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ACETYLCHOLINE RECEPTOR CHANNELS AND THEIR BLOCK BY CLONIDINE IN CULTURED BOVINE CHROMAFFIN CELLS

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SUMMARY

1. Acetylcholine (10–50 μM) applied to bovine chromaffin cells under whole-cell voltage clamp produced an inward current and an increase in the noise level of the current trace. At concentrations $\geqslant 20$ μM the ACh current desensitized with time. Spectral analysis of the ACh-induced increase in current noise showed that it could be fitted by a single Lorentzian component with a time constant, $\tau_{noise} = 11 \pm 0{\cdot}9$ ms ($V_m = -80$ mV).

2. Clonidine (2–30 μM) markedly reduced the size of the ACh-induced whole-cell current, and altered the shape of the noise spectrum. In the presence of clonidine, ACh noise spectra were fitted by two Lorentzian components with time constants which varied with clonidine concentration. The single-channel conductance from noise ($\gamma = 24$ pS) was unaltered by clonidine.

3. The reduction in the size of the whole-cell ACh current, produced by clonidine, was not mimicked by adrenaline (at concentrations up to 60 μM). GABA-induced whole-cell currents and spectra of GABA noise were also unchanged suggesting that the effect of clonidine was specific for the ACh receptor channel.

4. When applied intracellularly (from the patch pipette) clonidine had no apparent influence on the whole-cell ACh current. Furthermore, when clonidine was perfused over the cell surface at a concentration sufficient to block the whole-cell ACh response, single ACh channels could still be recorded under the patch-pipette tip (i.e. in cell-attached patches, not exposed to clonidine).

5. Clonidine block showed little voltage dependence; the peak ACh current remained linearly dependent on clamp potential. In addition, the fast and slow time constants derived from ACh noise spectra in the presence of clonidine did not show the sort of dependence on antagonist concentration expected for simple channel block; this suggests that clonidine has a complex blocking action.

6. Single ACh channels recorded in outside-out patches had a conductance of $\gamma = 39 \pm 0{\cdot}7$ pS, although a subpopulation of smaller and larger events also occurred in some patches. The mean single-channel conductance in outside-out patches was unaltered by clonidine.

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7. Under normal conditions the kinetics of single ACh channels were more complex than suggested from noise analysis. Burst length distributions could be described by two exponential components with fast and slow time constants of $\tau_f = 0{\cdot}69 \pm 0{\cdot}17$ and $\tau_s = 9{\cdot}51 \pm 0{\cdot}84$ ms. Clonidine reduced the time constants of both components of the burst length distribution and also reduced the frequency of opening of ACh channels.

8. It is concluded that the action of clonidine on nicotinic ACh receptors in chromaffin cells may involve a complex blocking mechanism, perhaps related to agonist-induced desensitization.

INTRODUCTION

Chromaffin cells of the adrenal medulla are electrically excitable cells (Douglas, Kanno & Sampson, 1967; Brandt, Hagiwara, Kidokoro & Miyazaki, 1976; Kidokoro & Ritchie, 1980) that are depolarized by acetylcholine (ACh) and secrete catecholamines by exocytosis (e.g. Fenwick, Marty & Neher, 1982; Neher & Marty, 1982). It was originally considered that the inhibitory effect of clonidine and similar α-adrenoceptor agonists, on the ACh-evoked secretion of catecholamines from bovine chromaffin cells, was mediated via an α-adrenoceptor (Gutman & Boonyaviroj, 1974; Greenberg & Zinder, 1982; Wada, Sakurai, Kobayashi, Yanagihara & Izumi, 1982; Sakurai, Wada, Izumi, Kobayashi & Yanagihara, 1983). However, more recent studies have suggested that this may not be the case (Collett, Rand & Story, 1981; Collett & Story, 1982; Powis, 1985) and rather that clonidine may act on the ACh receptor or its associated ion channel (Powis & Baker, 1986). This study was designed to test directly this latter suggestion. Preliminary results of part of this work have been published (Cull-Candy, Mathie & Powis, 1986).

METHODS

Preparation. Bovine chromaffin cells were prepared by the method of Knight & Baker (1983) with the inclusion of modifications (Powis & Baker, 1986). Cells were plated on collagen-coated cover-slips and used for experiments after 1–5 days.

Recording. Experiments were carried out using three variants of the patch-clamp recording technique (recording from whole cells, outside-out patches and cell-attached patches) (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Cells were found to have a capacitance in the range 5–20 pF. In the experiments described here, small spherical cells (diameter 10–20 μm cf. Clapham & Neher, 1984*a*) with capacitance values less than 10 pF were used. The access resistance in whole-cell recording was about 10–15 MΩ and the resistance of the patch electrodes varied from about 2 to 10 MΩ. Experiments were performed at room temperature: 20–23 °C.

Solutions. The external solution contained (in mM): NaCl, 150; KCl, 2·8; $MgCl_2$, 2; $CaCl_2$, 1; and Na-HEPES, 10 (pH 7·2). Pipette solution for whole-cell and outside-out patches contained (in mM): KCl (or CsCl), 140; $MgCl_2$, 2; $CaCl_2$, 1; K-EGTA, 10; and K-HEPES, 10 (pH 7·2). Acetylcholine chloride, clonidine hydrochloride, γ-aminobutyric acid and adrenaline bitartrate (all from Sigma) were applied to whole-cell or outside-out patches by continuous bath perfusion.

Analysis of noise. The variance and power spectra of the whole-cell current noise were obtained by filtering at 500 Hz, 1 kHz or 2 kHz (−3 dB, 8 pole Butterworth filter) and then digitizing the data at 1024, 2048 or 4096 Hz respectively. 512 point spectra were then calculated as previously described by Colquhoun, Dreyer & Sheridan (1979). The spectrum obtained in the absence of drug was then subtracted from that produced in the presence of drug to give the net noise spectrum of the drug effect alone. Net spectra were fitted either by one or two Lorentzian curves.

The amplitude of single-channel currents (i) was estimated from noise data as the ratio of the current variance to the mean current (I) for records with no visible desensitization induced by ACh (for concentrations of ACh of 20 μM or lower). The amplitude of single-channel currents was also calculated from the fitted spectra (Katz & Miledi, 1972; Anderson & Stevens, 1973) according to:

$$i = \frac{\pi G(0) f_c}{2I} \quad \text{or} \quad i = \pi \frac{(G_1(0) f_{c1} + G_2(0) f_{c2})}{2I}, \tag{1}$$

for one and two Lorentzian fits, respectively; where $G(0)$, $G_1(0)$ and $G_2(0)$ are the zero frequency asymptotes and f_c, f_{c1} and f_{c2} their half-power frequencies (see Dreyer, Walther & Peper, 1976; Dionne, 1981). There was always good agreement between the two methods.

