.4 pA for data obtained before, during and after exposure to halothane, respectively. Recording conditions as in Fig. 6.

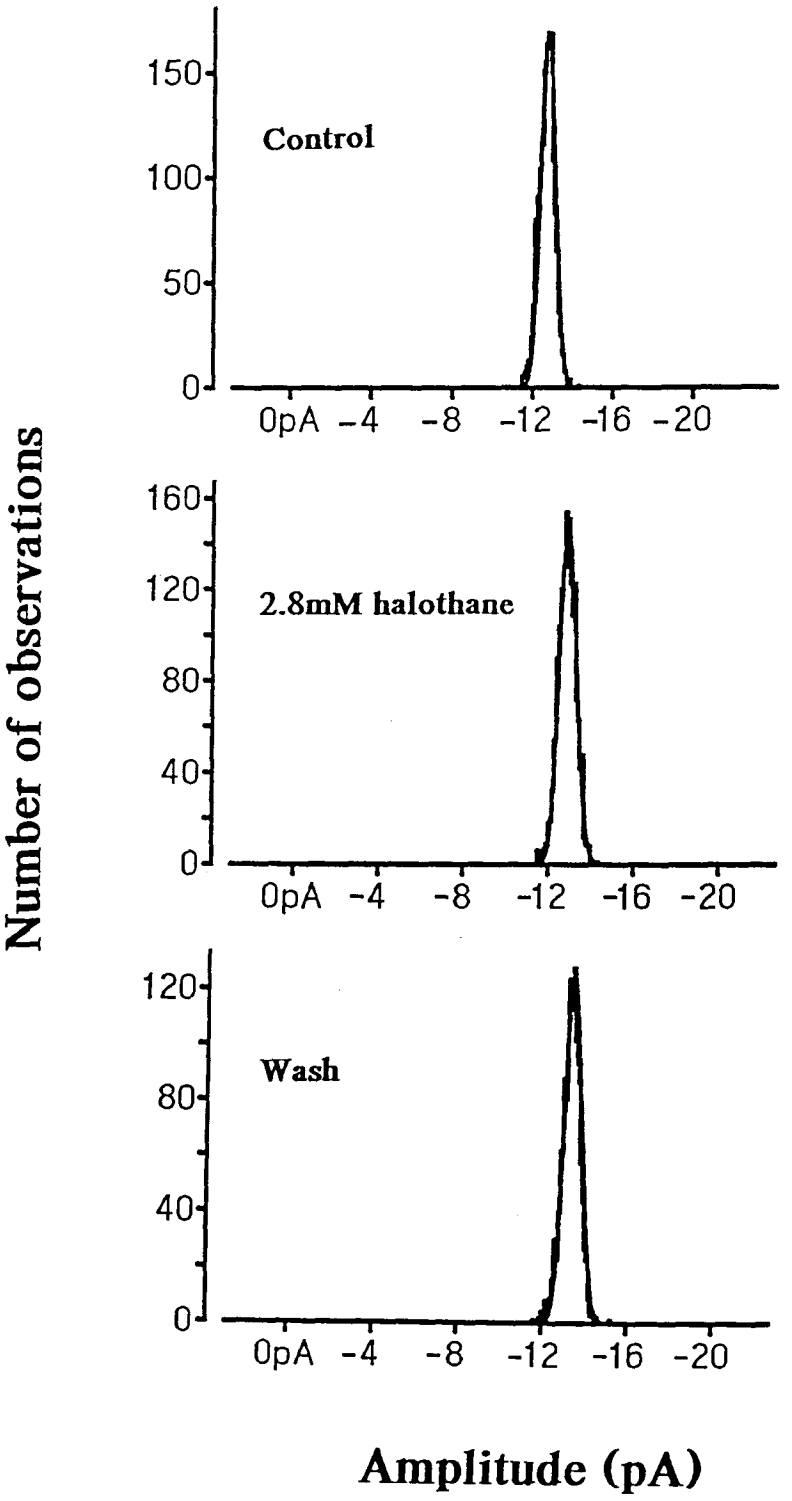

Number of observations
150
100
50
0
Control
0pA -4 -8 -12 -16 -20
160
120
80
40
0
2.8mM halothane
0pA -4 -8 -12 -16 -20
120
80
40
0
Wash
0pA -4 -8 -12 -16 -20
Amplitude (pA)

Fig. 10 illustrates the current-voltage relationship of BK channel currents obtained from a single membrane patch in the absence and presence of 2.8 mM halothane. Neither the slope of the current-voltage relationship (the conductance of BK channels, control, 217 pS, halothane, 219 pS), nor the interpolated reversal potential of the single channel current was altered by application of halothane.

The effects of 0.5, 1.6 and 2.8 mM halothane on the conductance of BK channels are summarized in Table 1. This table shows that halothane had no effect on the conductance of BK channels at any of the concentrations tested ($P > 0.05$).

2. Effect Of Halothane On The Open Probability Of The BK Channel.

Inspection of Figs. 6, 7 and 8 suggested that halothane application altered the open probability, P_O of BK channels in a dose-dependent manner. Confirmation of this effect is shown in Figure 11. At a concentration 0.5 mM, halothane had no significant effect on the open probability of BK channels ($P > 0.05$, $n=7$). However, application of 1.6 mM halothane reduced P_O by 14 % ($P < 0.01$, $n=11$). On increasing the anesthetic concentration to 2.8 mM, a larger reduction of 55 % was seen in P_O, ($P < 0.01$, $n=11$). This effect reversed on washing with drug-free solution.

3. Effects Of Halothane On The Open Time Distribution Of BK Channel currents.

The halothane-induced reduction in the open probability of BK channels could result from a decreased mean open time, an increased closed time, or both. In order to resolve this issue, the effects of halothane on the kinetic properties of BK channels were examined.

