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The mechanism by which procaine inhibits catecholamine secretion from bovine chromaffin cells

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1 We have investigated the action of procaine on stimulus-secretion coupling in bovine adrenal chromaffin cells.

2 Procaine inhibited the catecholamine secretion evoked by 500 μM carbachol (CCh) with an IC_{50} of 35 μM and the associated calcium influx (IC_{50} 60 μM). It inhibited the catecholamine secretion evoked by depolarization with high potassium by less than 20% even at the highest concentrations tested (3.2 mM).

3 The secretion evoked by CCh was associated with an increase in sodium influx. This evoked influx was also inhibited by procaine (IC_{50} 80 μM).

4 This selective action of procaine on the CCh-evoked catecholamine secretion was investigated further by patch-clamp techniques.

5 In agreement with the ion flux studies, procaine inhibited the inward current evoked by CCh. Procaine also altered the spectral characteristics of the noise associated with the agonist-induced current by adding an additional high frequency component. The amplitude of this component showed an e-fold increase for a 55 mV membrane hyperpolarization.

6 Data from cell-attached patches showed that increasing concentrations of procaine produced a progressive fall in the mean channel open time and an increase in mean blocked time. This combination led to a decrease in mean burst length. In addition, P_{open} was reduced by 50 μM procaine. These changes in channel conducting time were sufficient to account for the reduction in inward current. A limited study of the action of procaine on nicotinic channels in outside-out patches gave similar results.

7 The data were considered in relation to various schemes of anaesthetic-channel interactions. The data did not fit the sequential blocking model or the extended channel block model but could be fitted to a modified sequential blocking model in which the rate constant for channel reopening after block was itself subject to modulation by the anaesthetic and the blocked channel could close without passing through the open state.

Keywords: Anaesthetic; chromaffin cells; catecholamines; nicotinic receptor; patch-clamp; procaine; secretion

Introduction

Bovine adrenal chromaffin cells secrete catecholamines in response to nicotinic stimulation or to direct depolarization (Baker & Knight, 1984; Douglas & Rubin, 1963). We have previously shown that general anaesthetics such as pentobarbitone and halothane interfere with this secretion by depressing the influx of sodium and calcium ions (Pocock & Richards, 1987; 1988). Although local anaesthetics such as procaine are primarily used as nerve blocking agents they are also known to modify the response of the nicotinic acetylcholine receptors of the neuromuscular junction (Katz & Miledi, 1975; Adams, 1976; Ruff, 1977; Neher, 1983) and those of *Aplysia* neurones (Marty, 1978). In addition, procaine inhibits chemical transmission in the olfactory cortex (Richards, 1982) an action it shares with general anaesthetic agents (see Pocock & Richards, 1991 for a recent review).

To examine in greater detail the mechanism by which procaine modifies chemical transmission, we have investigated its action on stimulus-secretion coupling in bovine isolated adrenal medullary cells. These cells are derived from embryonic neural crest tissue and are homologous with sympathetic postganglionic neurones. Following isolation they retain the secretory characteristics of the intact tissue (Baker & Knight, 1984) and offer a suitable experimental model for detailed study of neurosecretion at the cellular and molecular level. Here we describe experiments in which we have made parallel measurements of ion fluxes and catecholamine secretion in intact cells and employed patch clamp recording techniques to characterize the actions of procaine on the events underlying stimulus-secretion coupling. We show that while procaine has little effect on secretion evoked by direct depolarization, it selectively inhibits the secretion evoked by nicotinic agonists such as carbachol (CCh). This inhibition of catecholamine secretion occurs at concentrations of procaine much less than those required for nerve block and can be fully accounted for by blockade of the channels gated by the nicotinic receptor. A preliminary account has been given to the Physiological Society (Charlesworth *et al.*, 1990).

Methods

Preparation and handling of cells

Bovine chromaffin cells were isolated by enzymic digestion as described previously (Pocock, 1983a). For tissue culture, the cell isolation procedure was carried out under sterile conditions and the resulting suspension was purified by differential plating as described by Waymire *et al.* (1983). For the patch-clamp experiments, cells were plated on glass coverslips at a density of 0.5×10^6 ml^{-1}. The coverslips were coated with poly-L-lysine or collagen prior to plating. A small number of experiments were performed with cells immobilised on cytodex beads. For these studies the cells were plated at a density of $2-5 \times 10^6$ ml^{-1} and maintained in culture in petri dishes. Cultures were maintained in a 1:1 mixture of Dulbecco's minimum essential medium and Ham's F12 medium supple-

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mented with newborn bovine serum (usually 10% but supplements of 2% or 5% were occasionally used) and kept at 37°C in a humidified atmosphere containing 5% CO_2.

Measurement of catecholamine secretion and ion fluxes in cell suspensions

Basal and evoked secretion of catecholamines and the associated ion fluxes were measured by centrifuging cell suspensions through a layer of oil as described previously (Pocock, 1983b; Pocock & Richards, 1987). To measure the ^{22}Na influx evoked by CCh it was necessary to inhibit the sodium efflux via the sodium pump and sodium influx via voltage-gated sodium channels respectively by including ouabain (20 μM) and tetrodotoxin (TTX; 10 μM) in the bathing medium. The catecholamine content of the cell suspension and supernatant was assayed by the method of von Euler & Floding (1955) using a Perkin-Elmer fluorescence spectrophotometer. Catecholamine secretion could then be expressed as a percentage of the total (see Pocock, 1983a and Pocock & Richards, 1987 for further details).

Continuous measurement of catecholamine secretion from immobilised cells

Cytodex beads carrying cells were mounted in a small perspex column (10 × 3 mm) and perfused continuously at 1 ml min^{-1} with sodium Locke solution. Where appropriate to the experimental design, procaine was included in the perfusate. The cells were stimulated by injecting either 100 μl or 2 ml of a 500 μM solution of CCh in sodium Locke solution into the perfusing stream via an injection loop. The injected solution contained procaine when required. The catecholamine content of the perfusate was monitored continuously by an electrochemical detector (Applied Chromatography Systems model 350/06). Neither procaine nor CCh interfered with the electrochemical assay of the catecholamine.

Electrophysiological methods

Experiments were carried out using three variants of the patch-clamp recording technique of Hamill *et al.* (1981). The configurations used were on-cell (cell attached) recording, whole cell recording under voltage clamp and recording from excised outside-out patches. All experiments were performed on small spherical chromaffin cells (10–15 μm diameter) which had a capacitance of less than 10 pF. Patch pipettes were fabricated from thick-walled borosilicate glass (Clarke Electromedical GC150) and had tip diameters of 1–2 μm. The electrode tips were fire polished on a microforge before use. Their resistance was 5–15 MΩ and the series resistance was always less than 60 MΩ and usually less than 20 MΩ. As the input resistance of chromaffin cells was more than 1 GΩ in all of our recordings and as the currents flowing through the pipette during the experiment were small, the voltage errors due to series resistance were also small and membrane holding potentials were not corrected for these errors. All experiments were performed at room temperature (18–22°C).

Recording and analysis Signals were recorded with either a List electronic L/M EPC-5 or Axopatch 1D patch clamp amplifier and were stored on magnetic tape (flat bandwidth d.c.-2.5 kHz or in later experiments d.c.-20 kHz). For analysis of whole cell current noise the signal from the tape recorder was filtered with a 7-pole Cauer eliptical filter (Frequency Devices model 675, bandpass d.c.-1 kHz) and digitized at 2.5 kHz. These anti-aliasing filters give a flat frequency – response (typically ± 0.02 dB) to their cutoff frequency with a phase shift similar to that of an eight pole Butterworth filter but have a much greater attenuation rate above the cutoff (−94 dB at twice the corner frequency). The residual high frequency ripple lies below the resolution of the least significant bit of our 12 bit analogue to digital converter. Records were divided into 0.4 s blocks prior to calculation of the spectral density. CCh or CCh plus procaine was applied for 60 s in order to acquire sufficient time blocks for averaging and each spectrum was derived from a single continuous application. The mean power spectrum was calculated by averaging at least sixty four of the spectra obtained from the 0.4 s time blocks. To obtain the noise spectrum due to application of agonist alone the spectrum obtained before application of agonist was subtracted from that obtained during its application. The resulting spectra were then fitted by either a single or a double Lorentzian function using a least-squares Levenberg-Marquart algorithm with proportional weighting of the data points. The quality of the fitted curve was judged from the value of χ^2 and the random distribution of residuals. The amplitude of single channel currents was estimated from the parameters derived from the fitted power spectra and the mean inward current (Anderson & Stevens, 1973; Cull-Candy *et al.*, 1988).

