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Sodium channel isoform-specific effects of halothane: protein kinase C co-expression and slow inactivation gating

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1 The modulatory effect of protein kinase C (PKC) on the response of *Xenopus* oocyte-expressed Na channel α-subunits to halothane (2-bromo-2-chloro-1,1,1-trifluroethane) was studied. Na currents through rat skeletal muscle, rat brain and human cardiac muscle Na channels were assessed using cell-attached patch clamp recordings. PKC activity was increased by co-expression of a constitutively active PKC α-isozyme.

2 Decay of macroscopic Na currents could be separated into fast and slow exponential phases.

3 PKC co-expression alone slowed Na current decay in neuronal channels, through enhancement of the amplitude of the slower phase of decay.

4 Halothane (1.0 mM) was without effect on any of the three isoforms expressed alone but, after co-expression of PKC, there was enhancement of Na current decay with reduction in charge movement through skeletal muscle and neuronal channels. Cardiac channels were relatively insensitive to halothane.

5 Enhanced Na current decay resulted from suppression of the slow phase, without effect on the faster phase or on either decay τ.

6 Suppression of Na current through skeletal muscle channels was concentration-dependent over the therapeutic range and was described by third order reaction kinetics, with an IC_{50} of 0.55 mM.

7 We conclude that the halothane suppresses skeletal muscle and brain Na channel activity in this preparation through a reduction in the slow mode of inactivation gating, but only after PKC co-expression. Cardiac Na channels were relatively insensitive to halothane. The mechanism is likely to involve phosphorylation of the channel inactivation gate, although phosphorylation of other sites in the channel may account for the isoform specific differences.

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Introduction

Voltage-gated Na channels are at the basis of neuromuscular excitability and are a potential target for general anaesthetics. Suppression of Na channel activity by clinically relevant concentrations of volatile general anaesthetics has been reported in a variety of tissues (Bean *et al*., 1981; Ruppersberg & Rudel, 1998; Eskinder *et al*., 1993; Rehberg *et al*., 1996) and Na channel blockade by local anaesthetics reduces the MAC of general anaesthetics (DiFazio *et al*., 1976; Himes *et al*., 1977; 1979).

Na channels are substrates for phosphorylation by multiple kinases. Among these protein kinase C (PKC) interacts with a site in the channel inactivation gate (Numann *et al*., 1991; Bendahhou *et al*., 1995; Murray *et al*., 1997). Volatile anaesthetics modulate PKC activity (Tsuchia *et al*., 1988; Lester & Baumann, 1991; Hemmings & Adamo, 1994; 1996; Tas & Koschel, 1997) and it is possible that the effects of volatile anaesthetics on Na channels may depend on PKC activity. Bosnjak and co-workers have recently demonstrated that modulation of cardiac Na channels by halothane is dependent on cyclic AMP-dependent protein kinase, whilst the effects of isoflurane are dependent on PKC (Weigt *et al*., 1998a,b). We have previously shown that Na current decay in *Xenopus* oocyte-expressed skeletal muscle Na channel α-subunits is enhanced by halothane, but only in the presence of concurrent over-expression of a PKC α-isozyme. This effect was dependent on the phosphorylation site in the Na channel inactivation gate known to be a PKC substrate. Halothane also caused a small reduction in Na current amplitude, which was independent of phosphorylation of this inactivation gate site (Mounsey *et al*., 1999). In view of the experiments of Bosnjak and co-workers, we set out to assess whether PKC sensitized cardiac and neuronal Na channel α-subunits to halothane in our preparation. We were also interested to probe the mechanism of halothane's actions further from the more resolved recordings available in cell attached multi-channel patch clamping.

Methods

RNA preparation

We obtained full length cyclic DNA encoding the rat skeletal muscle Na channel α-subunit (μ1, Dr Gail Mandel, SUNY, Stony Brook (Trimmer *et al*., 1989)), the human cardiac Na channel α-subunit (hH1, Dr A.L. George, Vanderbilt (Gellens *et al*., 1992)), and the rat brain IIa Na channel α-subunit (Dr A.L. Goldin, University of California, Irvine, (Auld *et al*.,

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1990)). We prepared mRNA using either the T7 or SP6 promoters for *in vitro* RNA synthesis as previously described (Mounsey *et al*., 1995). mRNA encoding a constitutively active PKC α-isozyme was generously supplied by Drs KP Lu and EN Olson. The expressed PKC lacks the coding region for the regulatory domain and is thus constitutively active. The biochemical evidence for enzymatic activity has been described (James & Olson, 1992).