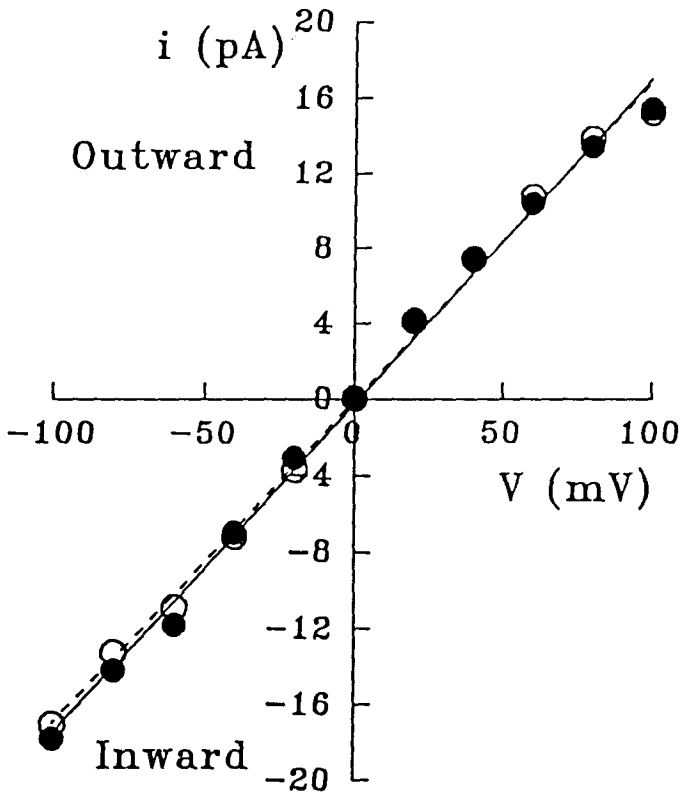
Table 1. The effects of 0.5, 1.6 and 2.8 mM halothane on the conductance of BK channels studied in inside-out patches. Patches were voltage-clamped to V = -60 mV and exposed to symmetrical 140 mM K^+ solutions, with $[Ca^{2+}]_i$ = 100 μM. The number of patches from which the mean value was determined is indicated by n.

NS indicates that the data obtained in the presence of halothane were not significantly different from control values ($P > 0.05$), as determined using a Paired *t*-test.

Table 1. Effect of halothane on the conductance of BK channels

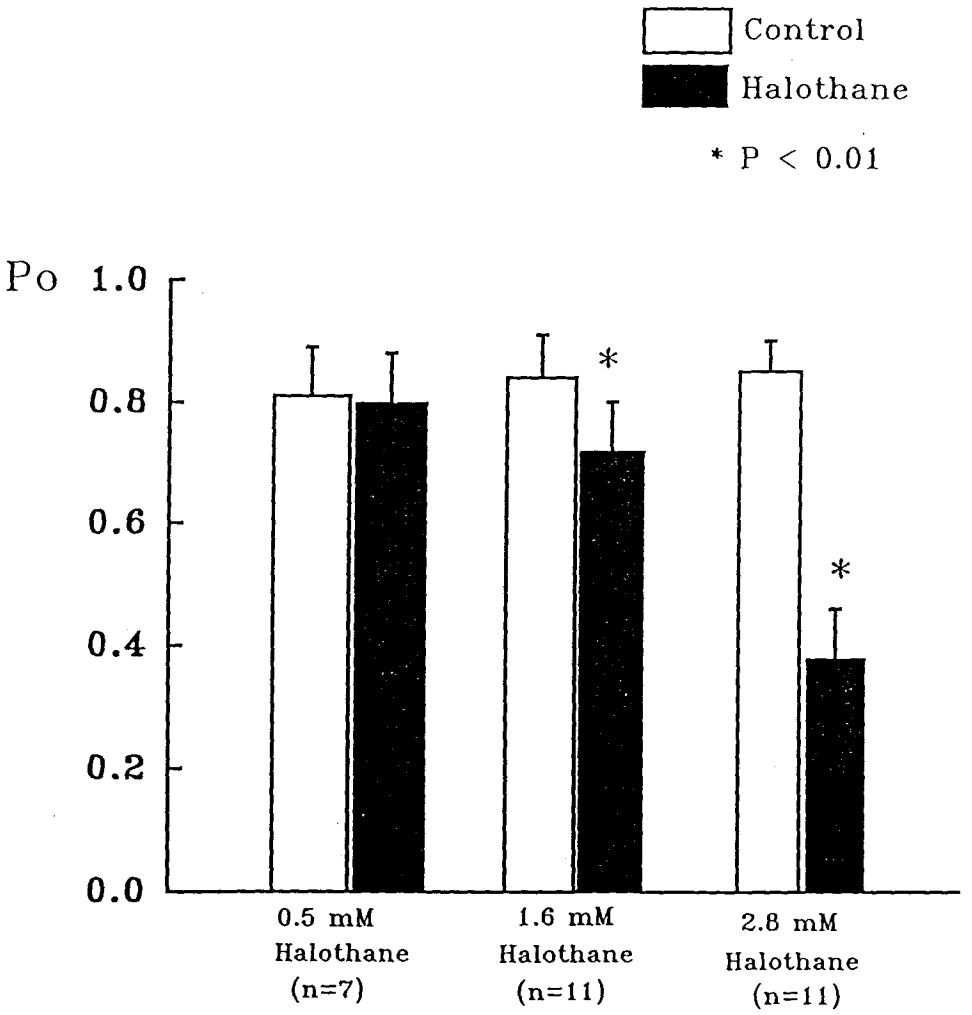
Concentration of halothane	CONDUCTANCE			
	Control	Halothane	Wash	P
0.5 mM (n=7)	179 pS (±8.9)	176 pS (±11)	177 pS (±11)	NS
1.6 mM (n=11)	193 pS (±8.3)	190 pS (±10)	189 pS (±11)	NS
2.8 mM (n=11)	202 pS (±11)	201 pS (±12)	202 pS (±12)	NS

Figure 10. Current-voltage relationship of single BK channel currents measured in the absence (open circles) and presence (closed circles) of 2.8 mM halothane. Both current-voltage relationships were well fitted by least squares regression to the indicated straight lines. These fits yielded single channel conductances of 217 pS (dashed line) and 219 pS (solid line) for control and halothane-containing salines, respectively. The interpolated reversal potentials for control and halothane-currents were +2 mV and +2.5 mV respectively. Recording conditions as in Fig. 6.



○ control ● 2.8 mM halothane

Figure 11. Effects of halothane at 0.5, 1.6 and 2.8 mM on the open probability, P_O of BK channels studied in inside-out patches of CVSMC membrane. Patch membranes were voltage-clamped to V = -60 mV. $[Ca^{2+}]_i$ = 100 μM and $[Ca^{2+}]_O$ = 50 nM. Halothane was applied to the cytoplasmic face of patches. * indicates that halothane significantly reduced P_O when compared to control values ($P < 0.01$). Significance was determined using a Paired *t*-test. The number of patches in each group is denoted by n.