Single channels. Records of single-channel currents, from outside-out or cell-attached patches, were stored on analog magnetic tape (tape speed 15 or 30 in/s). For analysis the data were filtered at 1·5–3 kHz (−3 dB, 8 pole filter with Bessel characteristics) then digitized at sampling rates ranging from 18 to 36 kHz. The opening and shutting transitions were measured by fitting the time course of the signal using the measured step response function of the recording system as has been described previously (Colquhoun & Sigworth, 1983; Colquhoun & Sakmann, 1985). The resolution of the system was usually set at 90 μs for openings and 60 μs for closings. Events shorter than these were deemed undetected. The idealized record obtained was then used to construct histograms of amplitudes, open times, closed times etc., which were fitted with appropriate probability density functions representing the sum of several geometric or exponential terms using the method of maximum likelihood (Colquhoun & Sigworth, 1983).

Definitions of single-channel parameters such as 'apparent openings' 'bursts' etc. were essentially as previously described (Colquhoun & Sakmann, 1985). The critical gap length, t_c, for bursts below which a gap is classified as a gap within a burst, was calculated by the method of Colquhoun & Sakmann (1985) to minimize the *proportion* of gaps misclassified as gaps within or between bursts.

Values obtained from the open-time distributions have to be corrected to take account of missed gaps – shorter than the imposed resolution of the recording system (Colquhoun & Sakmann, 1985). The total time spent in gaps within bursts, m_{TS} (i.e. gaps shorter than t_c, see above), including those that are undetected, can be estimated as

$$m_{TS} = N_g \int_0^{tc} t f(t)\, dt, \tag{2}$$

where N_g is the estimated total number of shut times and

$$f(t) = \Sigma a_i / \tau_i e^{-t/\tau_i}, \tag{3}$$

is the distribution of all shut times where a_i is the relative area of the ith component and τ_i is its time constant. Equation (2) can become:

$$m_{TS} = N_g[m_g - \Sigma a_i(t_c + \tau_i) e^{-t_c/\tau_i}],$$

where $m_g = \Sigma a_i \tau_i$ is the overall mean gap length. The total number of gaps within bursts ($N_{g1} + N_{g2}$) will equal $N_g a_{g1} + N_g a_{g2}$.

If the burst length distribution is fitted with two exponentials with time constants τ_{b1} and τ_{b2} and areas a_{b1} and a_{b2} (the total number of bursts being N_b) then the overall mean burst length is defined as $m_b = a_{b1}\tau_{b1} + a_{b2}\tau_{b2}$. The total time occupied by bursts is thus $N_b m_b$ and the corrected total open time is $m_{TO,corr} = N_b m_b - m_{TS}$ where m_{TS} is the total shut time within bursts (see above). The mean number of gaps per burst will be equal to $(N_{g1} + N_{g2})/N_b$, the number of openings per burst will be $((N_{g1} + N_{g2})/N_b) + 1$, and the corrected total number of openings will be:

$$N_{o,corr} = (((N_{g1} + N_{g2})/N_b) + 1) N_b. \tag{4}$$

Finally, the corrected mean open time is simply:

$$m_{o,corr} = m_{TO,corr}/N_{o,corr}. \tag{5}$$

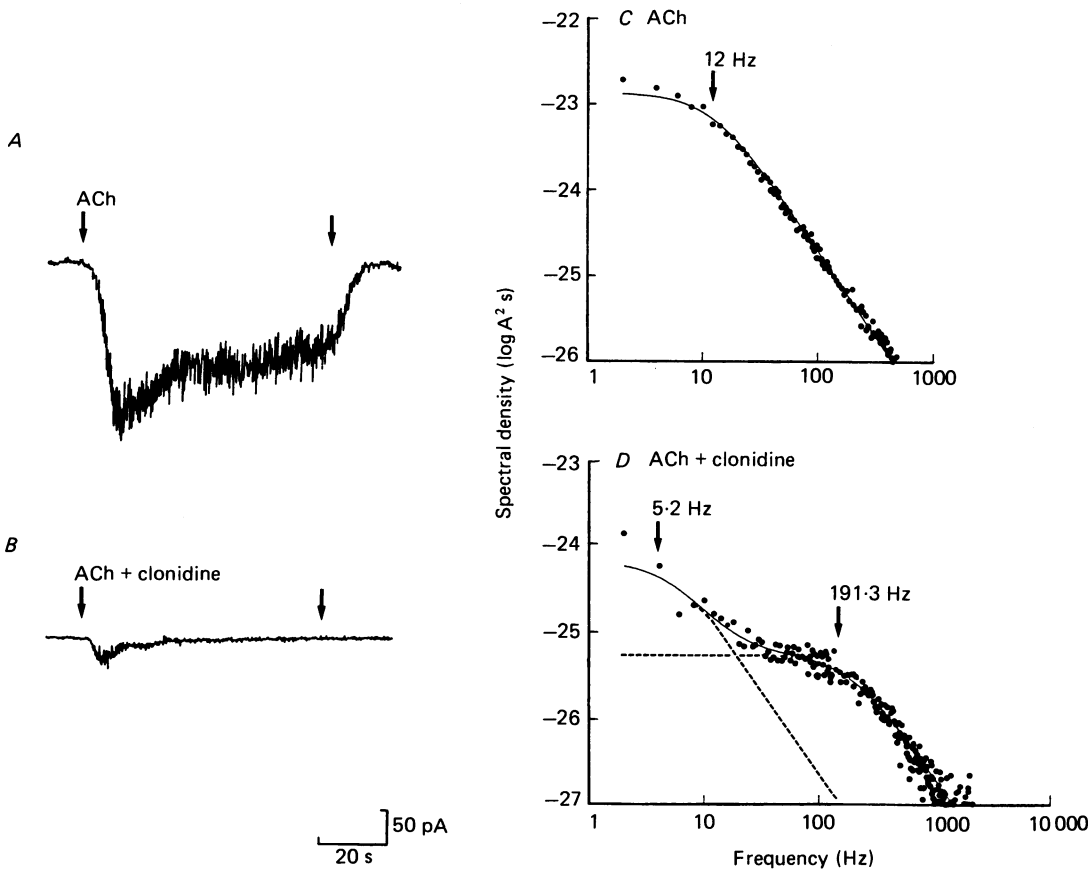

Fig. 1. *A*, membrane current change produced by steady application of 30 μM-ACh (applied between arrows) to a chromaffin cell under whole-cell voltage clamp at −80 mV. *B*, membrane current change from the same cell in response to 30 μM-ACh in the presence of 30 μM-clonidine. Note the reduction in both the inward current and the membrane noise associated with the ACh-activated current. Calibration, 50 pA and 20 s. *C*, spectral density of current noise produced by steady bath application of 30 μM-ACh to a chromaffin cell under whole-cell voltage clamp (−80 mV, T = 21 °C). ACh noise spectrum is fitted by a single Lorentzian with a half-power frequency (indicated by arrow) of f_c = 12 Hz, hence τ = 13·3 ms. The mean current I = 245·6 pA and the single-channel conductance estimated from the fitted curve is γ = 21 pS. *D*, spectral density of current noise produced by steady bath application of 30 μM-ACh in the presence of 30 μM-clonidine. Spectrum is fitted by the sum of two Lorentzians (dashed lines) with half-power frequencies of f_{c1} = 5·2 Hz and f_{C2} = 191·3 Hz, hence τ_s = 30·6 ms and τ_f = 0·83 ms. Mean current I = 12·2 pA and the single-channel conductance estimated from the fitted curve is γ = 21·9 pS.

RESULTS

ACh noise

Application of 10–50 μM-ACh to chromaffin cells held under whole-cell voltage clamp produced a fast inward current which rose to a well-maintained plateau. At ACh concentrations greater than about 20 μM the responses showed clear desensitization with time. Figure 1*A* shows a response from a cell held at −80 mV

in which 30 μM-ACh gave an inward current of about 200 pA accompanied by a large increase in the noise level of the current trace, due to fluctuations in the number of open channels (Katz & Miledi, 1972). In the concentration range used in these experiments (10–50 μM) ACh invariably gave noise spectra which were best fitted by

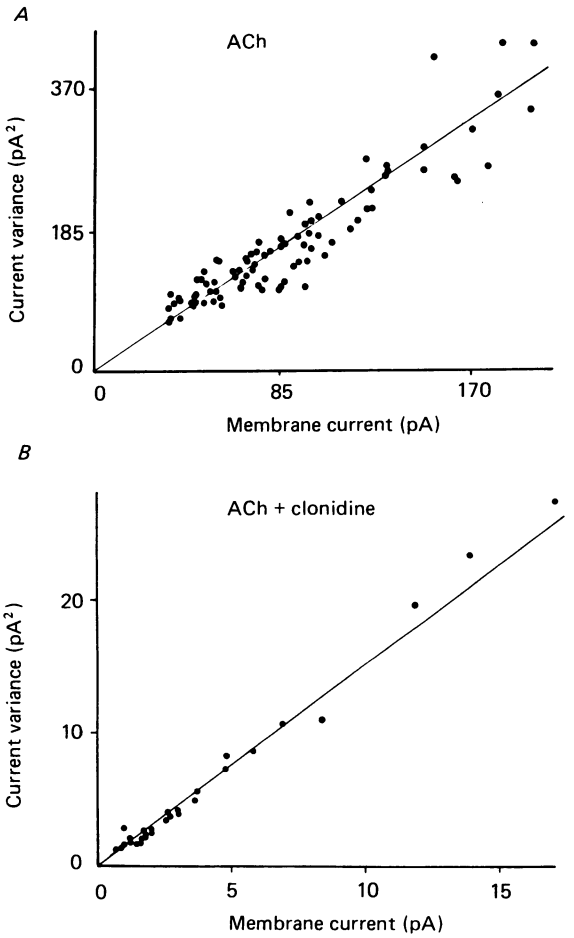

Fig. 2. Relationship between the membrane current change, I, and variance, var(I), of associated current fluctuations. A, 10 μM-ACh and B, 10 μM-ACh in the presence of 10 μM-clonidine (different cell from A). Holding potential = −80 mV; T = 22 °C. Straight lines drawn through the points by least-squares method give single-channel currents of i = 1·86 pA (A) and i = 1·74 pA (B).

a single Lorentzian component. The spectral density of this ACh-induced current noise is illustrated in Fig. 1C. The half-power frequency of the fitted Lorentzian (f_c) is 12 Hz; hence the time constant of the noise, τ_{noise}, was 13·3 ms (according to $\tau_{noise} = 1/(2\pi f_c)$). The mean value for seven cells was $\tau_{noise} = 11 \pm 0{\cdot}9$ ms (membrane potential, V_m, = −80 mV) (mean ± S.E.M.). Figure 1B shows the response (same cell as in Fig. 1A) to 30 μM-ACh after the cell had been exposed to 30 μM-clonidine for approximately 5 min. The ACh current is reduced to less than 10% of the control

amplitude and desensitizes rapidly. However, a small noise increase is still visible. The spectrum of ACh noise in the presence of clonidine (Fig. 1 *D*) was best fitted by the sum of two Lorentzians with fast and slow time constants of $\tau_f = 0{\cdot}83$ and $\tau_s = 30{\cdot}6$ ms.

At the 'low-concentration limit' (Anderson & Stevens, 1973) the current variance (var(I)) *vs.* the mean membrane current (I) induced by ACh should be linear, with the