For the analysis of the single channel events, signals were low-pass filtered at 1 kHz with a four-pole Bessel Filter and digitised at 10 kHz. To determine the frequency and duration of channel openings an all-points histogram of current amplitude was first constructed and Gaussian distributions fitted to the peaks in the histogram. The detection threshold for channel opening was then set at 50% of the amplitude for a single channel opening. Each transition was inspected and an idealized reconstruction of the open-closed transitions made. From this transition list we constructed frequency distribution histograms of open state and closed state durations. For analysis of the transition list, multiple channel openings were excluded from the formal data analysis together with openings of less than full amplitude (apparent substates), the excluded states were considered closed and their time values amalgamated with the preceding closed times. For the data obtained from some patches we also analysed the distribution of open and closed times taking the substate to be equivalent to a full opening. The time constants of the distributions of open and closed times derived in this way were little different from those obtained solely from full amplitude openings. The minimum resolvable time of single channel events for a low pass filter of 1 kHz was taken as 0.3 ms. Shorter events were considered unresolved. These unresolved openings and closures bias the recorded value in the first bin of frequency histograms leading to a consistent under-estimate of the true value. For this reason, the value of the first bin was always excluded from the curve fitting procedures

Very long openings in the cell attached configuration tended to show a distinct relaxation. As chromaffin cells have a high input resistance (> 1 GΩ) the current flowing through a single nicotinic channel may well be sufficient to depolarize the cell by a few mV, sufficient to account for the observed relaxation (seen Fenwick *et al.*, 1982). Nevertheless, the amplitude of these long openings rarely falls by more than 20% and so the openings do not fall below the 50% detection threshold. The relaxation of current amplitude will not contribute significantly to the all points current distribution as long openings are comparatively rare.

For the anlaysis of 'bursts' of openings we calculated a critical gap length for bursts below which a closure is classified as a gap within a burst (Colquhoun & Sakmann, 1983; Clapham & Neher, 1984). This is calculated so that the proportion of short intervals misclassified as closures between bursts was equal to the proportion of long intervals misclassified as closures within bursts. In practice the value of this time interval (t_{crit}) under control conditions was about 3 ms but the calculation of burst frequency and duration was not unduly sensitive to small changes in the chosen value.

The curve fitting procedure employed an unweighted least-squares Levenberg-Marquart algorithm as this procedure has previously been shown to be relatively insensitive to truncation of data sets (Dionne & Liebowicz, 1982).

To record whole cell calcium currents, cells were subjected to a depolarizing step of 100 ms duration from a holding

potential of −80 mV to zero at 10 s intervals. The mean inward current flowing in response to the voltage step was then plotted as a time sequence to give the final record. The pulse sequence was controlled by a Tandon PCA computer coupled to a Cambridge Electronic Design 1401 interface.

The spectral noise analysis (SPAN), voltage clamp (VCAN) and channel transition (PAT) detection programmes were kindly supplied by Dr John Dempster, University of Strathclyde, U.K.

Solutions

The external bathing medium (Locke solution) contained (mM): NaCl 140, KCl 5, $MgCl_2$ 1.8, $CaCl_2$ 1.0, HEPES 15, glucose 5.5; the pH was adjusted to 7.4 with NaOH. For the experiments designed to study the correlation between catecholamine secretion and ion movements procaine and CCh were dissolved in Locke solution at concentrations appropriate to the experimental design and cell suspensions were added to these solutions. For studies of the effects of procaine on secretion evoked by direct depolarization, cell suspensions were added to an equal volume of Locke solution in which the concentration of sodium and potassium ions had been reversed. The cells are therefore depolarized by a solution containing 75 mM K^+. Further experimental details can be found in the papers by Pocock & Richards (1987, 1988).

For the experiments on the nicotinic receptor we wished to define the equilibrium potentials for sodium and potassium. To achieve this the pipettes used for whole cell recordings contained (mM): KCl 120, NaCl 20, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 11; the pH was adjusted to 7.2 with KOH and free calcium was measured as less than 10^{-7} M with an Orion 93-20 calcium electrode. For recording from outside-out patches, KCl was 140 mM and NaCl was omitted and other ions were as for the whole cell recordings. For cell attached patches the electrode was filled with sodium Locke solution containing either 10 μM CCh or 10 μM CCh plus an appropriate concentration of procaine.

To maximize the amplitude of the calcium channel currents we used barium as the main charge carrier (see Nowycky *et al.*, 1985) and for these experiments the bathing medium contained mM: tetraethyl ammonium chloride 120, $CsCl_2$ 5, $BaCl_2$ 10, HEPES 10, glucose 10 and tetrodotoxin 10 μM pH 7.4. The pipette contained mM: $CsCl_2$ 120, EGTA 10, $MgCl_2$ 5, HEPES 40, ATP 2 and GTP 0.3, pH 7.2

In the whole-cell patch-clamp experiments procaine and CCh were applied by microperfusion by use of a U-tube similar to that described by Krishtal & Pidoplichko (1980) and for the outside-out patches procaine was applied by pressure from a micropipette. For cell-attached patch-clamp experiments both CCh and procaine were present in the patch pipette at appropriate concentrations. Both carbachol and procaine were obtained from Sigma Chemicals Co.

Statistical analysis

Combined values are shown as mean ± s.e.mean unless otherwise indicated. Differences between data sets were assessed using the *t* test and were considered statistically significant when $P < 0.05$.

Results

The action of procaine on catecholamine secretion

In agreement with our previous work (Pocock & Richards, 1987; 1988) the total catecholamine content of the cell suspensions was around 70 nmol/10^6 cells. In the absence of secretagogues, the catecholamine present in the medium after 10–15 min incubation was 3–5% of the total. Concentrations of procaine up to 200 μM produced no significant change in this basal secretion. Higher concentrations of anaesthetic (400–3200 μM) produced statistically significant increases ($P < 0.05$) in basal secretion e.g. from 4.43 ± 0.28% ($n = 14$; control) to 6.27 ± 0.60% (mean ± s.e.mean; for 1600 μM procaine; $n = 4$).

As described previously (Pocock & Richards, 1987) the chromaffin cells were stimulated to secrete by exposure to either 500 μM CCh or a solution nominally containing 75 mM potassium. These stimuli caused an additional 3.82 ± 0.18% of total catecholamine to be secreted following potassium depolarization ($n = 7$) and 3.83 ± 0.33% following stimulation by CCh ($n = 14$). Figure 1 shows the effects of procaine (5–3200 μM) on catecholamine secretion evoked by both CCh and high potassium. CCh-evoked secretion was strongly inhibited by the anaesthetic with an IC_{50} of 35 μM. In contrast, the secretion evoked by high potassium was scarcely inhibited (less than 20%) even at the highest concentrations of anaesthetic tested (3.2 mM). The Hill coefficient for the inhibition of CCh-evoked catecholamine secretion by procaine was 1.1.

Effect of procaine on the time-course of secretion

To determine the effect of procaine on the rate of catecholamine secretion, beads carrying chromaffin cells were mounted in a small column and continously perfused with Locke solution at approximately 1 ml min^{-1}. The effluent from the column was continuously monitored to determine the rate of catecholamine secretion (see Methods). In response to a brief pulse of 200 μM CCh (about 6 s duration) injected into the superfusate the catecholamine secretion from the immobilised cells rose to a peak within 15–20 s as shown in Figure 2a and declined over the ensuing 40–45 s. When cells were exposed to CCh for 80–120 s, secretion reached a plateau before declining slowly in the continuing presence of the agonist, with a half time of about 80 s. Repeated applications of identical pulses of CCh led to decreasing responses similar to the progressive decrease in the secretory response of intact glands (Douglas & Rubin, 1963; Holmes & Schneider, 1973).