Control
Halothane
* P < 0.01
Po
1.0
0.8
0.6
0.4
0.2
0.0
*
*
0.5 mM Halothane (n=7)
1.6 mM Halothane (n=11)
2.8 mM Halothane (n=11)

In both the absence and presence of halothane, open time distributions for BK channel currents were well fitted by the sum of two exponential functions, $y = N_{OS}.e^{-t/TAU_{OS}} + N_{OL}.e^{-t/TAU_{OL}}$ (Fig. 12). Here TAU_{OS} and TAU_{OL} were the time constants governing short-duration openings and long-duration openings respectively. The zero-time amplitudes of these components were N_{OS} and N_{OL} respectively. The total number of events in the fit components governed by TAU_{OS} and TAU_{OL} were calculated from the relationships, $A_{OS} = (N_{OS}/BW).TAU_{OS}$ and $A_{OL} = (N_{OL}/BW).TAU_{OL}$, where BW is the bin width of the plotted histograms.

Fig. 13 shows the influence of 0.5, 1.6 and 2.8 mM halothane on the mean values of TAU_{OS} and TAU_{OL} obtained from fitting the open time distributions of BK channel currents. It can be seen that halothane had no significant effect on the value of TAU_{OS} at any of the anesthetic doses tested. However, halothane caused a dose-dependent reduction in the value of TAU_{OL}. At a concentration 0.5 mM, halothane had no effect on the value of TAU_{OL}. On increasing the anesthetic dose to 1.6 mM and 2.8 mM, TAU_{OL} was reversibly reduced by 27 % (control, 26 ± 5.0 msec, halothane, 19 ± 4.1 msec, n = 11) and 54 % (control, 41 ± 8.5 msec, halothane, 19 ± 3.4 msec, n = 9), respectively.

The mean open time, T_{open} of BK channels was calculated from the fit parameters to the open time distributions using the relation: $T_{open} = A_{OS}/(A_{OS} + A_{OL}).TAU_{OS} + A_{OL}/(A_{OS} + A_{OL}).TAU_{OL}$. As shown in Table 2, 0.5 mM halothane had no significant effect on T_{open} ($P > 0.05$). However, halothane did significantly reduce T_{open} when applied at a concentration of 1.6 mM or of 2.8 mM, ($P < 0.05$). This effect reversed on perfusion with drug-free saline. These data indicate that the fall in the open probability, P_o in the presence of halothane was due in part to a decrease in the mean open time of BK channels. This change itself reflected a decrease in the time constant governing long-duration openings of the channel.

Figure 12. Effect of 2.8 mM halothane on the open time distribution of BK channel currents. Data were obtained from a single inside-out patch voltage-clamped at V = -60 mV with $[Ca^{2+}]_i$ = 100 μM. Note the use of a logarithmic time axis in all three plots, which converts each exponential function to a curve with peak at its time constant. Each distribution was well fitted by the sum of two exponential terms (smooth curves). In the control data, short-duration openings were governed by the time constant, TAU_{OS} = 0.5 msec and made up 6 % of total openings. Long-duration openings were governed by the time constant TAU_{OL} = 46 msec and made up the remaining 94 % of openings.

Corresponding values for the other two distributions were as follows. Halothane-containing saline: TAU_{OS} = 0.4 msec, 14 % of openings; TAU_{OL} = 20 msec, 86 % of openings. After wash with drug-free saline: TAU_{OS} = 0.5 msec, 3 % of openings and TAU_{OL} = 32 msec, 97 % of the total openings.

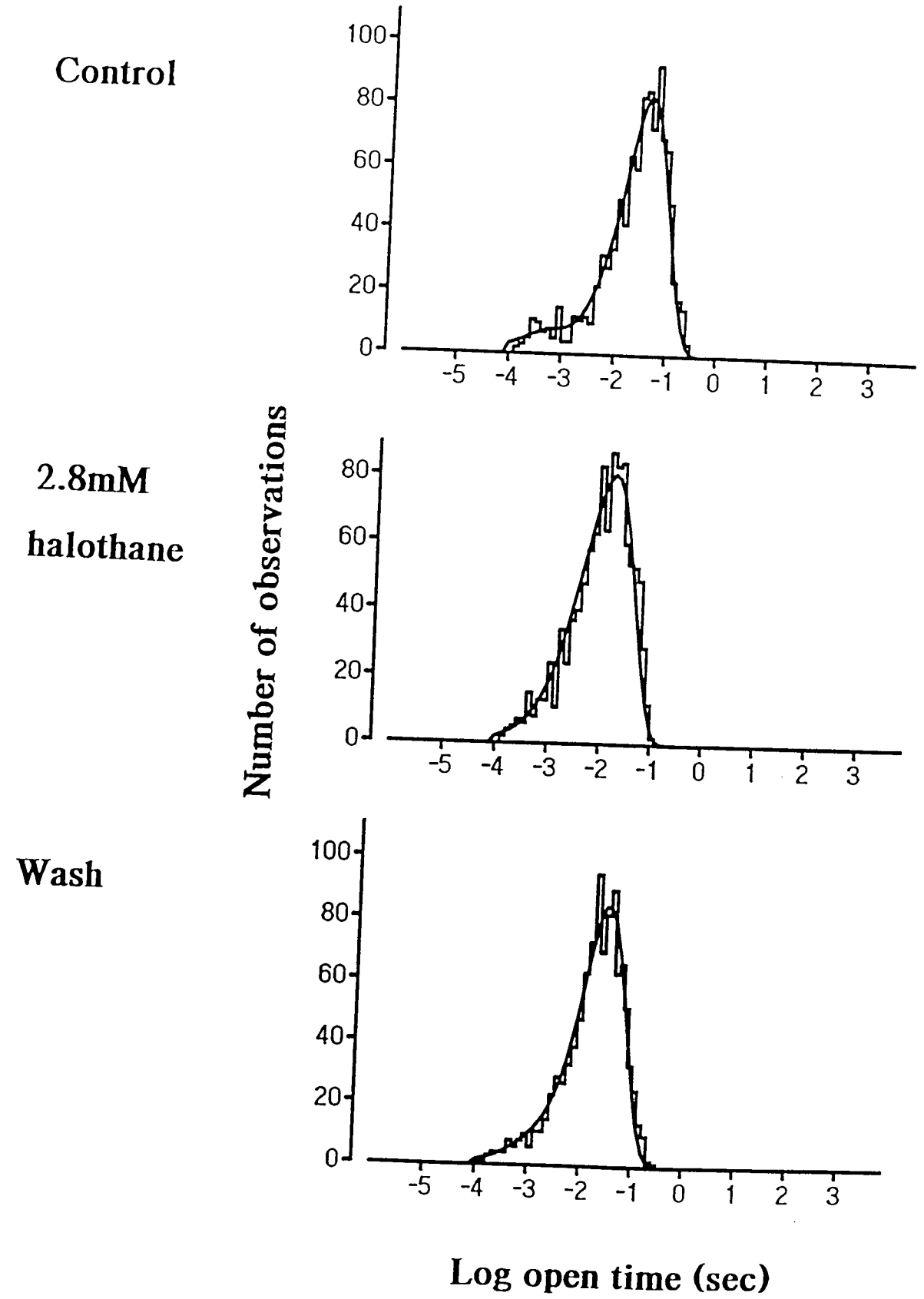

Control
2.8mM
halothane
Wash
Number of observations
Log open time (sec)
100
80
60
40
20
0
-5
-4
-3
-2
-1
0
1
2
3