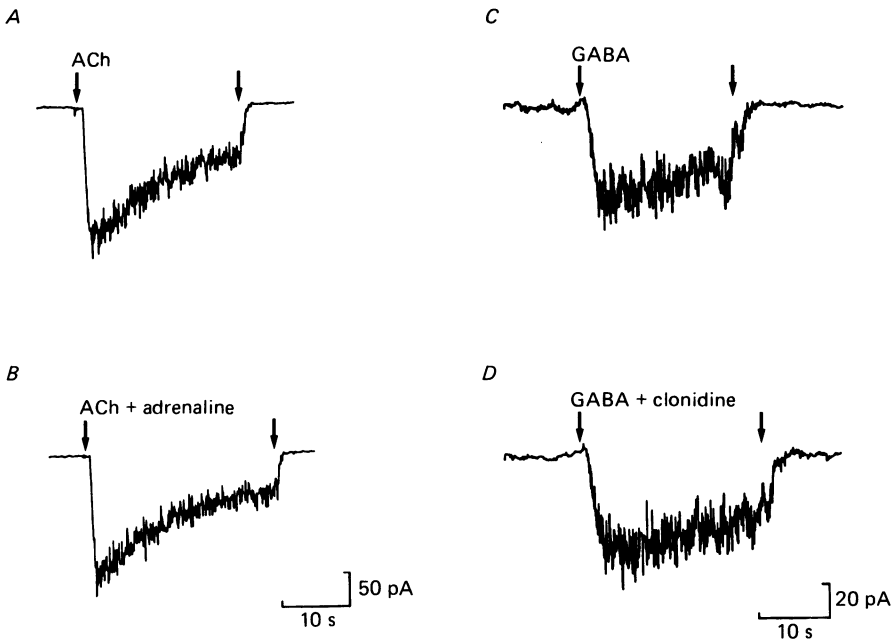

Fig. 3. Inward currents produced by steady application of ACh (*A* and *B*) and GABA (*C* and *D*), to chromaffin cells under whole-cell clamp. *A*, inward current response to 40 μM-ACh; *B*, response to ACh obtained from same cell in presence of 60 μM-adrenaline. Calibration, 50 pA and 10 s. *C*, inward current produced by 20 μM-GABA. *D*, response to GABA obtained in same cell in the presence of 30 μM-clonidine. Calibration 20 pA and 10 s. Holding potential = −80 mV, T = 22 °C.

slope of the plot giving an estimate of the apparent single-channel current, i, according to $i = \text{var}(I)/I$ (Katz & Miledi, 1972). Examples of variance to mean plots for ACh in normal medium, and in the presence of clonidine (10 μM), are illustrated in Fig. 2. Both relationships are reasonably linear and the unitary ACh currents estimated from the slopes were 1·86 pA ($V_m = -80$ mV) in normal medium, and 1·74 pA in the presence of clonidine. This yielded single-channel conductances of 23·3 and 21·8 pS respectively (for a reversal potential of 0 mV). So, although clonidine alters the kinetics of single channels opened by ACh, as indicated by the noise spectra, it does not appear to alter their conductance. However it should be noted that the mean ACh current and current variance values were much larger in the absence of clonidine.

Having established that clonidine has a marked effect on nicotinic ACh receptor channels it was of interest to determine whether the effect of clonidine was related

to its action as an adrenaline analogue. Figure 3*A* shows a whole-cell response to 40 μM-ACh in normal medium and in the presence of 60 μM-adrenaline (Fig. 3*B*). Adrenaline (20–60 μM) did not mimic the effect of clonidine, suggesting that clonidine does not exert its blocking action via α-adrenoceptors.

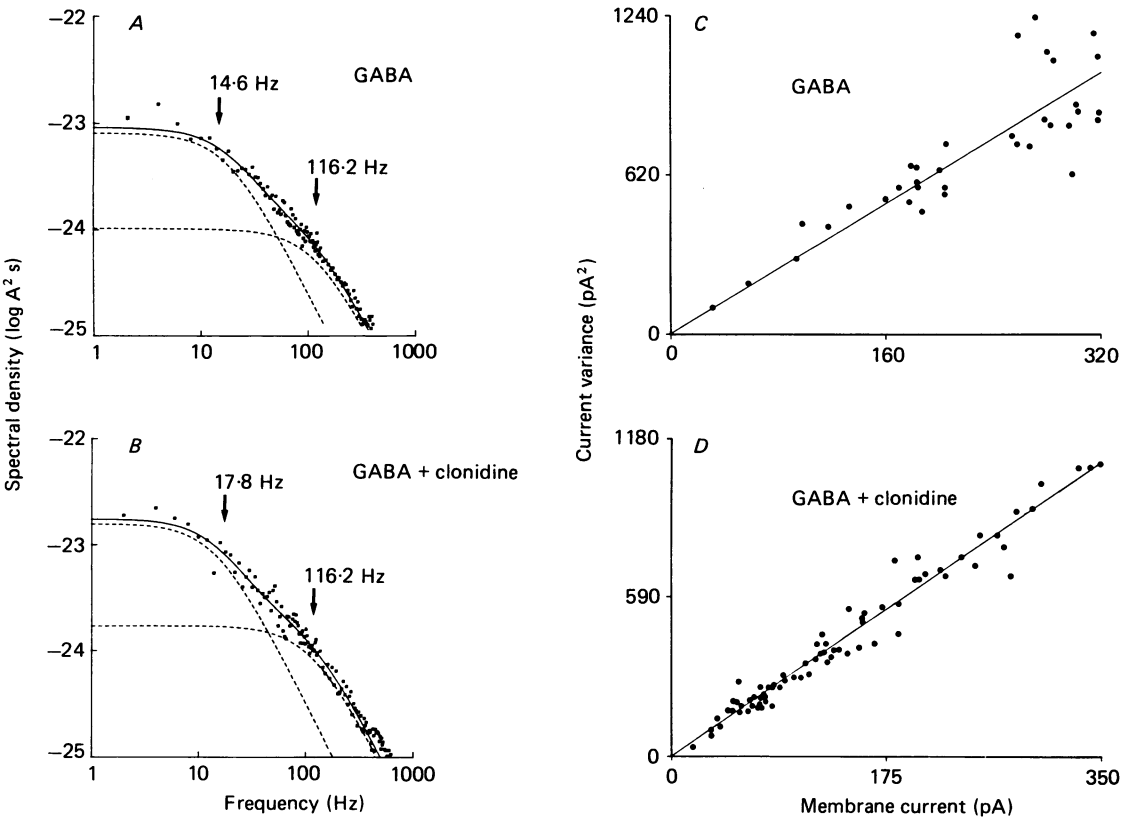

Fig. 4. *A*, spectral density of current noise produced by steady application of 20 μM-GABA to a cell under whole-cell clamp ($V_m = -100$ mV). Spectrum is fitted by two Lorentzian components with half-power frequencies (indicated by arrows) of $f_{c1} = 14{\cdot}6$ Hz and $f_{c2} = 116{\cdot}2$ Hz; hence $\tau_s = 10{\cdot}9$ ms and $\tau_f = 1{\cdot}37$ ms. *B*, spectral density produced by 20 μM-GABA in presence of 30 μM-clonidine ($V_m = -100$ mV). Half-power frequencies of two Lorentzians are $f_{c1} = 17{\cdot}8$ Hz and $f_{c2} = 116{\cdot}2$ Hz; hence $\tau_s = 8{\cdot}94$ ms and $\tau_f = 1{\cdot}37$ ms. *C* and *D*, relationship between the membrane current change and current variance produced by 20 μM-GABA (*C*) and 20 μM-GABA in the presence of 30 μM-clonidine (*D*). Lines fitted to the points by least-squares method give the single-channel currents 3·16 (*C*) and 3·12 pA (*D*). Data all obtained from a single cell.