Oocyte expression and electrophysiology

Our methods for isolation and mRNA injection of *Xenopus laevis* oocytes have been described (Mounsey *et al*., 1995; 1999). Oocytes were defolliculated manually the day of the electrophysiologic experiments, 2–5 days after injection. Oocyte patch clamping was performed by standard techniques. Briefly, cell attached patches were obtained at room temperature using thin-walled capillary glass (7502, Corning Inc, Corning NY, U.S.A.). The bath solution contained (mM): K aspartate 150, EGTA 10, HEPES 10, pH 7.4 (KOH) and the preparation was superfused at 3–4 ml min^{-1} (chamber volume 1.5 ml). The patch pipette solution was (mM): NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1.0, glucose 10, HEPES 10, pH 7.4 (NaOH). Currents were amplified and low pass filtered (2 kHz; 3900A Dagan Corp, Minneapolis, MN, U.S.A.), and sampled at 33 kHz (pCLAMP; Axon Instruments Inc, Foster City, CA, U.S.A.). The holding potential was −100 mV, and clamp pulses to 0 mV, duration 60 ms, were applied at 0.2 Hz. Capacitance and leak currents were compensated by subtracting records obtained over a potential range where Po for Na channels is low, using depolarizations from −50 mV to 50 mV.

Halothane solution preparation

Halothane- (2-bromo 2-chloro 1,1,1-trifluroethane, Halocarbon Laboratories, River Edge NJ, U.S.A.) containing solutions were prepared by bubbling halothane vapour through the solution used for superfusion of oocytes. Halothane was vaporized in a conventional clinical general anaesthesia vaporizer (OHIO Medical Products, Madison WI, U.S.A.) using compressed air at 5 l min^{-1}. Solutions were equilibrated with halothane for at least 30 min and supplied to the experimental chamber through Teflon tubing. The concentration of halothane present at the cell was determined from samples of flowing control solution equilibrated with halothane. Halothane standards were prepared from a saturated solution of halothane (17.4 mM determined by the solubility of halothane in water: 0.345% – the Merck Index 11th edition) and used to construct a calibration curve. Bath solution was equilibrated with halothane at each setting on the halothane vaporizer, and samples were taken at the experimental site using gas tight syringes. The concentration of the anaesthetic in the effluent was determined using a gas chromatograph (Hewlett Packard 5890A series II using a 30 m HP-1 capillary column). Injections into the gas chromatograph were performed manually using a 1 μl syringe. Samples were dissolved in a methanol solvent in order to determine the injection volume. All experimental results were compared to standard solutions made and analysed at the time of the unknown sample analysis.

Data analysis

Ionic currents were analysed in pCLAMP (Axon), and Origin (MicroCal Inc Amherst MA, U.S.A.). Leak subtraction and construction of ensemble average currents was accomplished in pCLAMP. Curve fitting of the current waveforms was performed in Origin. Charge movement was derived by integrating the current waveforms in Origin. Parametric statistical tests were used for normally distributed data (as determined by the Kolmogorov-Smirnov test), and non-parametric tests were used for non-normally distributed data (SigmaStat, Jandel). Statistical significance was defined as $P<0.05$.

Results

Effects of PKC co-expression on Na current decay

To separate any effects of halothane from the effects of PKC co-expression alone, we compared Na currents in oocytes expressing Na channel α-subunits alone with oocytes co-expressing PKC. PKC co-expression or activation is known to cause a reduction in Na current amplitude in all of the three Na channel isoforms tested here, but the effects on current decay are isoform-specific (Numann *et al*., 1991; Bendahhou *et*

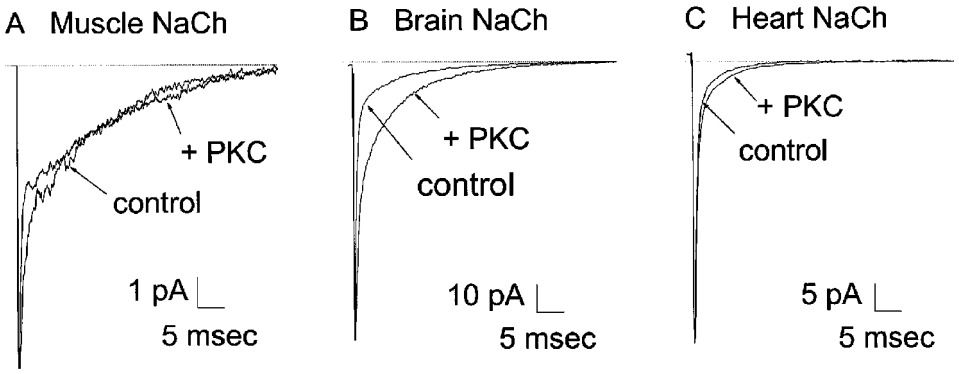

Figure 1 Effects of PKC co-expression on Na current decay kinetics in skeletal muscle (A), brain (B) and cardiac muscle (C) Na channel isoforms. Recordings were made from cell attached multi-channel patches. Each pair of currents were obtained from the same batch of oocytes injected at the same time with identical quantities of Na channel mRNA. Half of each batch was co-injected with PKC mRNA. Currents were recorded at 0 mV (holding potential −100 mV), and clamp pulses were applied at 0.1 Hz. The traces show the ensemble average of 100 depolarizing pulses. The current amplitudes in oocytes co-expressing PKC have been normalized to the amplitudes in oocytes expressing Na channels alone to emphasize effects on decay kinetics. Note that PKC co-expression induced a marked slowing of Na current decay in brain Na channels, but was without apparent effect in the other two isoforms.