Figure 13. Effects of halothane at 0.5, 1.6 and 2.8 mM on the time constants governing short-duration openings (TAU_{OS}) and long-duration openings (TAU_{OL}) obtained from fitting open time distributions of BK channel currents. n denotes the number of patches studied in each group. * indicates that halothane significantly decreased the mean value of the time constant, when compared to control ($P < 0.05$, *Sign*-test).

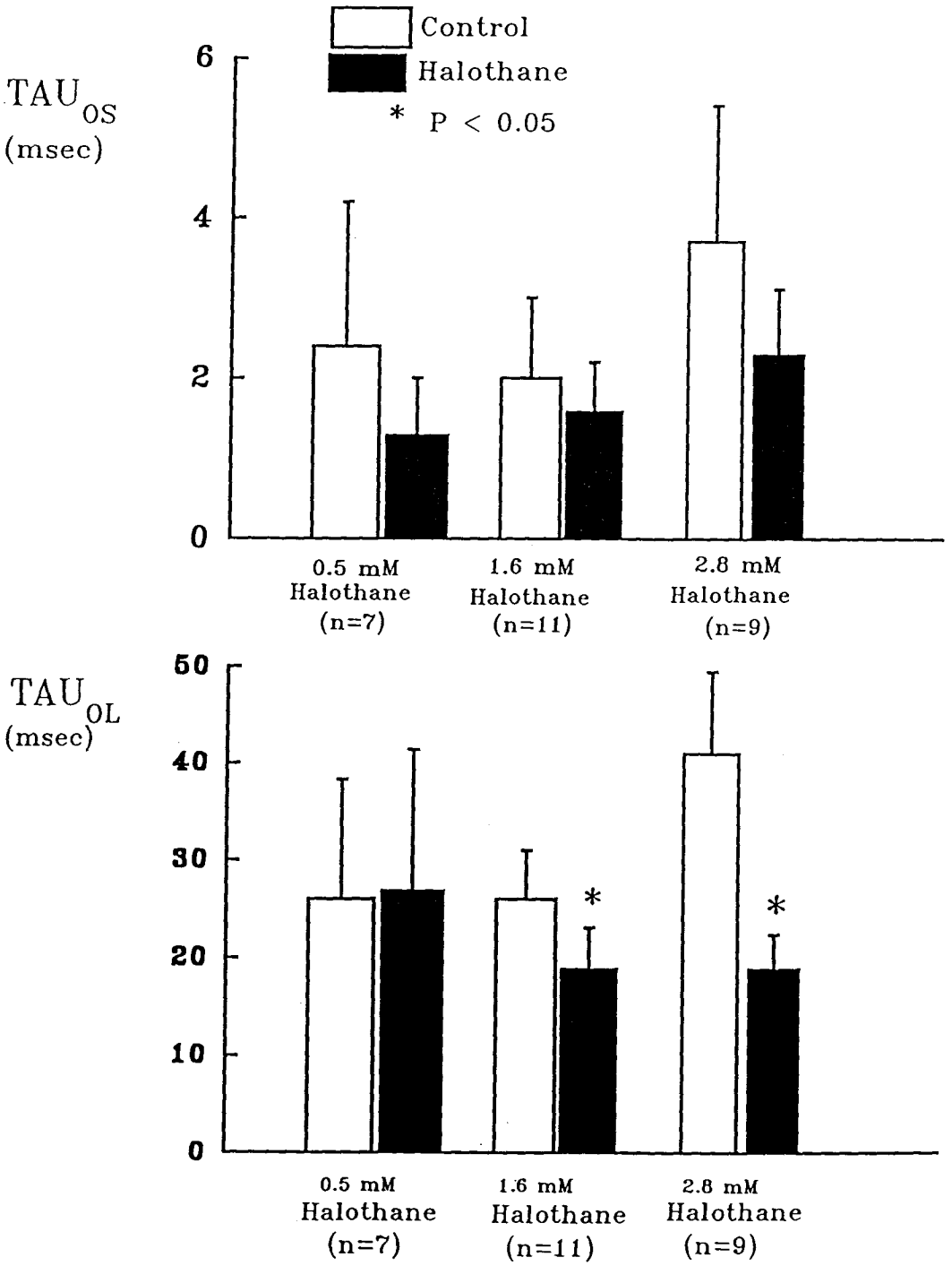

Control
Halothane
* P < 0.05
TAU OS (msec)
6
4
2
0
0.5 mM Halothane (n=7)
1.6 mM Halothane (n=11)
2.8 mM Halothane (n=9)
TAU OL (msec)
50
40
30
20
10
0
*
*
0.5 mM Halothane (n=7)
1.6 mM Halothane (n=11)
2.8 mM Halothane (n=9)

Table 2. Effect of halothane at 0.5, 1.6 and 2.8 mM on the mean open time of BK channels, T_{open}, as calculated from the fit parameters of open time distributions (for details, see text). Recording conditions as in Table 1. n denotes the number of patches studied in each group. * indicates mean values significantly lower than control data ($P < 0.05$, Paired *t*-test).

TABLE 2. Effect of halothane on the mean open time of BK channels (T_{open})

	T_{open}		
Concentration of halothane	Control	Halothane	Wash
0.5 mM (n=7)	20.4 msec (±10.0)	24.0 msec (±13.6)	23.0 msec (±11.1)
1.6 mM (n=11)	19.5 msec (±4.2)	15.1* msec (±4.1)	17.6 msec (±4.4)
2.8 mM (n=9)	29.4 msec (±7.9)	12.8* msec (±3.2)	24.2 msec (±7.2)

4. Effects Of Halothane On The Closed Time Distribution Of BK Channel Currents.

Closed time distributions of BK channel currents recorded in the absence and presence of 2.8 mM halothane are shown in Fig. 14. Each of these distributions was well fit by the sum of three exponential functions, $y = N_{CS}.e^{-t/TAU_{CS}} + N_{CM}.e^{-t/TAU_{CM}} + N_{CL}.e^{-t/TAU_{CL}}$. Here TAU_{CS}, TAU_{CM} and TAU_{CL} indicate the time constant governing the short-duration, medium-duration and long-duration closures, respectively. The number of events in each of these three components was calculated using the relationships: $A_{CS} = (N_{CS}/BW).TAU_{CS}$, $A_{CM} = (N_{CM}/BW).TAU_{CM}$ and $A_{CL} = (N_{CL}/BW).TAU_{CL}$.