In the presence of procaine (50 μM) both the rate of rise and the extent of secretion seen in response to CCh were depressed as shown in Figure 2. The rate of decline of secretion was accelerated during prolonged exposure to the agonist. The half time for decay decreased from 80 ± 1 s in control to 41 ± 2 s in the presence of 50 μM procaine ($n = 3$). Nevertheless, this effect was insufficient to account for the overall decrease in catecholamine secretion. These effects of procaine were partially reversible (see Figure 2).

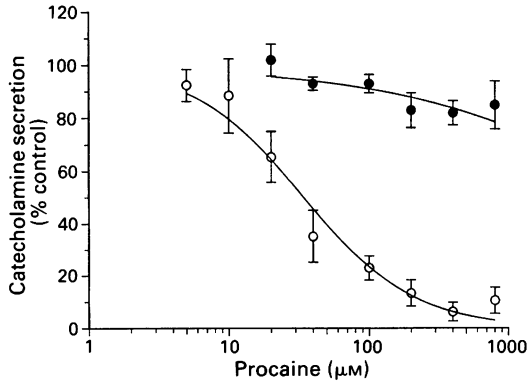


Figure 1 The concentration-dependence of the inhibition of catecholamine secretion by procaine. Freshly isolated bovine chromaffin cells were stimulated by either 75 mM potassium (●) or 500 μM carbachol (CCh, ○) as described in the Methods. The line for CCh-evoked secretion was fitted to the Hill equation with a coefficient of 1.1.

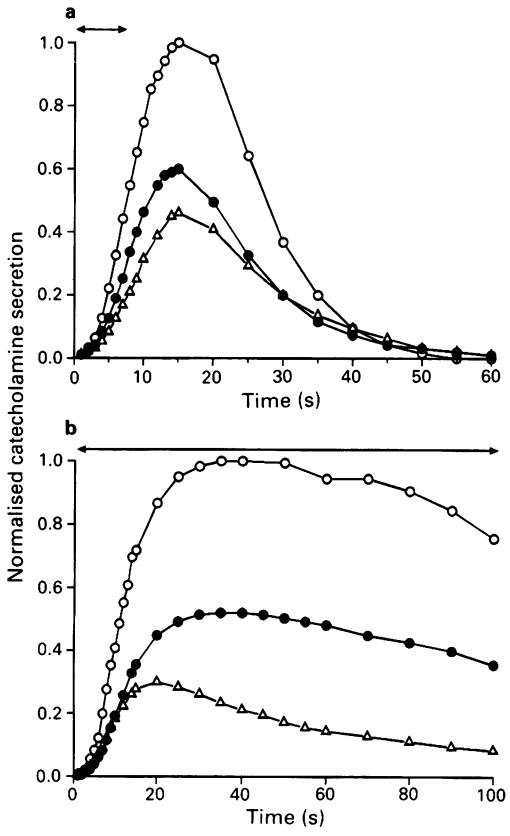


Figure 2 The effect of procaine on the time course of catecholamine secretion from cultured chromaffin cells attached to cytodex beads. The secretory response was normalised to the maximum seen following the initial challenge with carbachol (CCh). (a) The time course of catecholamine secretion seen in response to a 6 s pulse of 200 μM CCh as indicated by the double-headed arrow. (b) The time course of secretion during a 100 s application of CCh as indicated by the double-headed arrow. In (a) and (b): (○) control, (△) 50 μM procaine, (●) recovery.

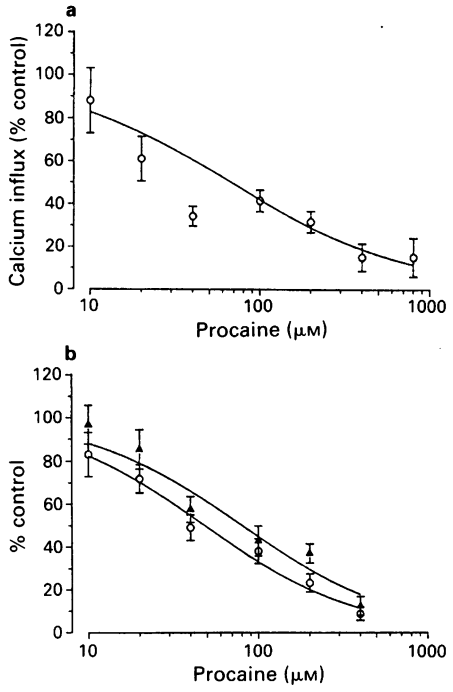


Figure 3 The concentration-dependence of the effect of procaine on carbachol on (CCh)-evoked ^{45}Ca influx (a) and on the CCh-evoked ^{22}Na influx and the associated catecholamine secretion (b) both expressed as a percentage of control. Lines were fitted to the Hill equation with coefficients of 0.82 for Ca^{2+} influx (a) and 0.97 and 0.95 for procaine inhibition of CCh-evoked catecholamine secretion (b, ○) and sodium influx (b, ▲).

The action of procaine on ^{45}Ca influx

Freshly isolated bovine chromaffin cells have a resting influx of ^{45}Ca of 21.1 ± 2.3 μmol (l cells)$^{-1}$ min^{-1}. When they were stimulated by either CCh or high potassium the increase in catecholamine secretion was paralleled by an increase in ^{45}Ca influx of 219 ± 39 and 581 ± 46 μmol (l cells)$^{-1}$ ($n = 6$) most of which occurred within the first minute of stimulation (see also Holz *et al.*, 1982; Pocock & Richards, 1987; 1988). Procaine at concentrations up to 800 μM had no significant effect on basal ^{45}Ca uptake but inhibited that seen in response to CCh with an IC_{50} of approximately 60 μM (see Figure 3a). In agreement with the data for secretion, procaine had little effect on the potassium-evoked ^{45}Ca influx. The inhibition by procaine of the ^{45}Ca influx evoked by CCh could be fitted to a Hill equation with a coefficient of 0.82.

The action of procaine on carbachol-evoked ^{22}Na influx and catecholamine secretion

It is generally agreed that activation of nicotinic receptors is associated with the inward movement of sodium ions (Fenwick *et al.*, 1982; Cull-Candy *et al.*, 1988; Jacobson *et al.*, 1991). In agreement with this we have shown previously a strong dependence of CCh-evoked secretion upon extracellular sodium ions (Pocock & Richards, 1987; 1988). Under the conditions for measuring sodium influx given in the Methods, procaine inhibited the CCh-evoked catecholamine secretion with an IC_{50} of approximately 50 μM (see Figure 3b). Concentrations of procaine up to 400 μM had no significant effect on resting ^{22}Na influx which was 3.02 ± 0.10 mmol (l cells)$^{-1}$ min^{-1} ($n = 6$). In the absence of procaine the CCh-evoked ^{22}Na influx was 13.5 ± 0.8 mmol (l cells)$^{-1}$ almost all of which occurred within the first minute of stimulation. The ^{22}Na influx evoked by CCh was inhibited by the anaesthetic with an IC_{50} of approximately 80 μM (Figure 3b), in parallel with the inhibition of secretion. As for the experiments with ^{45}Ca influx, the inhibition of both catecholamine secretion and ^{22}Na influx by procaine could be fitted by a Hill equation with a coefficient close to unity (0.97 and 0.95 respectively).