Table 1 Effects of PKC co-expression and halothane on Na channel decay kinetics

	τ1		*τ2*		*Prop. channels gating with τ2**		*Prop reduction charge movement*	
		+halothane		*+halothane*		*+halothane*	*+halothane*	n
Brain NaCh	0.7±0.1	0.9±0.2	8.9±1.1	10.7±1.5	0.11±0.04	0.09±0.04	−0.05±0.18	7
Brain NaCh+PKC	1.1±0.3	0.7±0.1	9.3±1.6	7.4±1.7	0.21±0.02†	0.11±0.04‡	−0.5±0.08‡	6
Heart NaCh	0.7±0.1	0.8±0.1	5.4±1.7	4.0±1.2	0.14±0.06	0.2±0.06	−0.07±0.11	4
Heart NaCh+PKC	0.6±0.1	0.6±0.2	4.1±1.4	4.1±1.2	0.25±0.08	0.29±0.06	−0.2±0.18	4
Muscle NaCh	0.8±0.2	0.9±0.3	9.6±2.7	11.2±2.9	0.31±0.1	0.28±0.1	−0.03±0.14	6
Muscle NaCh+PKC	1.1±0.4	0.5±0.05	12.6±2.0	12.5±2.1	0.34±0.08	0.03±0.01‡	−0.88±0.06‡	5

Results of fitting current waveforms (I) to a sum of two exponentials model: $I = A1e^{-t/\tau1} + A2e^{-t/\tau2}$, where: τ1, τ2: Fast and slow decay time constants. A1, A2: proportion of channels gating with each time constant. †$P<0.05$ for the comparison between NaCh and NaCh+PKC; ‡$P<0.05$ for the effect of halothane; *Proportion of channels gating with the slower time constant = A2/(A1+A2); NaCh: Na channels.

al., 1995; Murray *et al*., 1997). PKC co-expression was without effect on Na current decay in skeletal or cardiac muscle channels (Figure 1A,C) but decay in brain Na channels was slowed (Figure 1B). In these ensemble average recordings Na current decay could be separated into fast and slow phases, caused by fast and slow inactivating modes of Na channel gating. Qualitatively, the major effect of PKC co-expression in brain channels was to enhance the amplitude of the slower phase of current decay. To quantify effects on current decay, the current waveforms were fit to a sum of two exponentials model, and the parameters from this model for each of the three Na channel isoforms tested are listed in Table 1. The model confirmed that the effect of PKC on current decay in brain channels was not through reduction of the fast or slow decay time constants, but rather through a significant enhancement of the proportion of Na channels gating with the slower decay time constant. PKC co-expression had no significant effects on current decay kinetics in the other two Na channel isoforms.

Effects of halothane on Na currents

In the absence of PKC co-expression halothane at a concentration of 1.0 mM was without significant effect on any of the Na channel α-subunit isoforms tested (Figure 2A–C). With PKC co-expression, however, there was enhancement of Na current decay with halothane, an effect that was apparent qualitatively in all three isoforms (Figure 2D–F). The effect appeared to be largest in skeletal muscle and brain channels. In cardiac muscle channels the effect was small.

To explore the single channel mechanism of the halothane effect in more detail we examined currents from individual depolarizing clamp pulses, and Figure 3 shows representative examples from skeletal muscle channels. Figure 3A was taken from an oocyte expressing Na channels alone. This was a multi-channel patch, so there was an initial rapid component of current. This is clipped in the figure because the traces have been scaled to show single channel activity. The initial burst of activity is followed in some traces by later bursts of channel openings, caused by the slow mode of inactivation gating. These fast and slow components of channel activity are reflected in the biphasic decay of the ensemble average currents (lowest panel). Halothane (1.0 mM) had little effect on either channel activity or the current waveform in this oocyte. In an oocyte expressing Na channels and PKC, however (Figure 3B), halothane induced a reduction in the number of sweeps showing late channel openings, and this is reflected in the greatly enhanced rate of decay of the ensemble average trace. So the major effect of halothane on muscle Na channels in this preparation appeared to be a reduction in the slow mode of inactivation gating. This effect required co-expression of PKC and resulted in a reduction in charge movement without much effect on current amplitude.