GABA (γ-aminobutyric acid) noise

To decide whether clonidine acts non-specifically on membrane-bound receptors in chromaffin cells we have looked at the properties of GABA-receptor channels in the absence and presence of clonidine. Figure 3*C* and *D* shows an inward current and associated noise increase produced by bath application of GABA (20 μM) to a

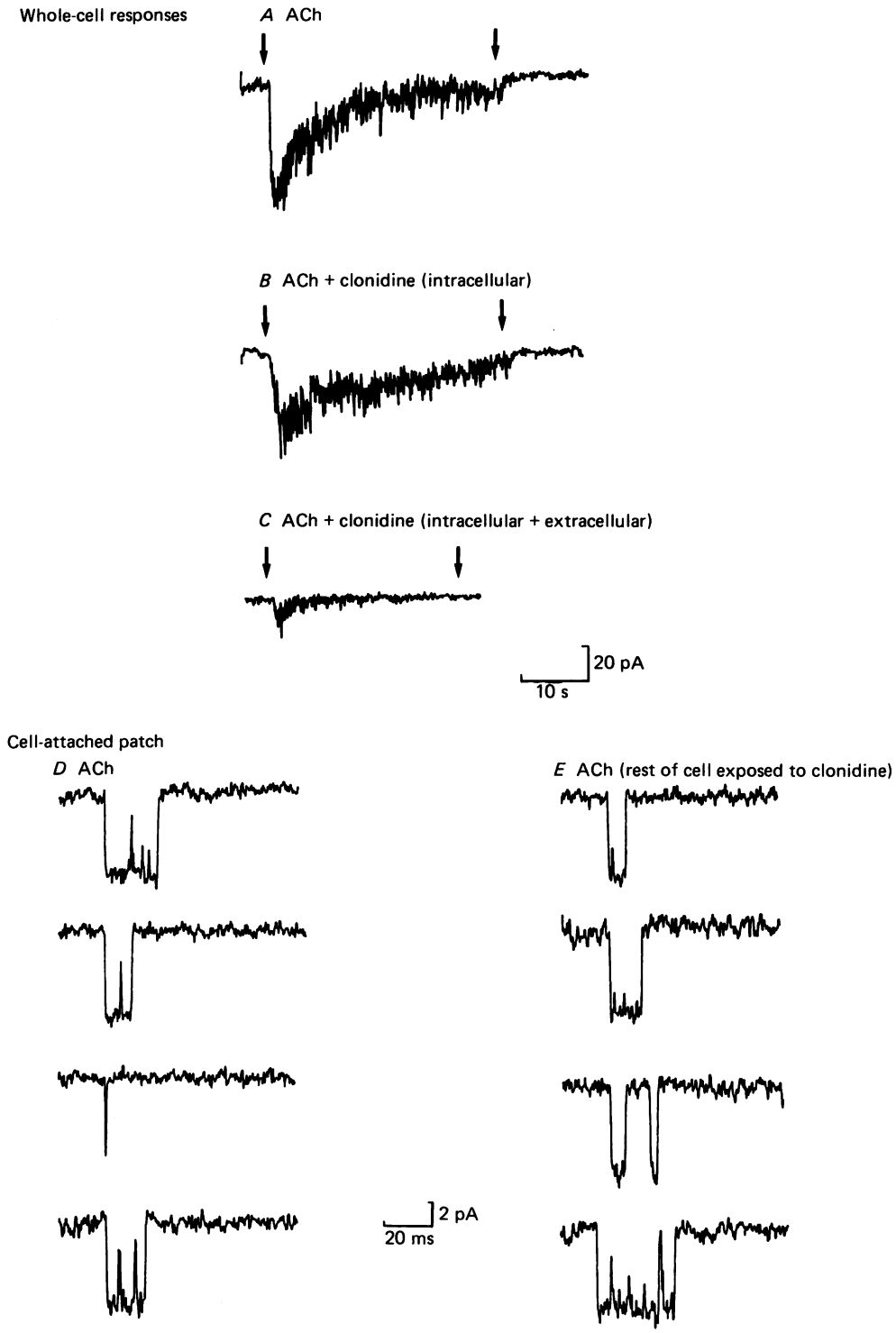

Fig. 5. For legend see opposite.

chromaffin cell under whole-cell clamp. The size of the response was not appreciably altered by 30 μM-clonidine (Fig. 3*D*).

The spectral density of GABA-induced current noise was usually well fitted by the sum of two Lorentzian components. In the example illustrated in Fig. 4 the time constants of the GABA noise are $\tau_f = 1{\cdot}37$ and $\tau_s = 10{\cdot}9$ ms in normal medium and $\tau_f = 1{\cdot}37$, $\tau_s = 8{\cdot}94$ ms in the presence of 30 μM-clonidine. Furthermore, the current variance *vs.* mean current plots for GABA noise in normal medium and in the presence of clonidine are similar (Fig. 4*C* and *D*) giving estimates of the apparent single-channel current of respectively, 3·16 and 3·12 pA (at $V_m = -100$ mV). For a reversal potential of 0 mV (see Methods) the conductance of the single GABA-activated channel in this cell was 31·6 pS in normal medium, and 31·2 pS in 30 μM-clonidine. The GABA-induced channels possess multiple-conductance states in chromaffin cells (e.g. Bormann & Clapham, 1985; Cottrell, Lambert & Peters, 1985). The apparent single-channel conductance obtained from noise is, therefore, a weighted mean of these conductances. It is clear from Fig. 4 that the apparent conductance is unaltered and it seems likely that the same conductance levels, in the same relative proportions, are being opened by GABA in the presence and absence of clonidine.

Clonidine acts at an extracellular site, near the ACh receptor

Figure 5 describes two experiments designed to determine the site in the membrane at which clonidine influences the ACh response. Figure 5*A* is a typical whole-cell response from a small cell ($\sim$ 10 μm diameter) to 20 μM-ACh. The response of a similar-sized cell to 20 μM-ACh was unaffected by 20 μM-clonidine added to the intracellular pipette solution and therefore dialysed into the cell (Fig. 5*B*). When clonidine was applied to both the extracellular and intracellular surfaces of this cell the response to ACh was clearly reduced (Fig. 5*C*). Clonidine therefore appears to act from the outside but not the inside of the cell.