Electrophysiological analysis of the effect of procaine

Action of procaine on carbachol-evoked inward current By use of the whole cell variant of the patch clamp technique it is possible to record an inward current in response to application of CCh to adrenal chromaffin cells. This inward current is the electrical parallel of the agonist-evoked ^{22}Na influx. Figure 4 shows typical current records observed in single chromaffin cells in response to a 5 s pulse of 200 μM CCh. There is a fast initial transient followed by a rapid decline, reflecting receptor desensitization. The initial amplitude varied from cell to cell and tended to desensitize with repeated applications. For the control tests the peak inward current had a mean value of 261 pA and ranged from 458 pA to 35 pA (6 tests in three cells). During a 5 s application of 200 μM CCh the total charge carried ranged from 40 to 721 pC and had a mean value of 347 pC. The decay of the inward current could be fitted by a single exponential with a mean time constant of 1.06 ± 0.06 s. In paired tests, co-application of procaine (50 μM) reduced the total charge carried to 46 ± 7% of control (mean ± s.e.mean; 6 paired tests in three cells). This decrease resulted both from a reduction in peak amplitude (59 ± 7% of control) and from a reduction in the time constant of current decay (66.5 ± 9.3%

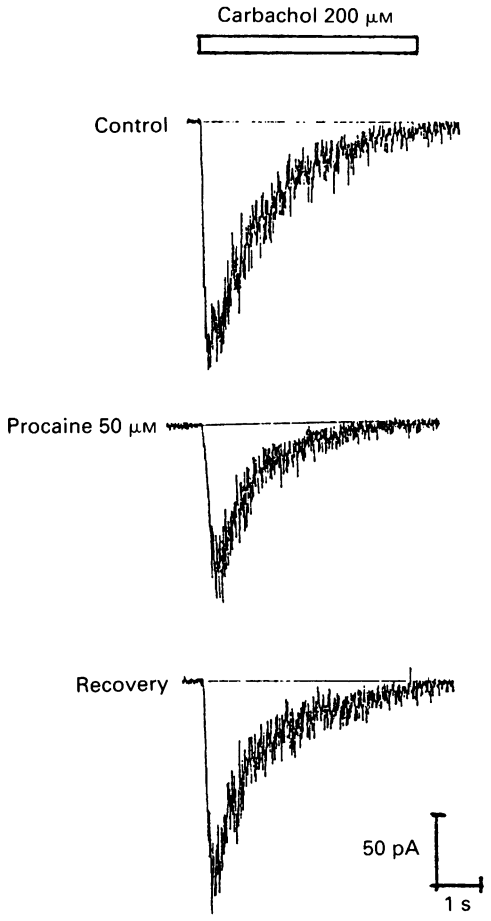


Figure 4 The action of 50 μM procaine on the carbachol (CCh)-evoked inward current. The record was obtained by the whole cell voltage clamp technique and shows the response of a single chromaffin cell to a 5 s pulse of 200 μM CCh before, during and after exposure to 50 μM procaine. Holding potential was −80 mV. The interval between each record was 30 s. Inward currents are shown by downward deflections.

of control). These effects of procaine on the CCh-evoked inward current were largely reversed on washing. After recovery the total charge carried was 87 ± 9%, the peak amplitude was 80 ± 9% and time constant of decay was 90 ± 9% of the paired control values. An example of the action of procaine on the inward current is shown in Figure 4.

Low concentrations of CCh (10 μM) evoke a small inward current that does not show an obvious time-dependent decay. With a holding potential of −80 mV the steady-state inward current evoked by 10 μM CCh had a mean value of 1.58 ± 0.22 pA. Co-application of 20 μM procaine with 10 μM CCh reduced the inward current to 0.66 ± 0.12 pA (seven cells). When expressed as a percentage of the paired control the inward current was inhibited by 56 ± 6% ($n = 7$). In two cells, 50 μM procaine reduced the steady-state inward current by 80%.

Noise analysis An increase in noise is associated with the inward current (see Figure 4) and this noise can be attributed to activation of nicotinic channels (Anderson & Stevens, 1973; Fenwick *et al.*, 1982; Cull-Candy *et al.*, 1988). In the experiments described here the CCh concentration was kept low (10 μM) to avoid significant desensitization of the response and the mean inward current at a holding potential of −80 mV was very low at 1.58 ± 0.22 pA. With this holding potential a spectral analysis of the agonist-induced noise yielded a frequency distribution that was adequately fitted by a single Lorentzian function with a corner frequency of 15.6 ± 1.1 Hz ($n = 7$). This corner frequency corresponds to a time constant for channel closure of 10.2 ms for the simple model of channel behaviour proposed by Anderson & Stevens (1973). Similar values were found at other holding potentials (e.g. Figure 5a,c). A slightly better fit could be obtained by the sum of two Lorentzian functions in some instances (see also Jacobson *et al.*, 1991). The main component had a corner frequency around 15 Hz and the second a corner frequency around 100 Hz but the low-frequency intercept (S(0)) value of the high frequency component was usually less than 1% of the low frequency component. As the high frequency component was very small and the errors in estimating its parameters correspondingly large we have fitted our control data to single Lorentzian functions (Figure 5). The single channel conductance estimated from the noise spectra and the mean inward current was 32 ± 1.5 pS ($n = 7$). The reversal potential of CCh-evoked inward current was found to be −13 mV by extrapolation, a value close to the expected value of −3 mV assuming the nicotinic channel to be equally permeable to both sodium and potassium ions.

In addition to its depressant action on inward current, procaine caused a second, higher frequency, component to

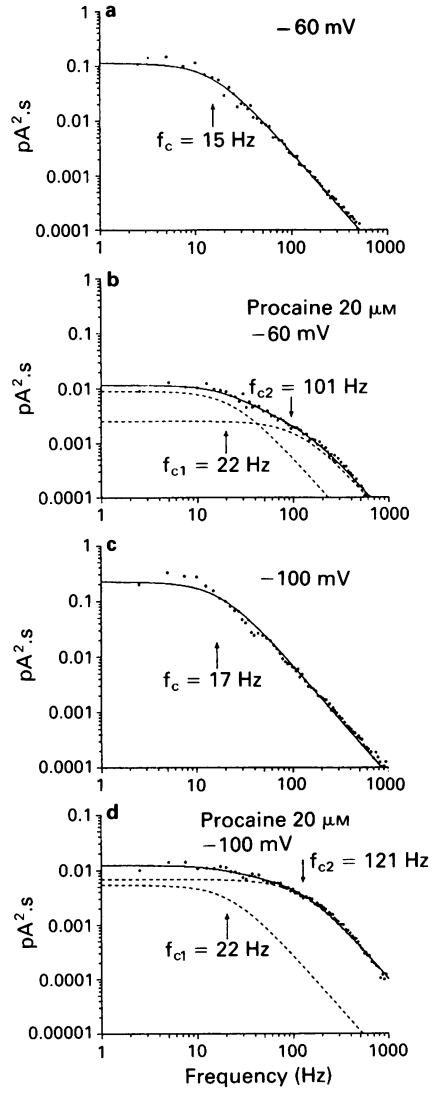


Figure 5 Spectral analysis of carbachol (CCh)-induced current noise in the presence and absence of 20 μM procaine. (a, c) Show the control spectra for holding potentials of −60 mV (a) and −100 mV (c). Panels (b, d) show spectra for the same holding potentials in the presence of anaesthetic. All data are from a single chromaffin cell.

appear prominently in the noise spectrum (see also Katz & Miledi, 1975). With a holding potential of −80 mV, co-application of 10 μM CCh and 20 μM procaine gave noise spectra that were best fitted by the sum of two Lorentzians with corner frequencies of 22.5 ± 1.2 Hz and 122 ± 6 Hz ($n = 7$) corresponding to time constants for channel closure of 7.1 and 1.3 ms respectively. The S(0) intercept of the low frequency component was on average 2.4 times that of the high frequency component when the holding potential was −80 mV. A similar pattern is seen at other holding potentials (see Figure 5b,d). In two cells, co-application of 10 μM CCh with 50 μM procaine the corner frequencies were higher at 36 Hz and 280 Hz (corresponding to time constants of 4.4 and 0.6 ms). At this concentration of procaine the relative amplitude of the S(0) value of the high frequency component was also increased. Procaine had no effect on the apparent single channel conductance calculated from the noise spectra. The conductance was 32.6 ± 1.3 pS ($n = 7$) in 20 μM procaine and 36 and 35 pS for two determinations in 50 μM procaine.