Halothane did not significantly alter the mean value of TAU_{CS} at any of the concentrations tested. TAU_{CS} averaged 0.5 ± 0.1 msec under control conditions. However, halothane did increase the mean value of both TAU_{CM} (control = 2.9 ± 0.4 msec, halothane = 25 ± 8.7 msec) and TAU_{CL} (control = 36 ± 6 msec, halothane = 168 ± 45 msec) when applied at a concentration of 2.8 mM ($P < 0.05$, Fig. 15).

The mean channel closed time, T_{closed} of BK channels was calculated from fit parameters to closed time distributions using the equation: $T_{closed} = A_{CS}/(A_{CS} + A_{CM} + A_{CL}).TAU_{CS} + A_{CM}/(A_{CS} + A_{CM} + A_{CL}).TAU_{CM} + A_{CL}(A_{CS} + A_{CM} + A_{CL}).TAU_{CL}$. As shown in Table 3, halothane increased T_{closed} by factors of 2.1 and 9.3 on application of 1.6 mM and 2.8 mM halothane, respectively ($P < 0.05$). At 0.5 mM, halothane had no effect on the mean closed time of BK channels. These data indicate that higher concentrations of halothane (1.6 and 2.8 mM) reduce the open probability of BK channels by increasing the mean channel closed time, in addition to exerting a depressant effect on mean channel open time at these concentrations.

Figure 14. Closed time distributions for BK channel currents recorded from a single patch in the absence and presence of 2.8 mM halothane. The patch membrane was voltage-clamped to V = -60 mV. $[Ca^{2+}]_i$ = 100 μM. Note the use of a logarithmic time axis in each of these plots.

Each distribution was well fitted by the sum of three exponential terms (smooth curves). In the control distribution, the short-duration closures were governed by the time constant, TAU_{CS} = 0.6 msec and made up 80 % of total closures. Medium-duration closures were governed by the time constant, TAU_{CM} = 2.3 msec and composed 19.8 % of total closures. Long-duration closures were governed by the time constant, TAU_{CL} = 19.9 msec and made up the remaining 0.2 % of closures.

Corresponding values of these parameters in the other distributions were as follows. Halothane-containing saline: TAU_{CS} = 0.5 msec, 39 % of closures; TAU_{CM} = 2.9 msec, 44% of closures; and TAU_{CL} = 52 msec, 17 % of closures. After wash-out of drugs: TAU_{CS} = 0.7 msec, 61.3 % of closures; TAU_{CM} = 2.9 msec, 37.6 % closures; and TAU_{CL} = 23.1 msec, 1.1 % of closures.

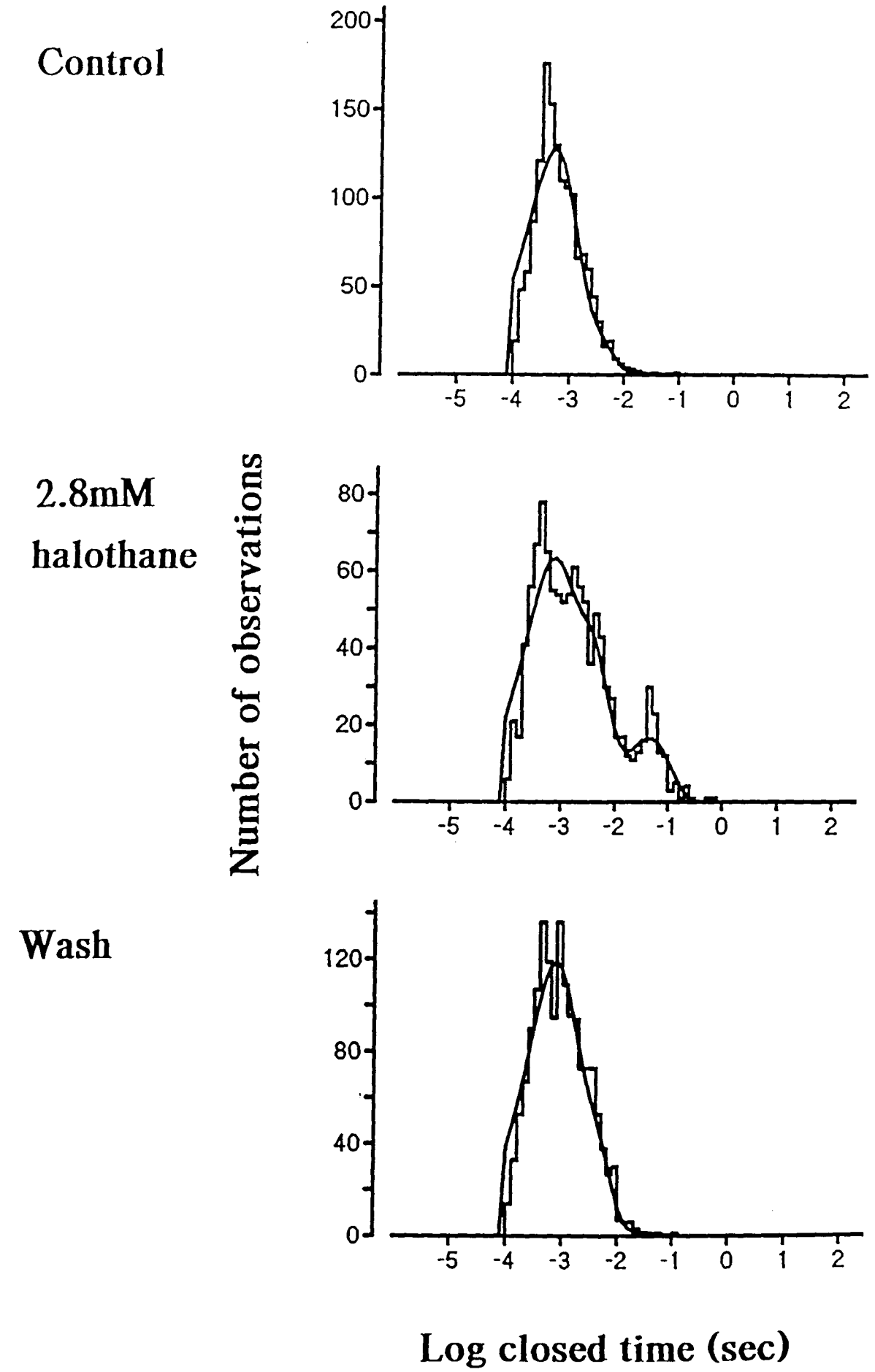

Control
2.8mM
halothane
Wash
Number of observations
Log closed time (sec)
200
150
100
50
0
80
60
40
20
120
-5
-4
-3
-2
-1
0
1
2

Figure 15. Effects of halothane at 0.5, 1.6 and 2.8 mM on the time constants governing medium-duration closures (TAU_{CM}) and long-duration closures (TAU_{CL}), obtained from fitting closed time distributions of single BK channel currents. n denotes the number of patches studied in each group. * indicates mean values significantly higher than control data ($P < 0.05$, *Sign*-test).