To quantify the effects of halothane on the slow mode of inactivation gating, ensemble average Na current waveforms were fitted to a sum of two exponentials model, and the parameters of this model derived from for 4–7 patches in each group of oocytes are summarized in Table 1. Halothane had no effect on time constants for the fast or slow phases of current in any of the three isoforms tested. Rather, as suggested by examination of the single channel activities, the effect of halothane was to reduce the proportion of channels gating with the slower time constant, an effect that was confined to oocytes co-expressing PKC. The effect was significant for brain and skeletal muscle channels. In brain channels, where PKC expression alone resulted in enhanced slow modal inactivation gating, the effect of halothane was to reduce the proportion of channels gating with the slower time constant. In muscle channels, where PKC co-expression alone had no effect, halothane resulted in a significant reduction in the proportion of channels gating slowly. For this isoform, almost all the channels gated with the faster time constant after exposure to halothane. In cardiac muscle channels the proportional change in slow inactivation gating induced by halothane was not statistically significant.

Halothane appeared to reduce current amplitude, but the effects were small. To assess the combined effect of these small changes in amplitude and the changes in current decay, the ensemble average current waveforms were integrated and the proportional changes in charge movement induced by halothane in the three Na channel isoforms are given in Table 1. Halothane had no effect on charge movement in any of the isoforms expressed alone, but in brain and skeletal muscle channels co-expressed with PKC, there was a significant reduction in charge movement, which was larger in skeletal muscle channels. In cardiac muscle channels, there were no significant effects on charge movement.

The halothane concentration-response relationship in muscle Na channels

The concentration-response relationship for halothane was measured in cell attached multi-channel patches in oocytes co-expressing skeletal muscle Na channel α-subunits and PKC (Figure 4). At the lowest concentration tested (0.1 mM) halothane was without effect (Figure 4A), but as the concentration was increased to 0.4, 0.5 and 1.0 mM, Na currents were progressively more suppressed (Figure 4B–D). Qualitatively the mechanism of the halothane effect at lower