To determine whether clonidine acts at an extracellular site close to the ACh receptor, single ACh channels were first recorded from cell-attached patches of chromaffin cells bathed in normal medium (Fig. 5*D*). Clonidine (40 μM) was then perfused over the cell surface for about 1 min. This concentration of clonidine is sufficient to block the whole-cell response to ACh within a few seconds. However, the single-channel currents under the pipette tip appeared unaffected (Fig. 5*E*). This suggests that clonidine either acts on the receptor–channel complex itself, or at a site nearby.

Fig. 5. *A*, membrane current changes produced by 20 μM-ACh (applied between arrows), recorded under whole-cell clamp ($V_m = -70$ mV). *B*, membrane current change produced by 20 μM-ACh recorded with a patch pipette containing 20 μM-clonidine. *C*, membrane current change produced by 20 μM-ACh with 20 μM-clonidine in bathing medium (patch pipette also contains 20 μM-clonidine, same cell as *B*). Calibration 20 pA and 10 s. *D*, single-channel currents recorded from a cell-attached patch with a pipette containing 5 μM-ACh. *E*, single ACh channels (same patch as *D*); 40 μM-clonidine has been perfused over the rest of the cell surface but is not contained within the patch pipette. Calibration 2 pA and 20 ms.

Channel kinetics from ACh noise

From noise analysis it is clear that there are differences in the acetylcholine noise spectra when clonidine is present (Fig. 1), suggesting that one possible action of clonidine may be to block the passage of ions through the channel itself. The observation that in the presence of clonidine the ACh noise spectrum is fitted by the sum of two Lorentzians, rather than the single Lorentzian obtained in normal control

TABLE 1. Properties of single ACh channels from noise analysis

	Channel conductance (γ, pS)	
ACh (7)	23·9 ± 1·7	
ACh + clonidine (11)	22·8 ± 1·5	
	Time constant of fitted Lorentzians	
	f_c (Hz)	τ (ms)
ACh (7)	14·5 ± 1·2	11·0 ± 0·9†
ACh + 2 μM-clonidine (3)	6·4 ± 1·0	25·9 ± 3·7
	40·1 ± 7·1	4·25 ± 0·90
ACh + 5 μM-clonidine (3)	3·4 ± 0·3	46·7 ± 4·4
	75·4 ± 11·7	2·23 ± 0·39
ACh + 10 μM-clonidine (4)	5·3 ± 1·8	37·4 ± 7·8
	102·8 ± 14·9	1·76 ± 0·4
ACh + 20 μM-clonidine (4)	4·2 ± 0·8	43·6 ± 10·3
	139·0 ± 26·6	1·25 ± 0·18
ACh + 30 μM-clonidine (3)	6·3 ± 1·15	26·0 ± 4·7
	151·3 ± 40·0	1·13 ± 0·3

Single-channel conductance and noise time constants were obtained in response to ACh or ACh plus clonidine. Numbers of cells used for each estimate are indicated in parentheses. Time constants were obtained from single-component ACh spectra†, or two-component (ACh plus clonidine) spectra, giving two estimates of τ at each clonidine concentration. The larger number in each pair is the slow time constant (τ_s); the smaller number is the fast time constant (τ_f).

condition, implies that the channels exist in three rather than two kinetically distinguishable states (cf. Colquhoun & Hawkes, 1977; Neher & Stevens, 1977). To investigate the channel kinetics more fully in the presence of clonidine, the time constants of the spectra have been calculated as the concentration of clonidine was raised from 2 to 30 μM (see Table 1).

The results presented in Table 1 confirm that the conductance of the single ACh channel is unaltered by clonidine. These conductance values were calculated from the fitted spectra (see Methods), and are in good agreement with values obtained from variance to mean plots. It is also apparent from Table 1 that at all concentrations of clonidine used, the ACh noise spectra were best fitted by two Lorentzian components. The simplest mechanism to explain this phenomenon would be that clonidine blocks the open channel, as described for local anaesthetics etc. (Adams, 1976, 1977; Ruff, 1977; Neher & Steinbach, 1978).

If this mechanism adequately describes the action of clonidine then a plot of $\tau_f^{-1} + \tau_s^{-1} - \tau_{con}^{-1}$ against antagonist concentration is expected to be linear (where τ_f^{-1} and τ_s^{-1} are the reciprocals of the fast and slow time constants in clonidine, and

τ_{con}^{-1} is the reciprocal of the control time constant) (see Colquhoun & Sheridan, 1981; Ogden, Siegelbaum & Colquhoun, 1981). The data presented in Table 1 are plotted in this form in Fig. 6. While $\tau_f^{-1}+\tau_s^{-1}-\tau_{con}^{-1}$ increases with antagonist concentration the relationship clearly is not linear. Furthermore, as can be seen from inspection of Table 1, while τ_f gets consistently faster as the antagonist concentration is increased, as expected for channel block, there is no consistent relationship between τ_s and antagonist concentration. A more complex mechanism of action for clonidine is likely (see Discussion).

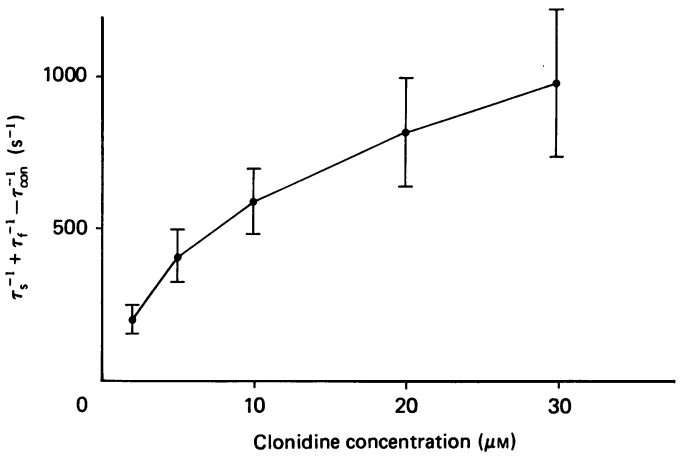

Fig. 6. Sum of the fast and slow rate constants ($\tau_f^{-1}+\tau_s^{-1}$) in clonidine, minus the control rate constant in ACh alone (τ_{con}^{-1}), as a function of clonidine concentration (at $V_m = -80$ mV). Standard errors are indicated by bars.

Voltage dependence of ACh response and noise parameters with clonidine

The effect of membrane potential on the degree of clonidine block was examined in two ways. In Fig. 7 responses to ACh in normal medium, and in the presence of 10 μM-clonidine, were recorded at different holding potentials. The graph (Fig. 7*C*) is a plot of the peak ACh current against membrane potential, before and after the addition of clonidine. Values are normalized to those obtained in the cell for the same concentration of drug(s) when it was clamped at −80 mV. The peak current in the presence of clonidine does not depart from linearity, in the range examined (−30 to −100 mV), indicating that the block was not voltage dependent. Interpretation of this type of experiment is complicated by inaccuracies in measuring the peak current when desensitization occurs and because the application of a constant ACh concentration to the whole cell cannot be regarded as instantaneous.