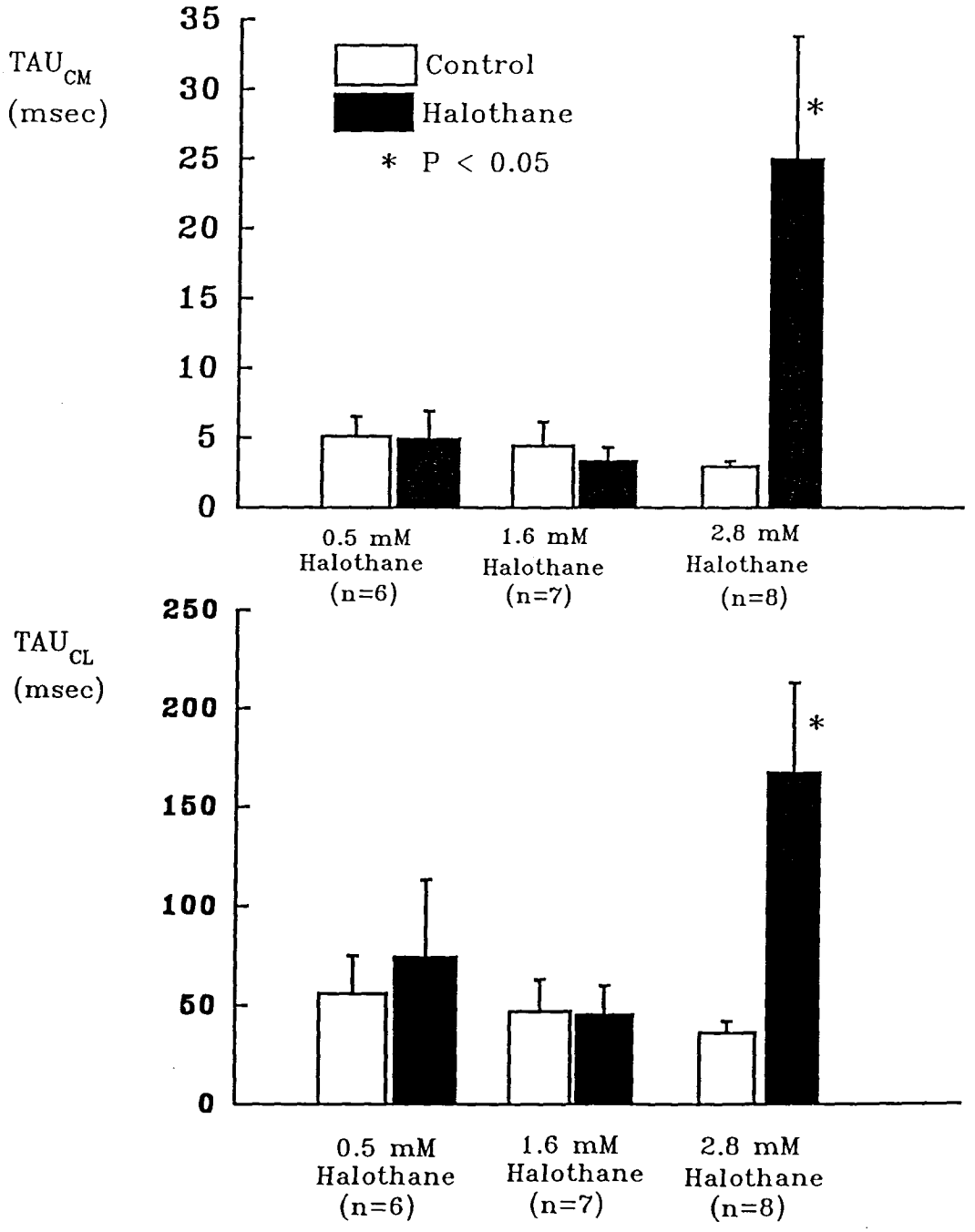

TAU$_{CM}$
(msec)
35
30
25
20
15
10
5
0
Control
Halothane
* P < 0.05
*
0.5 mM
Halothane
(n=6)
1.6 mM
Halothane
(n=7)
2.8 mM
Halothane
(n=8)
TAU$_{CL}$
(msec)
250
200
150
100
50
0
*
0.5 mM
Halothane
(n=6)
1.6 mM
Halothane
(n=7)
2.8 mM
Halothane
(n=8)

Table 3. Effect of halothane at 0.5, 1.6 and 2.8 mM on the mean closed time of BK channels, T_{closed}, as calculated from the fit parameters of closed time distributions (see text for details). Recording conditions as in Table 1. n denotes the number of patches studied in each group. * indicates mean values significantly larger than control data ($P < 0.05$, *Sign*-test).

TABLE 3. Effect of halothane on the mean closed time of BK channels (T_{closed})

	T_{closed}		
Concentration of halothane	Control	Halothane	Wash
0.5 mM (n=6)	3.9 msec (±1.7)	5.1 msec (±2.1)	4.4 msec (±2.0)
1.6 mM (n=7)	6.7 msec (±0.7)	14.1* msec (±10.0)	8.0 msec (±5.9)
2.8 mM (n=8)	3.9 msec (±1.2)	36.2* msec (± 13.0)	9.1 msec (±5.1)

DISCUSSION

Halothane Directly Depresses Activity Of BK Channels

The major finding of this study is that halothane reduces the open probability of BK channels in rat cerebrovascular smooth muscle cells. Reduction of the open probability of BK channels could occur by a number of mechanisms, including receptor-coupled G-protein-induced inhibition (Cole & Sanders, 1989), decreased availability of free calcium for activation of BK channels (Kolb, 1990; Wang & Mathers, 1993) and direct pharmacological blockade (Castle et al., 1989). Since our measurements were carried out in a cell-free system, it seems unlikely that the inhibitory effect of halothane on BK channels was due to an alteration of G-protein function.