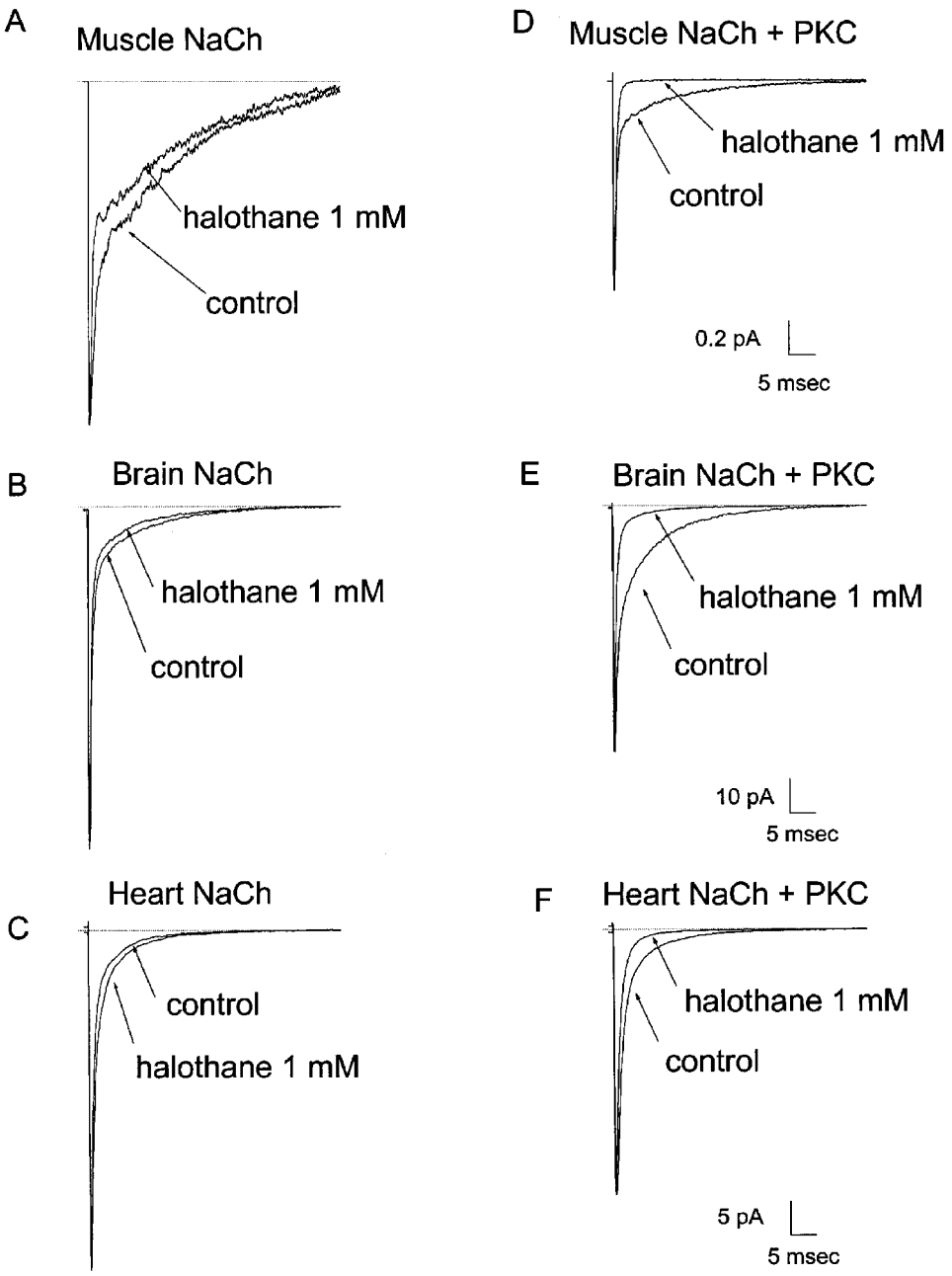

Figure 2 The effects of PKC co-expression on the response of Na channels to halothane. Currents were recorded at 0.1 Hz before and 10 min after exposure to halothane (1 mM), and the traces show superimposed the ensemble average of 100 sweeps. A, B and C respectively show recordings obtained in the absence of PKC co-expression for skeletal muscle, brain and cardiac muscle Na channels. D, E and F show similar recordings in the presence of PKC co-expression. Skeletal muscle, and to a lesser extent brain channels co-expressed with PKC were suppressed by halothane. Cardiac muscle channels, and all three isoforms in the absence of PKC co-expression were much less sensitive to halothane. The current amplitudes have been normalized to the control (pre-halothane) amplitude in each panel. Experimental details otherwise as in Figure 1.

concentrations appeared similar to the mechanism at a concentration of 1.0 mM – there was a concentration-dependent reduction in the amplitude of the slower phase of current decay. To quantify the halothane effect, the current waveforms were integrated, so that the combined effects on current amplitude and current decay could be assessed together, and the proportional reduction in charge movement at each concentration was used to construct a concentration-response curve (Figure 4E). The data were not adequately fit by a simple hyperbolic relationship, and the line in Figure 4E assumes third order kinetics. The IC_{50} for halothane on these skeletal muscle channels was 0.55 mM.

Discussion

We studied the effects of halothane and PKC co-expression on *Xenopus* oocyte-expressed skeletal muscle, cardiac muscle and rat brain Na channel α-subunits. Halothane had no effect on any of the three Na channel isoforms when expressed alone. With co-expression of PKC, however, halothane caused a reduction in charge movement and an enhancement in Na current decay in skeletal muscle and brain Na channels, but had no significant effect on cardiac Na channels. Na current decay could be resolved into fast and slow phases. The enhancement of Na current decay