Voltage-dependence of procaine action The voltage-dependence of the noise spectra was examined in seven cells. In the absence of procaine the corner frequency showed no consistent change as the holding potential was varied between −100 mV and −50 mV (see Figure 5a,c). This is somewhat at variance with the weak voltage-dependence of corner frequency reported by Cull-Candy *et al.* (1988). The reason for this discrepancy is not clear. In the presence of 20 μM procaine, neither the corner frequency of the low frequency component nor that of the high frequency component showed any statistically significant trend with membrane potential. In contrast, the S(0) intercept of the high frequency component increased approximately linearly with increasing hyperpolarization showing an e-fold increase for a 55 mV hyperpolarization (Figures 5 and 6). At a holding potential of −100 mV the S(0) values for both components were almost equal.

Single channel studies

The lignocaine derivatives QX222 and QX314 have previously been shown to produce an increase in the high-frequency components of the noise induced by acetylcholine at the neuromuscular junction (Ruff, 1977; Neher & Steinbach, 1978). This behaviour has been explained in terms of a simple sequential blocking model (see Ruff, 1977; Neher & Steinbach, 1978; Colquhoun & Hawkes, 1983) which makes a number of predictions for anaesthetic-channel interactions. The most readily tested predictions are:

(1) That the mean channel open time should decrease as the anaesthetic concentration increases.

(2) That the mean time for which the channel is blocked should be independent of the anaesthetic concentration.

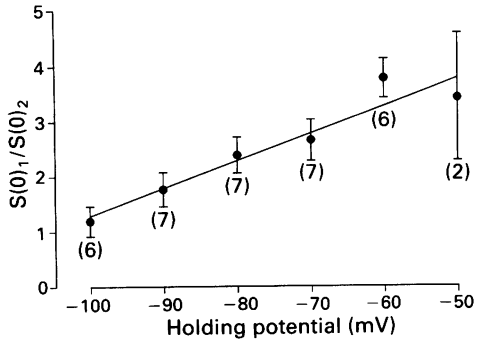


Figure 6 The influence of holding potential on the ratio between the zero-frequency intercepts ($S(0)_1/S(0)_2$) observed during the application of 20 μM procaine. The numbers in parentheses give the number of observations for each data point. The regression line was fitted to the equation $y = 0.05(x) + 6.27$ by the method of least squares.

(3) That the total channel open time within a burst of openings should be independent of the anaesthetic concentration.

(4) That the mean burst length should increase with increasing anaesthetic concentration.

The single channel studies described below were performed both to provide a detailed description of the action of procaine on a neuronal type of nicotinic channel and to test these predictions.

Cell attached patches With the cell attached configuration, CCh (10 μM) was always present in the patch pipette to activate the channels. In this configuration, application of procaine to the region outside the patch was ineffective in modifying the kinetics of channel opening so, where appropriate, procaine was included in the solution within the patch pipette at 2–50 μM.

When activated by CCh alone, the channel openings were seen as inward currents of approximately 2pA (Figure 7). The individual channel openings were usually single events and were not interrupted by brief closures (see below). In 6 out of 7 patches examined, the distribution of current amplitudes showed a second peak corresponding to a channel with 70% of the conductance of a full channel opening. These events appear to represent a substate of the nicotinic channel as direct transitions between the full and low conductance states were occasionally observed. The substate accounted for about a quarter of all transitions and, although we found no difference between the action of procaine on the low conductance state and its action on the full conductance state, we have excluded low conductance states from the formal analysis presented here (see Methods). The amplitude of the channel openings was unchanged by 2 and 10 μM procaine but 50 μM caused a statistically significant reduction in amplitude when assessed by the t test ($P < 0.05$; see Table 1). At this concentration, however, the channel openings are very brief with an open time distribution time constant of approximately 0.8 ms (see below). About two thirds of these fast openings will be shorter than 0.8 ms and will be attenuated by our low-pass filter (−3 dB at 1 kHz). In addition, when channel openings are very brief, it is not easy to distinguish substates from attenuated full openings. This apparent reduction in channel conductance can, therefore, be fully explained by the filter characteristics of the recording system.

In the absence of procaine the duration of channel open times was best fitted by the sum of two exponentials with time constants of 1.07 ms and 6.94 ms. The combined data for seven separate patches are shown in Figure 7a. The probability of the channel being in the open state (P_{open}) was low with a mean value of 0.0068 (Table 1).

Addition of procaine (2–50 μM) to the patch pipettes reduced the number of long duration openings and shifted the distribution of open times progressively to shorter intervals as the concentration of procaine increased (see Figure 7b–d). At the highest concentration tested, the open time distribution could be fitted by a single exponential with a time constant of 0.78 ms. The open time distributions of Figure 7 represent the combined data of between five and seven separate patches. The mean open time of the channels progressively decreased as the concentration of procaine increased (Figure 9) as predicted by the sequential blocking model. The channel blocking rate constant was estimated from these data as $2 \times 10^7\ M^{-1}\ s^{-1}$ (the value determined from the noise spectra was about $6 \times 10^6\ M^{-1}\ s^{-1}$). This decrease in mean channel open time was associated with marked oscillations of the open state which increased in frequency as the procaine concentration was increased from 2 to 10 μM (Figure 7). The open state probability was not significantly different from control for 2 and 10 μM procaine but was reduced in the presence of 50 μM procaine (see Table 1).

The closed times were distributed in a biphasic manner with a high incidence of brief closures (<2 ms) and a broad

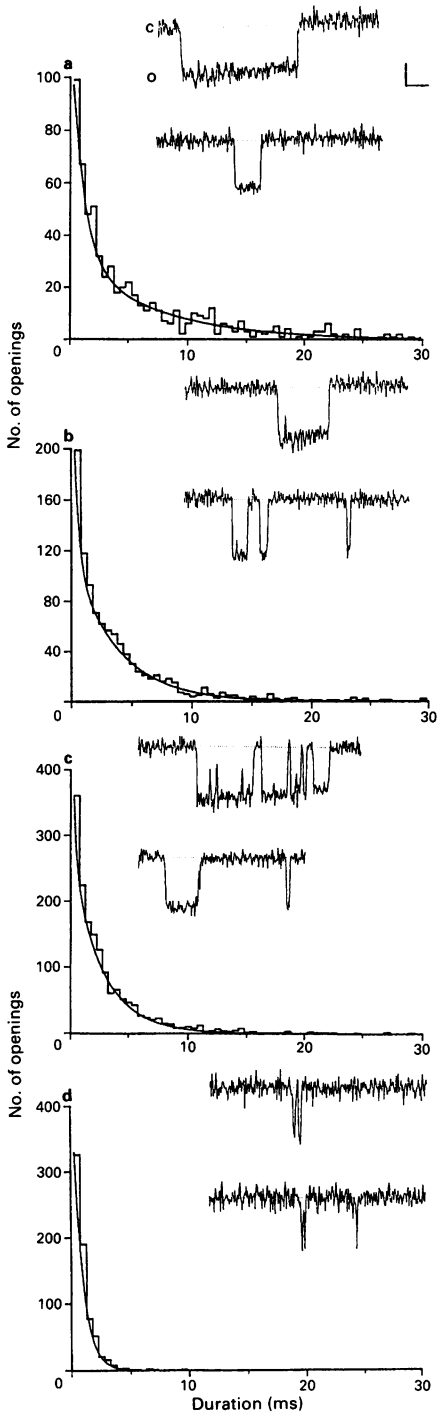


Figure 7 The effect of increasing concentrations of procaine on the distribution of the open times of the channels activated by 10 μM carbachol (CCh). Each panel shows combined data for 5–7 cell attached patches. The pipette potential was set at zero. The smooth curves are the calculated exponential functions fitted to the frequency distribution of open times greater than 0.5 ms (see Methods). The histograms shown are (a) control, (b) 2 μM procaine, (c) 10 μM procaine and (d) 50 μM procaine. The original records are examples of channel openings and bursts recorded under the stated conditions. Calibration bars in (a) apply to all traces and are 1 pA (vertical bar) and 10 ms (horizontal bar). The open state of the channel (reflecting *inward* current) is shown by downward deflections (o) from the closed state (c).

distribution of longer closed times which could be fitted with a time constant of about 500 ms (not shown). We interpret this biphasic distribution as follows: the brief closures represent closures within bursts and the longer closures are intervals between bursts (see also Colquhoun & Sakmann, 1983; Clapham & Neher, 1984). Application of procaine (2–50 μM) caused a progressive increase in the proportion of brief closures and their distribution was then best fitted by two exponentials (Figure 8 and Table 1). As the time constant for the distribution of the long closures reflects the gap between bursts and as the number of channels in a patch is not known, the long time constants are difficult to interpret and have therefore been ignored in our analysis.