The present data were obtained under conditions in which internal free calcium concentration was kept constant. The results therefore demonstrate that halothane directly depresses activity of BK channels in cerebral artery smooth muscle cells.

Biochemical studies utilizing $^{86}Rb^{+}$ have shown that ion flux through BK channels in rat glioma C6 cells is significantly reduced by halothane at clinical concentrations (Tas et al., 1989). In isolated hippocampal neurons, the fast after-hyperpolarization phase of the action potential, which is mediated by BK channels (Lancaster et al., 1991), is also readily suppressed by halothane, resulting in an increase in excitability (Fujiwara et al., 1988; Southan & Wann, 1989). In both of these cases, halothane did not alter the entry of calcium into these cells. Therefore, these observations also support the view that halothane exerts a direct inhibitory effect on the activity of BK channels, independent of the drug's actions on calcium dynamics (Tas et al., 1989; Fujiwara et al., 1988).

Effects Of Halothane On The Kinetics And Conductance Of BK Channels

The present studies showed that halothane decreased the open probability of BK channels in cerebrovascular smooth muscle cells in a concentration-dependent manner. Halothane also affected closed-open transitions of BK channels. In the absence of halothane, BK channels remained open most of the time and only brief closures occurred. During exposure to the anesthetic, BK channels closed more rapidly and spent more time in the closed state. The halothane-induced fall in open probability was due to a decreased mean channel open time and to an increased mean channel closed time. In contrast to these effects on BK channel kinetics, halothane did not alter the conductance of BK channels at any of the concentrations tested. These findings may be compared with previous reports on the actions of halothane and of the structurally related anesthetics enflurane and isoflurane on ion channels in other tissues (Pancrazio et al., 1992; Antkowiak & Kirschfeld, 1992; Brett et al., 1988).

In bovine adrenal chromaffin cells, single channel studies revealed that 3.5 % enflurane suppressed the open probability of BK channels to 68 % of control value, resulting from a shortened open time and from an increased closed time, without alteration of the mean conductance of single BK channels (Pancrazio et al., 1992).

In contrast, in the algal species *Chara australis*, 2 % enflurane decreased not only the open probability but also the mean conductance of single BK channels. In the presence of enflurane, BK channels of *Chara australis* stayed closed for periods of several seconds, and exhibited both an increased closed time and a decreased open time (Antkowiak & Kirschfeld, 1992).

Interestingly, the kinetic properties of acetylcholine receptor channels in clonal BC3H-1 cells and embryonic *Xenopus* skeletal muscle cells were also altered

by isoflurane and halothane, leaving the mean conductance of these channels unchanged (Brett et al., 1988; Lechleiter & Gruener, 1984). Isoflurane shortened both the mean channel open time and mean channel closed time, giving rise to a flickering of current flow through acetylcholine receptor channels (Brett et al., 1988).

These results indicate the existence of some diversity in the structure of BK channels among various tissues.

Concentration Dependence Of Halothane Action On BK Channels

The anesthetic EC_{50} value or minimum alveolar concentration (MAC) of halothane is defined as the dose which prevents movement in response to surgical incision in 50 % of patients or animals, and this is generally accepted to be 0.75 % for humans, and 1.03 % for rats (Marshall & Longnecker, 1990). These doses correspond to aqueous concentrations of 0.21 mM and 0.29 mM, respectively, at 37^{o}C (Steward et al., 1973). During maintenance of anesthesia, 0.11 - 0.42 mM halothane is typically employed. However, for induction of anesthesia, a higher concentration (0.42 - 0.63 mM) of halothane is necessary (Atkinson et al., 1987). In surgical cases involving the risk of severe hemorrhage, unusually high doses of halothane (0.42 - 0.84 mM) may be administered to patients as a maintenance concentration, with a view to exploiting the marked hypotensive effect these drug doses produce (Quail, 1989).

The present study showed that halothane at very high concentrations (1.6 mM and 2.8 mM) significantly inhibited the activity of BK channels in cerebrovascular smooth muscle cells. However, the failure of 0.5 mM halothane to directly affect BK channels in cerebrovascular muscle was unexpected, in view of the reportedly high sensitivity of this channel class in other tissues. For example, Tas et al. (1989) showed that cation flux through activated BK channels in a glioma cell line was reduced by 50

% in the presence of 0.5 mM halothane. Similarly, 0.5 mM halothane markedly reduced the amplitude of the fast after-hyperpolarization, which terminates the action potential in rat hippocampal neurones (Fujiwara et al., 1988). This after-hyperpolarization is probably due to the activation of BK channels by Ca^{2+} which enters during the spike (Hille, 1992). As noted previously, the suppressive effect of halothane seen in these studies could not be attributed to a reduced availability of calcium for activation of BK channels (Tas et al., 1989; Fujiwara et al., 1988).

The differing sensitivities of BK channels to halothane in these cell types also implies the existence of structural diversity, in either the channel proteins themselves or in their lipid environments. This view is also supported by known pharmacological differences among the BK channels of different cells. In skeletal muscle, BK channels are blocked by internal TEA^+ with a high K_d (> 30 mM) (Vergara & Latorre, 1983; Blatz & Magleby, 1984). In contrast, BK channels from rat cerebral artery (Wang & Mathers, 1993) and clonal pituitary cells (Wong & Adler, 1986) are much more sensitive to blockade by internal TEA^+ (K_d < 1 mM).

Physiological Significance Of BK Channel Inhibition By Halothane

Potassium efflux through BK channels contributes a major portion of the macroscopic outward potassium current recorded in vascular smooth muscle cells (Benham et al., 1986; Ohya et al., 1987; Beech & Bolton, 1989a). Activation of these large conductance K^+ channels under physiological conditions results in outward membrane current, which hyperpolarizes smooth muscle cells, closing voltage-dependent calcium channels. This action terminates the action potential and inhibits contraction in vascular smooth muscle (Wilde & Lee, 1989). BK channels therefore act as an endogenous dilatory mechanism to regulate vascular smooth muscle tone (Brayden & Nelson, 1992; Asano et al., 1993).