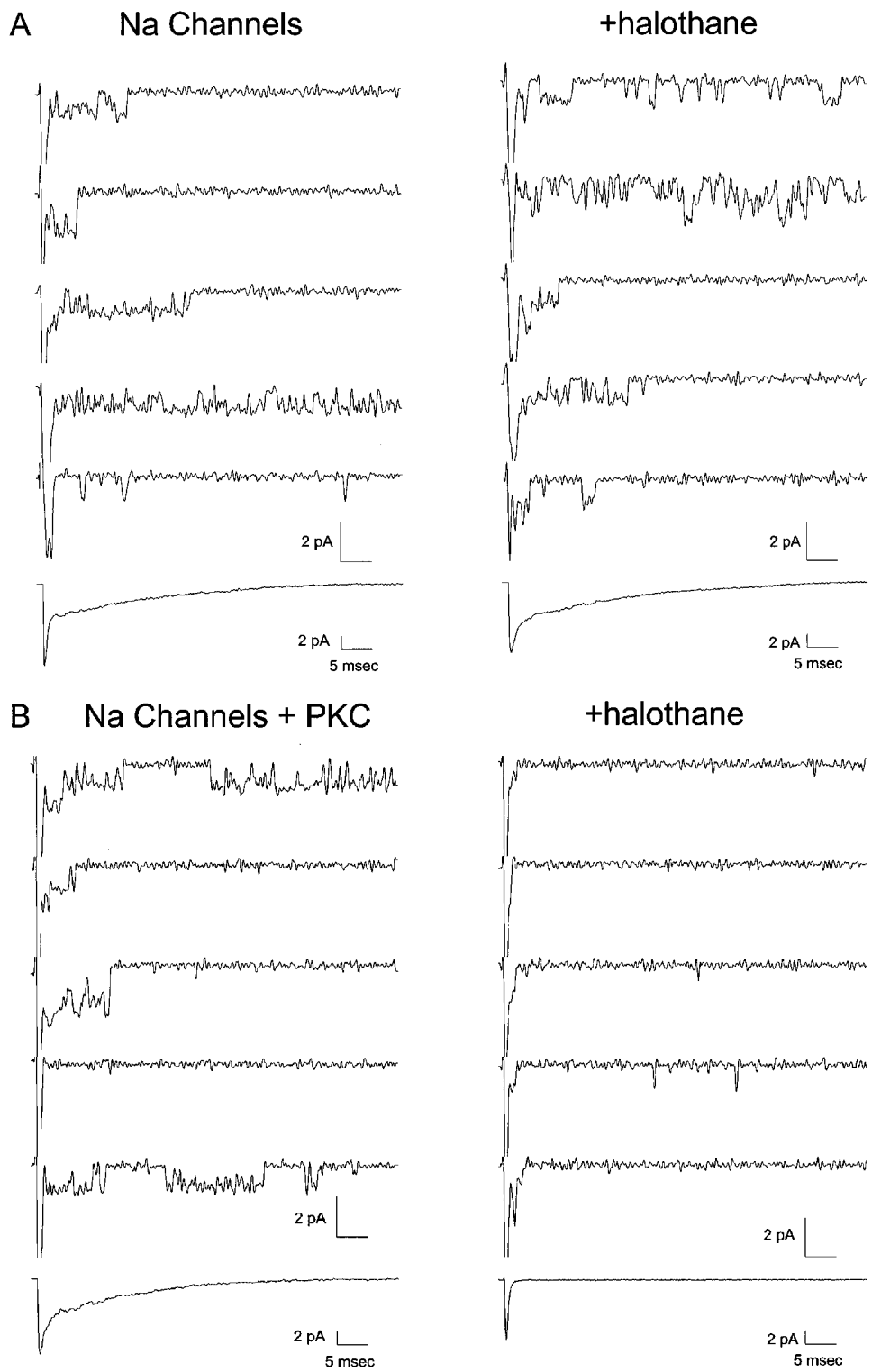

Figure 3 Effects of halothane on single channel activity on oocyte-expressed skeletal muscle Na channels (top five traces in each panel), and on the ensemble average current (bottom traces). In each trace there is an initial burst of channel activity, which results in macroscopic current in these multi-channel patches. This is followed by bursts of late channel openings in some of the traces. In oocytes expressing Na channels alone, (A), halothane (1 mM) was without effect, but in oocytes expressing PKC, (B), halothane resulted in a dramatic reduction in late Na channel openings. These effects are reflected in the ensemble average traces. The macroscopic currents have been clipped as the currents are scaled to emphasize single channel activity. Experimental details are as for Figure 1.

induced by halothane in skeletal muscle and rat brain Na channels was the result of a decrease in the amplitude of the slow phase. Both the effects on charge movement and Na current decay were concentration-dependent over the therapeutic range of halothane.

Modulation of Na channels by protein kinases is critically important in the regulation of channel function. Protein kinase C interacts with a site in the channel inactivation gate. This site is highly conserved and is present in all the isoforms tested here (Yang & Barchi, 1990; Numann *et al*., 1991; Bendahhou *et al*.,