Effects of procaine on bursting behaviour In the absence of procaine, the channel open times were exponentially distributed and there were few short channel closures. The distribution of burst length was therefore very similar to the distribution of channel open time. In the presence of procaine, channel openings were more frequently interrupted by brief closures so giving a characteristic pattern which is typical of burst behaviour. A similar effect on burst behaviour has been reported for the action of benzocaine and the lignocaine derivative QX222 at muscle nicotinic receptors (Ogden *et al.*, 1981; Neher, 1983). Nevertheless, even in the presence of 50 μM procaine the majority of channel openings were single events. For the experiments described here the classification of groups of openings as bursts was determined according to the criteria given by Clapham & Neher (1984) (see Methods). In the absence of procaine the distribution of burst length could be described adequately by the sum of two exponentials but as the concentration of procaine increased the time constants of the distributions became shorter and mean burst length decreased e.g. from 7.94 ms in control to 5.21 ms in 2 μM and 2.91 ms in 50 μM procaine.

As the distribution of closed times progressively changed with increasing procaine concentration (see Figure 8 and Table 1) the t_{crit} for calculating burst characteristics also changed. This will bias the calculation of mean blocked time. To compensate for this we have also analysed the data with a fixed value for t_{crit} of 8 ms which is the value appropriate for 50 μM procaine. Although this reanalysis gives different mean values for burst length and blocked time at other concentrations of procaine (e.g. 8.4 ms burst length in control compared to 7.94 ms with a t_{crit} of 5 ms), the tendency for burst length to decrease and blocked time to increase with increasing procaine concentration remains (Table 1). Overall, increasing concentrations of procaine caused an increase in the number of gaps per burst and an increase in the mean blocked time but a reduction in mean burst length (Figure 9).

Excised outside out patches To gain better control of the membrane potential during drug application and to permit acquisition of data from the same channels in the presence and absence of anaesthetic, we have also examined the effect of procaine in three excised outside-out patches. With a holding potential of −70 mV the channel openings are seen as inward current movements of 2.72 ± 0.098 pA amplitude. As with the cell attached patches, the distribution of channel open times was best fitted by the sum of two exponentials with time constants of 0.47 ms and 6.7 ms (combined data from three patches). Application of procaine (50 μM) reduced the amplitude of single channel events to 2.52 ± 0.71 pA, abolished long duration openings and shifted the open time distribution curve to the left. As for the studies with cell attached patches, the distribution of channel open times in the presence of 50 μM procaine was best fitted by a single exponential which, in this case, had a time constant of 1.33 ms.

As for channels activated by 10 μM CCh in cell attached patches, the distribution of channel closed times was skewed with a large number of brief closures (<2 ms) and a broad distribution of longer closed times with a time constant close to 500 ms. The distribution of brief closures could be fitted by the sum of two exponentials with time constants of 0.65 ms and 17 ms. Application of procaine caused a higher incidence of brief closures (from 12.5% of closures of 2 ms or less in control to 26% of closures of 2 ms or less duration

Table 1 Procaine modulation of the kinetic constants of currents induced by 10 μM carbachol (CCh) in cell attached patches

	Control	*Procaine*		
Parameter		2 μM	10 μM	50 μM
Open state				
Unit current (pA)	2.35 ± 0.03	2.29 ± 0.05	2.28 ± 0.04	1.98 ± 0.03*
τ_{fast} (ms)	1.07 ± 0.11	0.36 ± 0.03	0.27 ± 0.04	—
τ_{slow} (ms)	6.94 ± 0.68	3.64 ± 0.09	2.63 ± 0.06	0.79 ± 0.01
$Area_f/Area_s$	0.54	0.35	0.73	—
Mean open time (ms)	7.32 ± 0.59	4.26 ± 0.18*	2.67 ± 0.12*	1.07 ± 0.04*
P_{open} (%)	0.68 ± 0.03	0.70 ± 0.06	1.1 ± 0.02*	0.21 ± 0.04*
F_{open} (s^{-1})	1.05 ± 0.12	1.83 ± 0.19*	4.06 ± 0.54*	2.16 ± 0.28*
Closed state				
τ_{fast} (ms)	0.75 ± 0.03	0.50 ± 0.03	0.96 ± 0.03	0.85 ± 0.27
τ_{slow} (ms)	—	13 ± 4	11 ± 3	8.8 ± 2.6
$Area_f/Area_s$	—	1.89	2.65	1.09
Burst behaviour				
Burst length (ms)	8.4 ± 0.67	5.62 ± 0.20*	5.13 ± 0.56*	2.91 ± 0.24*
Mean blocked time (ms)	0.81 ± 0.05	1.11 ± 0.05*	1.21 ± 0.06*	1.82 ± 0.06*
Gaps/burst	0.18 ± 0.04	0.33 ± 0.04*	0.63 ± 0.19*	0.75 ± 0.17*
τ_{fast} (ms)	0.74 ± 0.08	0.47 ± 0.06	0.36 ± 0.08	0.74 ± 0.06
τ_{slow} (ms)	7.31 ± 0.53	5.01 ± 0.29	4.30 ± 0.28	4.13 ± 0.83
$Area_f/Area_s$	0.43	0.69	0.47	1.17

The time constants for open times and bursts were calculated using a bin width of 0.5 ms. Those for closed times were calculated for closures of less than 50 ms using a bind width of 1 ms. The burst characteristics were calculated assuming a t_{crit} of 8 ms (see text). The mean open time, burst length, blocked time, P_{open} and F_{open} for 5–7 patches are given ± s.e.mean. Statistical comparisons were made where appropriate (* denotes $P<0.05$ for the difference between control patches and those exposed to procaine). The time constants are calculated form amalgamated data and are given together with the standard error of the estimate. F_{open} is frequency of opening.

when 50 μM procaine was applied). The distribution of brief closures during application of procaine was best fitted by exponentials with time constants of 0.71 ms and 4.37 ms. In the absence of procaine, the distribution of burst length was well fitted by the sum of two exponentials which had time constants of 0.88 ms and 14.6 ms. In agreement with the studies in cell-attached patches, procaine decreased mean burst length from 7.34 ms to 4.31 ms, this decrease being evident in each of the patches studied. The data are summarised in Table 2.

Effect of procaine on voltage-activated calcium currents

Under whole cell voltage-clamp and with the pipette and bathing solutions given in the Methods, a voltage step from a holding potential of −80 mV to 0 mV elicits an inward current which has the characteristics of the calcium currents previously described in chromaffin cells by Bossu *et al.* (1991a,b) and Charlesworth *et al.* (1991). We have conducted a brief study of the effect of procaine on these currents in eight cells. In agreement with the calcium influx data, 200 μM procaine had a barely detectable effect on the inward calcium current, 500 μM procaine produced a 23% reduction while a concentration of 2 mM procaine reduced the current by about half (see Figure 10). Procaine did not alter the rate of decay of the inward current.