An alternative method for looking at the possible voltage dependence, particularly if clonidine block occurs slowly, is to measure the voltage dependence of the time constants from noise at a constant clonidine concentration. This is illustrated in Fig. 8. The normal ACh response in these cells shows voltage dependence with an e-fold increase in τ_{noise} for roughly a 115 mV hyperpolarization. This is similar to values found for ACh channels in frog, mouse and human end-plates (Magleby & Stevens,

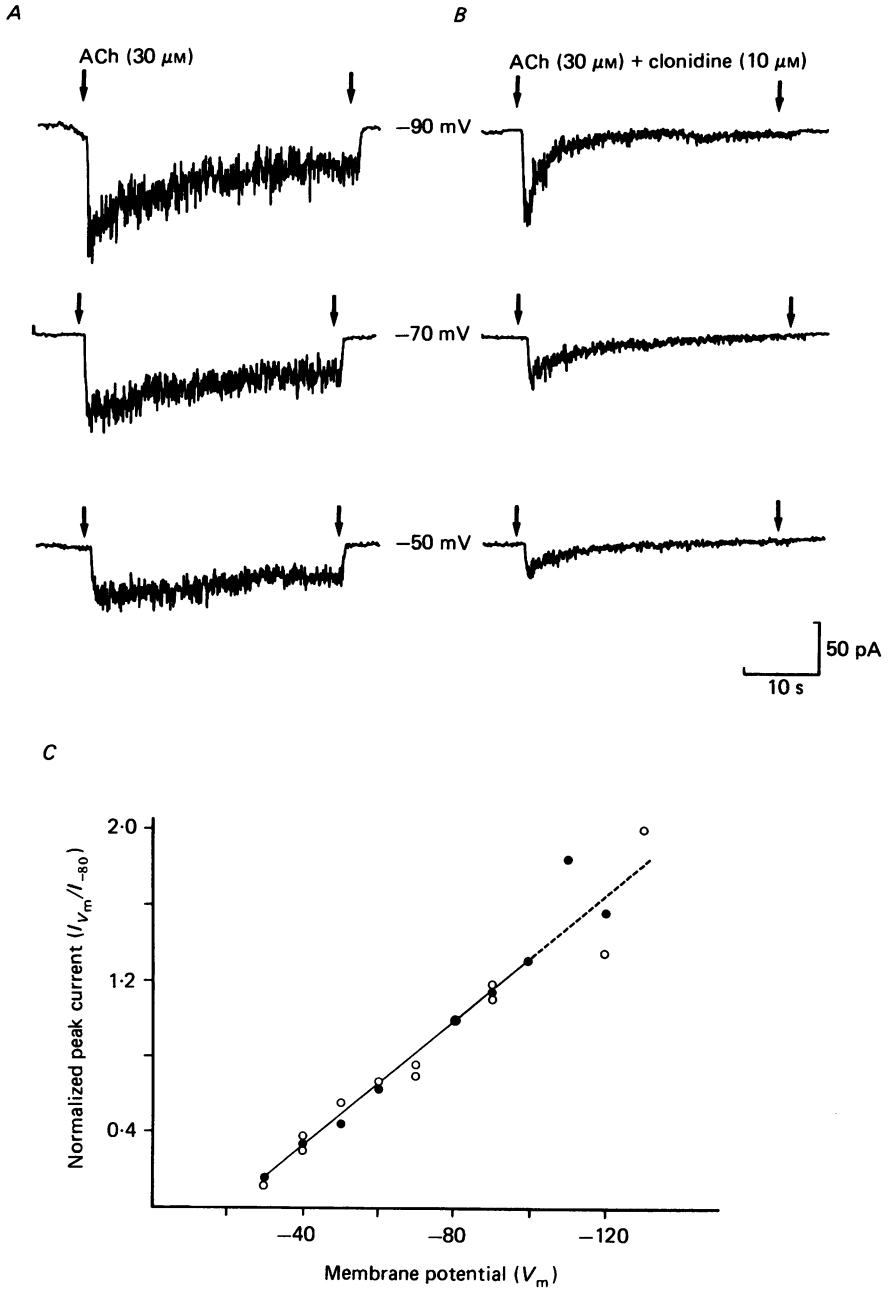

Fig. 7. *A* and *B*, inward currents produced by steady application of ACh (30 μM) to a chromaffin cell at various whole-cell clamp potentials in normal medium (*A*), and 10 μM-clonidine (*B*). All records from the same cell; $T = 22$ °C. Calibration 50 pA and 10 s. *C*, dependence of peak ACh current on membrane potentials in normal medium (●) and 10 μM-clonidine (○). Numbers on ordinate are normalized with respect to the value obtained at −80 mV in the same cell. Data are from two cells; line drawn by eye. At potentials more negative than −100 mV (dashed line) values show greater scatter.

1972; Mallart, Dreyer & Peper, 1976; Cull-Candy, Miledi & Trautmann, 1979). Figure 8*A* shows a typical control ACh noise spectrum ($V_m = -70$ mV), and three other spectra (Fig. 8*B*, *C* and *D*) from the same cell, in the presence of 10 μM-clonidine with the cell held at -50, -70 and -90 mV.