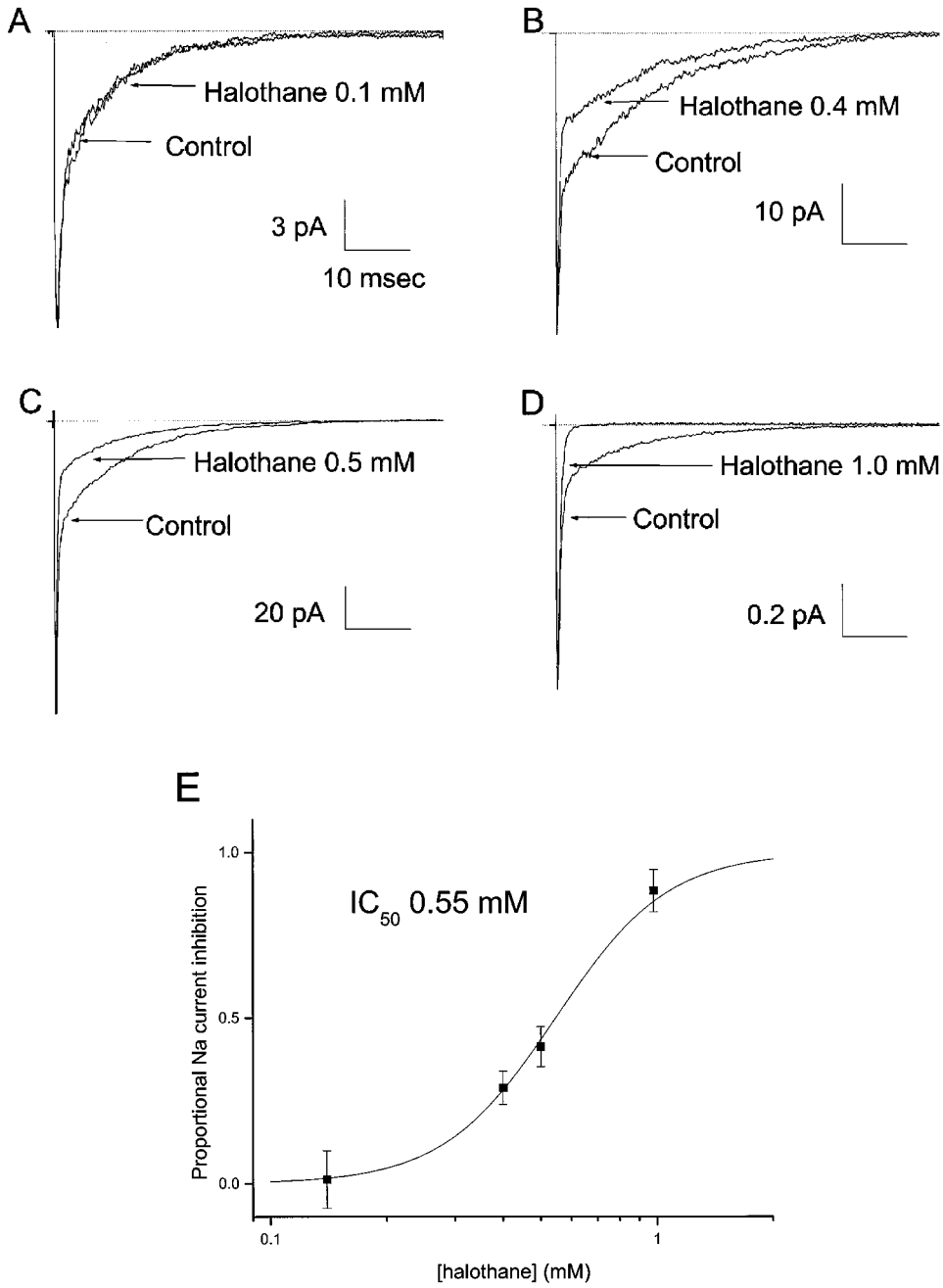

Figure 4 Effects of increasing concentrations of halothane on Na currents in oocytes co-expressing skeletal muscle Na channels and PKC. A–C show exemplary recordings. At 0.1 mM, halothane was without effect (A). As the concentration was increased to 0.4 mM (B), 0.5 mM (C) and 1.0 mM (D), there was a graded decrease in the amplitude of the slow component of Na current decay, and a consequent reduction in charge movement. At the highest concentration there is a small reduction in Na current amplitude. Experimental details as in Figure 2. The concentration response curve (E) shows data from 5–7 patches at each concentration. The magnitude of the halothane effect was assessed as the proportional reduction in charge movement at each concentration. Data points are mean ± s.e.mean The line is fit to the data assuming third order kinetics, and the calculated IC_{50} was 0.55 mM.

1995; Murray *et al.*, 1997). This inactivation gate phosphorylation site is significant in the response of Na channels to halothane. We have previously shown that the enhancement of Na current decay induced by halothane in oocytes co-expressing Na channels and PKC was absent in a Na channel mutant lacking this phosphorylation site (Mounsey *et al.*, 1999). In the experiments reported here the mechanism of this effect was found to be a decrease in the amplitude of the slow phase of Na current decay, which resulted in a reduction of charge movement, and hence suppression of Na current. We suggest that the reduction in the amplitude of the slow phase of Na current decay was the result of suppression of the slow mode of inactivation gating by halothane.

In skeletal and cardiac muscle, the decay of macroscopic Na current can be resolved into fast and slow components. Single channel recordings of Na channels in skeletal muscle (Patlak & Ortiz, 1986), and cardiac muscle (Patlak & Ortiz, 1985; Kunze *et al.*, 1985; Grant & Starmer, 1987) sometimes manifest prolonged bursts of openings. The slower component of current decay arises because of these channel re-openings, and this has been called slow inactivation gating. Neuronal Na currents normally decay rapidly with a single exponential time course and so, for the most part, do not display slow inactivation gating. This difference is apparent in brain and heart cells studied under identical experimental conditions (Kirsch & Brown, 1989). In the current study, neuronal