Discussion

We have examined the effect of the local anaesthetic, procaine, on stimulus-secretion coupling in bovine adrenal chromaffin cells. At micromolar concentrations this agent inhibits both the catecholamine secretion and the ionic fluxes (Na^+ and Ca^{2+}) evoked by CCh. At comparable concentrations, procaine neither inhibits the K^+-evoked catecholamine secretion and the associated ^{45}Ca influx nor does it inhibit the voltage-gated Ca^{2+} currents. Our previous studies have shown that catecholamine secretion in response to nicotinic agonists is directly related to the Na^+ influx (Pocock & Richards, 1987; 1988) and we have shown here that the inhibition of Na^+ influx can quantitatively account for the reduction in catecholamine secretion (Figure 3b). This suggests that procaine exerts its action at the nicotinic receptor-channel complex without significantly inhibiting either the secretory pathway beyond the level of the plasma membrane or the voltage gated Ca^{2+} channels.

Procaine could depress catecholamine secretion by simply increasing the rate of desensitization of the activated nicotinic receptors. This possibility has been investigated in experiments in which catecholamine secretion was monitored continuously and in studies of the action of procaine on the CCh-evoked inward current. These experiments showed that procaine accelerated the rate of receptor desensitization (see Figures 2 and 4). Nevertheless, this effect is modest and, as the experiments with low CCh concentrations show, procaine is able to reduce steady-state inward currents without inducing a time-dependent desensitization.

The electrophysiological studies of Bossu *et al.* (1991a,b) and of Charlesworth *et al.* (1991) have shown that voltage-activated Ca^{2+} channels of chromaffin cells appear to be of the N and L subtypes originally described in dorsal root ganglion cells by Nowycky *et al.* (1985). Procaine had little effect on the voltage-activated Ca^{2+} currents whether measured directly or indirectly by ^{45}Ca flux measurements (see Results). Nevertheless, agonist-evoked Ca^{2+} entry was powerfully inhibited by procaine. What is the mechanism of this inhibition? A reduction of Na^+ influx caused by a reduction in extracellular Na^+ leads to a decrease in both catecholamine secretion and the agonist-induced Ca^{2+} influx (Pocock & Richards, unpublished observations). Morever, Mulle *et al.* (1992) found that Ca^{2+} current of neuronal nicotinic channels recorded in Na^+ -free media with 5 mM Ca^{2+} , was only 2% of the Na^+ current. This suggests that Ca^{2+} entry through the nicotinic channel is likely to be very small under physiological conditions. Instead it is more likely that voltage-activated Ca^{2+} channels open following depolarization by the CCh-evoked Na^+ influx. The depressant action of procaine on the Na^+ movement gated by the nicotinic receptor would therefore inhibit both Ca^{2+} entry and catecholamine secretion.

The decrease in agonist-evoked ion fluxes is paralleled by a

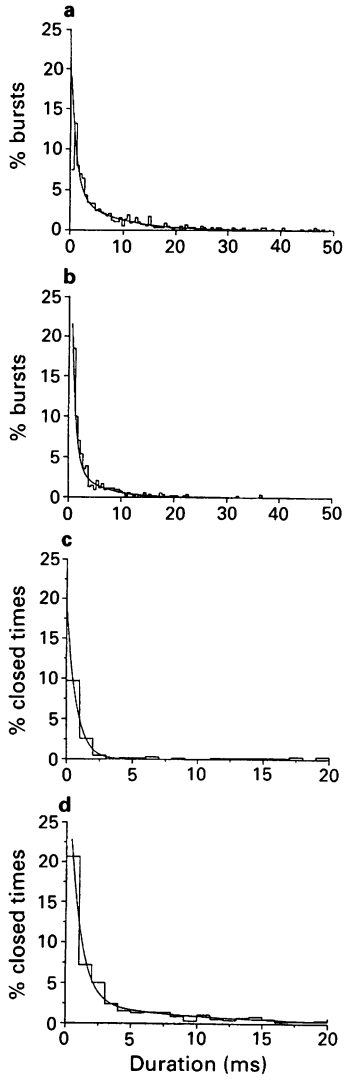


Figure 8 The effect of 50 μM procaine on the frequency distribution of burst length (a, b) and closed times (c, d). In each case the ordinates are expressed as a percentage of the total number of events. Combined data from 5–7 cell attached patches are shown. The smooth curves are the calculated exponential functions fitted to the data points. The time constants of the burst length distributions (τ_f and τ_s) were 1.03 ms and 7.93 ms in control (a), 0.74 ms and 5.0 ms in 50 μM procaine (b). The distribution of brief closed times was fitted by a single exponential with a time constant of 0.75 ms under control conditions (c), and by two exponentials with time constants of 0.85 and 8.8 ms in 50 μM procaine (d). The total number of bursts was 578 for (a) and 524 for (b). The total number of closures was 668 for (c) and 914 for (d). Note that in this figure t_{crit} was determined separately for each condition and was 5 ms for control and 8 ms for 50 μM procaine (see text).

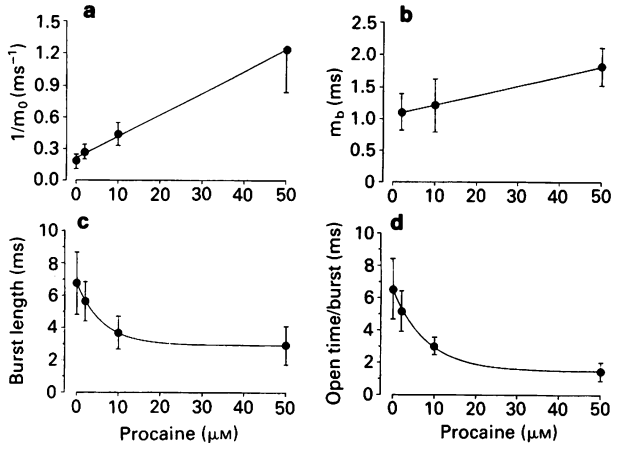


Figure 9 Tests of the principal predictions arising from the sequential blocking model (see text for details). (a) A plot of the reciprocal of mean channel open time (m_o) against procaine concentration. The line is fitted to the equation $y = 0.02(x) + 0.21$ by the method of least squares. (b) A plot of mean blocked time against procaine concentration. (c) A plot of mean burst length against procaine concentration (burst length calculated using a critical time of 8 ms (see text and Table 1)). (d) A plot of open time per burst against procaine concentration.

Table 2 Effect of procaine on the kinetic properties of currents induced by 10 μM carbachol (CCh) recorded from outside-out patches

	Control	*Procaine* 50 μM
Open state		
Unit current (pA)	2.72 ± 0.098	2.52 ± 0.071*
τ_f (ms)	0.47 ± 0.05	—
τ_s (ms)	6.70 ± 0.11	1.33 ± 0.07
$Area_f/Area_s$	1.61	—
Mean open time (ms)	5.8 ± 1.2	1.44 ± 0.15*
Closed state		
τ_f (ms)	0.65 ± 0.04	0.71 ± 0.12
τ_s (ms)	17 ± 12	4.37 ± 0.67
$Area_f/Area_s$	1.58	0.58
Burst behaviour		
Burst length (ms)	7.34 ± 1.79	4.31 ± 1.44
Mean blocked time (ms)	1.07 ± 0.05	1.8 ± 0.14*
Gaps/burst	0.18 ± 0.02	0.73 ± 0.30
τ_f (ms)	0.88 ± 0.04	0.92 ± 0.05
τ_s (ms)	14.6 ± 4.8	11.7 ± 4.6
$Area_f/Area_s$	2.14	2.59

Mean values ± s.e.mean are calculated from data obtained from three outside-out patches and statistical comparisons made where appropriate (* denotes $P<0.05$). The fitted time constants were calculated for amalgamated data from three patches and are given together with the standard error of the estimate.

decrease in the agonist-evoked inward current measured electrophysiologically. Neglecting any changes in receptor desensitization, there are two possible ways in which procaine could inhibit the inward current: it could reduce the probability of the channel being open or it could reduce the conductance of the channel. Direct measurement of the channel openings shows that procaine does not significantly reduce the conductance of the channel when the effects of filtering are taken into account (see Results). Although low concentrations of procaine shift the distribution of channel open times to shorter values, the probability of the channel being in the open state is not significantly affected by 2 μM and 10 μM procaine. At these concentrations procaine had little effect on catecholamine secretion or Na^+ influx. In the presence of 50 μM procaine, however, the probability of the channel being open is reduced by about two thirds. This is sufficient to account for its action in reducing the inward current evoked by low concentrations of CCh. (The P_{open} was reduced by approximately 70% and the mean steady-state inward current was reduced by 80% by 50 μM procaine, the slight difference being readily attributable to sampling error, see Results).