The present study showed that 0.5 mM halothane, a concentration typically used to maintain surgical anesthesia, had no significant effect on the activity of BK channels in CVSMCs. Higher anesthetic concentrations (1.6 mM and 2.8 mM) significantly reduced the opening probability of BK channels in these cells. The marked cerebral vasodilation, which is in fact observed during surgical anesthesia using halothane (Quail, 1989; Wood & Wood, 1990), must therefore result from a variety of other drug actions on vascular muscle cells. These actions include reduction in calcium influx through voltage-dependent calcium channels (Murat, 1990; Eskinder et al., 1991) decreased accumulation of intracellular free Ca^{2+} (Katsuoka et al., 1989), and lowered sensitivity of the contractile proteins to intracellular free calcium (Housmans, 1990).

As reported by Hirst et al. (1986), Ca^{2+}-activated potassium currents contribute to outward currents in CVSMCs. Therefore, anesthesia induced by high doses of halothane might be expected to be associated with reduced outward potassium current and enhanced contraction of cerebral blood vessel walls. This factor may be important in the disordered cerebral autoregulation seen during exposure to high doses of volatile anesthetics (Michenfelder & Theye, 1975).

Mechanism Of Action Of Halothane On BK Channels In VSMCs

It remains unclear whether the primary target sites for volatile anesthetics are membrane lipids (Haydon et al., 1986; Elliott & Haydon, 1986) or ion channel proteins (Franks & Lieb, 1986; 1987). In favour of the former target site, one may cite the Meyer-Overton rule, which shows the remarkable correlation between the potency of general anesthetics and their lipid solubilities (Smith et al., 1980). It has been suggested that volatile anesthetics disorder the lipid bilayer, resulting in an increased membrane fluidity (Trudell et al., 1973; Gage & Hamill, 1975; Lenaz et al.,

1979). Such a perturbation may in turn alter the conformation of channel proteins embedded in the lipid bilayer, thereby changing the behavior of these proteins. Application of high pressure reverses halothane-induced fluidization of the lipid bilayer and also reverses anesthesia produced by volatile agents (Mastrangelo et al., 1978).

At concentrations appropriate for surgical anesthesia, halothane does indeed increase the fluidity of lipids in a variety of biological and artificial membranes (Trudell et al., 1973; Gage & Hamill, 1976; Lenaz et al., 1979; Koblin, 1990). Bolotina et al. (1989) directly measured the effect of altering membrane fluidity on the properties of BK channels in aortic smooth muscle cells. The results indicated that alteration of membrane fluidity affected the kinetic properties, but did not change the conductance of this channel. Decreased membrane fluidity reduced channel open probability, by decreasing the mean channel open time and increasing the mean channel closed time. These effects are very similar to those produced by halothane at concentrations of 1.6 mM and 2.8 mM in the present study. Furthermore, similar changes in BK channel kinetics have been observed in studies using standard anesthetic doses of another volatile anesthetic, enflurane (Pancratzio et al., 1992; Antkowiak & Kirschfeld, 1992).

Thus, it seems unlikely that the reduced open probability of BK channels observed in the presence of volatile anesthetics is simply due to the fluidization of membrane lipids *per se*, since this would be expected to increase the open probability of the BK channel. Rather, these data imply that halothane and related agents also alter the function of the BK channel protein itself, either by binding directly to hydrophobic domains within the channel (Franks & Lieb, 1987), or by disrupting protein-lipid interactions in the membrane (Lenaz et al., 1979).

In cardiac myocytes of the guinea-pig, 1.1 mM halothane has no effect on the inward rectifier potassium current, at doses which significantly depress the time- and

voltage-dependent outward potassium current and also the slow inward calcium current (Hirota et al., 1989). While several membrane currents found in clonal GH_3 pituitary cells are inhibited by clinically appropriate concentrations of halothane, these currents show markedly different sensitivities to the anesthetic. For example, 0.8 mM halothane is sufficient to reduce rapidly inactivating potassium currents (A currents) by 50 %, while 2.6 mM halothane is required to block the sodium current by the same amount (Herrington et al., 1991). These observations also support the view that partially selective, drug-channel interactions are involved in the suppressive effects of volatile anesthetics on membrane currents.

Summary And Future Directions

General anesthesia induced by halothane is associated with increased cerebral blood flow and intracranial pressure, resulting from dilatation of cerebral blood vessels (Fleischer & Inni, 1989; Sato et al., 1988; Kitazawa et al., 1991). This action restricts the usefulness of volatile anesthetics for many neurosurgical procedures (Eintrei et al., 1985). Thus the mechanisms which underlie the cerebrovascular actions of volatile anesthetics are worthy of investigation.

Although it is assumed that volatile anesthetics influence the activity of transmembrane ion channels in muscle cells (Haydon et al., 1988), little is known of how anesthetics do so and what kinds of ion channels are sensitive to these anesthetic agents. The current study revealed that halothane directly and reversibly decreased the open probability of BK channels in vascular smooth muscle cells of rat cerebral artery in a dose-dependent manner. However, under the same conditions, halothane did not affect the unitary conductance of these channels.

Kinetic analysis showed that halothane reduced the mean channel open time and increased the mean closed time of BK channels. These changes in the kinetic

properties of BK channels caused by halothane in CVSMCs are similar to those caused by a decrease in the fluidity of vascular smooth muscle cell membranes (Bolotina et al., 1989).

However, halothane are reported to increase the fluidity of biological membranes (Trudell et al., 1973; Gage & Hamill, 1976; Lenaz et al., 1979; Koblin, 1990). Therefore, it is unlikely that alteration of membrane fluidity itself can account for the inhibitory effects of halothane on BK channel function. Rather, the binding of volatile anesthetic agents to hydrophobic domains within the BK channel protein or to sites at the lipid-protein interface is probably involved in producing the observed effects of halothane on BK channels (Franks & Lieb, 1987; Lenaz et al., 1979).

The gating kinetics of BK channels have been shown to be regulated by the intracellular concentrations of free calcium ions (Barrett et al., 1982; Moczydlowski & Latorre, 1983; McManus & Magleby, 1988). Whether halothane exerts its effects on the gating of BK channels by interfering with the binding of calcium ions to these channels should therefore be investigated. This could be done by quantitatively studying the action of halothane on the dependence of the open probability of BK channels on the intracellular free calcium concentration.

At the intact cell level, studies on the effect of halothane on the hyperpolarizing phase of action potentials in CVSMCs should also be performed, using intracellular recording techniques. As indicated earlier, the amplitude of this hyperpolarization should be suppressed by the anesthetic, at doses which suppress BK channel activity in these cells (Hirst et al., 1986).

Reconstitution of BK channel proteins into artificial lipid bilayers would potentially allow a better understanding of the interactions between volatile anesthetics and lipid bilayers, and the influence of these interactions on the function of the channel proteins.

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