channels only displayed a slow component of current decay after co-expression of PKC, at which time slow inactivation gating appeared, as has previously been reported by Catterall and co-workers (Numann *et al*., 1991). Slow Na channel inactivation gating is particularly prominent when Na channel α-subunits from brain and skeletal muscle are expressed alone in *Xenopus* oocytes (as in the current study and in (Moorman *et al*., 1990; Zhou *et al*., 1991)), and is dramatically reduced when β subunits are co-expressed (Isom *et al*., 1992; Bennett *et al*., 1993; Cannon *et al*., 1993; Chang *et al*., 1996). In contrast, cardiac Na channel α-subunits expressed in oocytes produce rapidly decaying Na currents, and decay rate is unaffected by co-expression of the β-subunit (Marban *et al*., 1998). In this study we examined α-subunits expressed alone in order to isolate the effect of PKC and halothane on slow inactivation gating. Similarly, studies of the voltage-dependence of this slow gating process purposely omitted the β subunit co-expression (Ji *et al*., 1994). We have previously demonstrated that, at least in skeletal muscle channels, the effects of halothane on current decay remain significant with co-expression of the β-subunit (Mounsey *et al*., 1999). The effects we observed were smaller than for α-subunits expressed alone, but it should be noted that small changes in the balance between excitatory and inhibitory influences can have large effects on excitable cells. For example in myotonic muscular dystrophy, a persistent Na current which amounts to 0.8% of the peak Na current can lead to myotonia and paralysis (Franke *et al*., 1990). By analogy, a small reduction in excitatory Na current could lead to significant diminution of impulse formation and propagation.

Halothane could exert its effects in our preparation either through preferential interaction with phosphorylated channels, through direct activation of PKC by halothane, or through effects of halothane on the lipid composition of the oocyte membrane. The experiments contained within this report do not distinguish these possibilities, although our previous work favours the first possibility (Mounsey *et al*., 1999). This is because, although halothane can directly modulate PKC (Lester & Baumann, 1991; Slater *et al*., 1997), the PKC isozyme used here was constitutively active, and so should be fully active already when expressed in the oocyte. Alteration of the lipid properties of the membrane is a credible explanation of the data. However, since the effects of halothane on skeletal muscle channels was dramatically different in a point mutant Na channel in which the inactivation gate phosphorylation site had been disabled, but in which the lipid milieu was identical, we have previously argued (Mounsey *et al*., 1999) that this effect was unlikely to be significant.

If the mechanism of the effect of halothane in this preparation is a preferential interaction with phosphorylated channels, how can the isoform specific differences be accounted for? Although the inactivation gate phosphorylation site is present in all isoforms tested here, the neuronal Na channel isoform has a unique 200 amino acid segment in the cytoplasmic linker between domains I and II of the molecule that contains multiple extra phosphorylation sites. This segment is absent in cardiac and skeletal muscle channels, but these channels too contain multiple other consensus sites for phosphorylation by PKC (Yang & Barchi, 1990). It is possible to account for all of the effects of PKC on neuronal channels through an interaction with the inactivation gate site (West *et al*., 1991; Numann *et al*., 1991; West *et al*., 1992), but this is not true for cardiac or skeletal muscle channels (Bendahhou *et al*., 1995; Murray *et al*., 1997; Mounsey *et al*., 1999). The discrepancy between the response to halothane of neuronal and muscle Na channels may relate to these structural differences between the two isoforms. For example, it is possible that activation of the I to II cytoplasmic linker phosphorylation sites in neuronal channels modulates the phosphorylation status of the inactivation gate site to PKC (Li *et al*., 1993), and this may affect the response to halothane.

Oocyte-expressed Na channels may also be phosphorylated by endogenous oocyte kinases, and this may differentially affect the response of the three isoforms tested here to exogenous PKC. For example, if neuronal channels were less phosphorylated by endogenous kinases than skeletal muscle channels, exogenous PKC may be required to shift gating of the channels to the slower time constant, thus rendering the channels susceptible to halothane. Since neither the neuronal or skeletal muscle isoforms were sensitive to halothane in the absence of exogenous PKC, it would be necessary to hypothesize that any endogenous kinase activity would exert its principal effect on sites other than the inactivation gate site, phosphorylation of which is essential for the effect of halothane on channel gating.

The relative insensitivity of cardiac channels to halothane in the presence of exogenous PKC is consistent with data from Bosnjak and co-workers who also demonstrated that the effect of halothane on cardiac Na channels was independent of PKC. They demonstrated that the effect of halothane on cardiac channels was dependent rather on activation of cyclic AMP dependent protein kinase (Weigt *et al*., 1997; 1998a). Interestingly in their preparation the action of isoflurane was dependent on PKC, and it will be of interest to assess the effects of PKC co-expression on the response of our preparation to isoflurane.

In conclusion, we have assessed the effects of halothane on skeletal muscle, cardiac muscle and neuronal Na channel isoforms in the absence and presence of PKC co-expression. Cardiac channels were relatively insensitive to halothane in this preparation. Halothane suppressed skeletal muscle and neuronal Na currents in the presence of PKC through suppression of the slow mode of inactivation gating.

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