By what mechanism does procaine modulate the nicotinic receptor complex to reduce the inward current? As discussed above, increasing concentrations of procaine shorten mean channel open time and alter the spectral characteristics of the channel noise by inducing an excess of high frequency noise

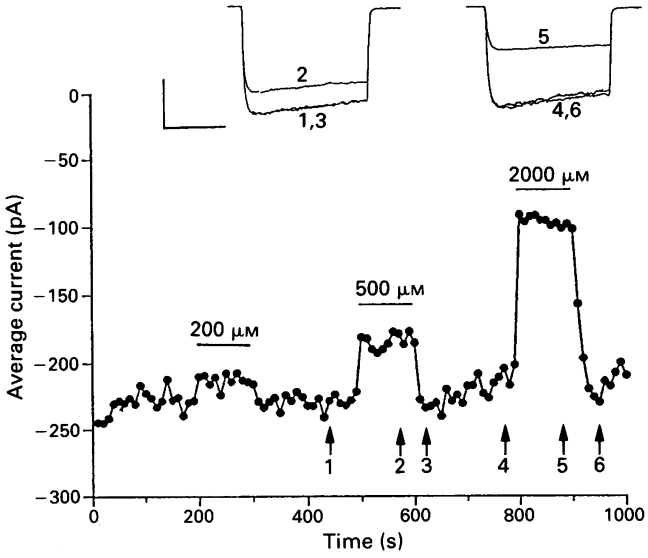


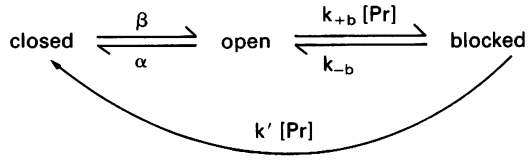
Figure 10 The effect of successive applications of 200, 500 and 2000 μM procaine on the inward calcium current. Each point is the mean current of four successive voltage steps from a holding potential of −80 mV to zero (see Methods for further details). Left inset shows the effect of 500 μM procaine and that on the right shows the effect of 2000 μM procaine on the mean inward Ca^{2+} current. The numbered arrows relate the traces shown in the insets to the overall timecourse. Calibration bars are: vertical 100 pA, horizontal 100 ms.

(Figure 5). Similar effects on channel noise have previously been reported for the action of local anaesthetics at the neuromuscular junction (Ruff, 1977; Neher & Steinbach, 1978; Ogden *et al.*, 1981) and have been interpreted in terms of a simple sequential blocking model in which transitions between closed, open and blocked states must occur in strict sequence. The dose-response curves for the action of procaine on catecholamine secretion and the CCh-evoked influx of Na^+ and Ca^{2+} have Hill coefficients close to unity. This suggests that the action of procaine is not cooperative and that a simple model such as the sequential blocking model outlined above may be sufficient to explain its action at the molecular level. This model has been proposed to account for the action of barbiturates and mepacrine at the neuromuscular junction (Adams, 1976; Adams & Feltz, 1980) and has been discussed by Gurney & Rang (1984) for the action of methonium compounds at ganglionic cholinoceptors.

In the absence of anaesthetic, the time constant for channel closure estimated from the noise is about 9 ms which corresponds to the long time constant for the distribution of burst length. When procaine was applied, a second high frequency component appeared in the noise spectrum and the low frequency time constant decreased. The time constants estimated from the spectra decreased as the procaine concentration increased from 20 to 50 μM and those estimated in the presence of 50 μM procaine corresponded to the time constants for burst length determined from the single channel studies (τ_f 0.6 and 0.74 ms; τ_s 4.4 and 5 ms respectively). The voltage-sensitivity of the high frequency component of the noise suggests that the positively charged procaine ions enter the channel to cause an open channel block. This interpretation is consistent with the sequential blocking model.

More stringent tests of the model have been made by investigating the effect of procaine on single channel currents. The results show that, while increasing concentrations of procaine progressively reduce the mean open time as predicted, they also increased the mean blocked time, decreased the mean open time within a burst and decreased burst length. As a burst may be terminated either by channel closure or by the channel entering a long-lived blocked state, a more detailed analysis of burst behaviour is unhelpful. Nevertheless, it is clear that the basic three state model is inadequate to account for our experimental findings. Gage & Wachtel (1984) and Gage & MacKinnon (1985) have previously reported similar results for the action of both procaine and pentobarbitone at the motor endplate. The three state model also fails to account for the action of the positively charged lignocaine derivative, QX222, at concentrations greater than 40 μM (Neher, 1983).

A model that could provide a reasonable framework for explaining our data is the extended block model discussed by Murrell *et al.* (1991), Murrell & Haydon (1991) and more fully by Dilger & Brett (1991). These authors propose the existence of a long-lived blocked state beyond the blocked open state. This modification of the original scheme correctly predicts the reduction in burst length and the increase in the number of openings per burst observed with procaine, alkanols and the volatile anaesthetics, enflurane and isoflurane, but it does not predict the concentration-dependence of the mean blocked time which increases in a concentration-dependent manner. In the cell-attached patches the closed time distributions in the presence of procaine reveal an increase in the proportion of brief closures (<2 ms) and an additional kinetically distinguishable component with a time constant of 8 ms. This latter component becomes more evident as the concentration of procaine increases. These data suggest that there are at least two blocked states that lead directly from the open state and there may be other blocked states we are unable to resolve. Moreover, the open state probability density function in the absence of procaine suggests the existence of at least two open states. A minimum of five discrete states are therefore required to describe the action of procaine on the nicotinic channel. In view of the complexity of the kinetics and the absence of any ready means of differentiating between the various blocked states, we have elected to describe the action of procaine (Pr) in terms of the simple three state model shown below:



These states are not regarded as unique but encompass blocked, closed and open states which may themselves be modulated by the anaesthetic. In essence our model proposes that procaine both blocks the open channel and binds to an allosteric site to modulate the rate at which the blocked channel can close without passing through the open state. Once the channel has entered the blocked state, the probability that it will close without passing through the open state rises as the concentration of procaine increases.

The simplified model accounts for the following observations: (1) The fall in mean open time with increasing concentration of anaesthetic. (2) The increase in mean blocked time with increasing concentration of anaesthetic. (3) The decrease in mean open time per burst as anaesthetic concentration increases and (4) the non-linear decrease in mean burst length with increasing concentration of anaesthetic. From the mean open times we are able to estimate the rate constant for channel closure in the absence of procaine as $176 \pm 26\ s^{-1}$ and the apparent rate constant for channel block as approximately $2 \times 10^7\ M^{-1}\ s^{-1}$. Comparable values have been obtained by Dilger & Brett (1991) for the higher alcohols in their action on the nicotinic receptors of BC3H-1 cells. Short chain alkanols and volatile agents such as enflurane and isoflurane have somewhat lower values close to $10^6\ M^{-1}\ s^{-1}$ (see also Wachtel & Wergrzynowicz, 1991). The rate con-

stants for unblocking can be estimated from the high resolution closed time distribution as 1000–2000 s^{-1} for the fast component and 77–114 s^{-1} for the slow component.

To conclude, procaine inhibits CCh-evoked catecholamine secretion from bovine chromaffin cells by depressing the agonist-evoked inward current. This depression can be largely explained by a dual action of procaine on the nicotinic receptor-channel complex.

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