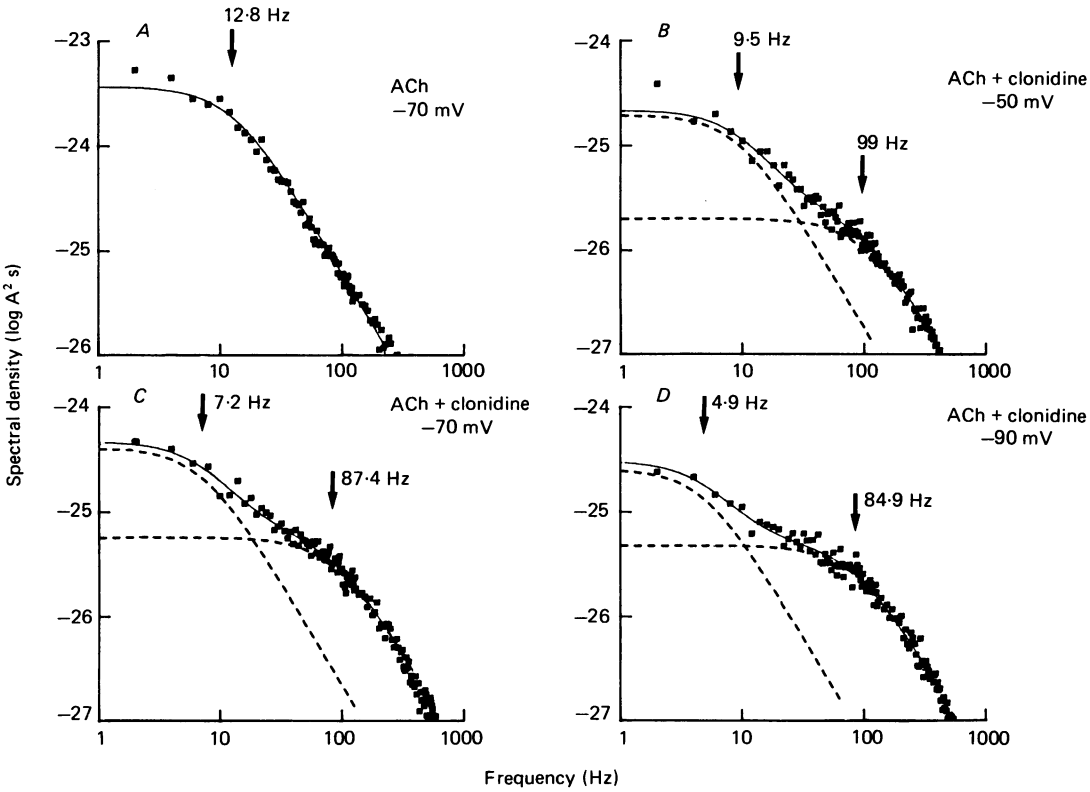

Fig. 8. *A*, spectral density of ACh (30 μM) current noise recorded under whole-cell clamp. Spectrum is fitted by a single Lorentzian component with $f_c = 12{\cdot}8$ Hz; hence $\tau = 12{\cdot}4$ ms (holding potential $= -70$ mV). *B*, *C* and *D*, spectral densities of ACh (30 μM) in the presence of 10 μM-clonidine at various clamp potentials in the same cell. Spectra are fitted by two Lorentzian components. *B*, $V_m = -50$ mV, $f_{c1} = 9{\cdot}5$ Hz, $f_{c2} = 99$ Hz ($\tau_s = 16{\cdot}8$ ms, $\tau_f = 1{\cdot}61$ ms). *C*, $V_m = -70$ mV, $f_{c1} = 7{\cdot}2$ Hz, $f_{c2} = 87{\cdot}4$ Hz ($\tau_s = 22{\cdot}1$ ms, $\tau_f = 1{\cdot}82$ ms). *D*, $V_m = -90$ mV, $f_{c1} = 4{\cdot}9$ Hz, $f_{c2} = 84{\cdot}9$ Hz ($\tau_s = 32{\cdot}5$ ms, $\tau_f = 1{\cdot}87$ ms).

The fast time constant has weak voltage dependence with an e-fold increase for a 250 mV hyperpolarization, while the slow time constant gave an e-fold increase for a 60 mV hyperpolarization.

Single-channel currents activated by ACh

In chromaffin cells the density of ACh channels is low (Fenwick *et al.* 1982) making it difficult to obtain channels in outside-out and cell-attached patches (see Clapham & Neher, 1984*a*, *b*). This was particularly problematical in the presence of clonidine. However, we obtained five control patches, and two patches in the presence of

clonidine, in which the number of openings was sufficient to allow a quantitative kinetic analysis; in addition ten patches were obtained where the single-channel conductance could be measured.

Single-channel conductance. The conductance of the predominant ACh channel in

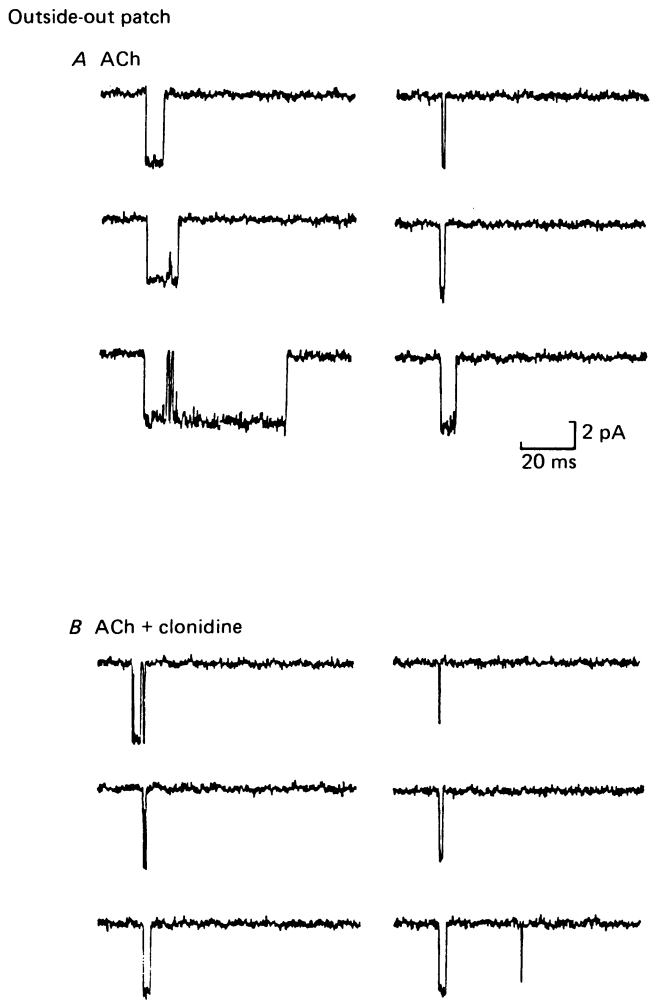

Fig. 9. Single-channel currents activated by ACh in an outside-out patch from a chromaffin cell. *A*, currents produced by 5 μM-ACh (holding potential = −110 mV). *B*, ACh-activated currents (same patch as *A*) in the presence of 2 μM-clonidine. Calibration 2 pA and 20 ms.

outside-out patches from chromaffin cells was 39·0 ± 0·7 pS (mean ± S.E.M., $n = 10$). In four patches where channels were recorded both before and after the application of clonidine the amplitude of the single-channel current was unaltered (see Fig. 9), confirming the observations from the ACh noise experiments. It should be noted, however, that the amplitude of the single-channel conductance is larger than estimated from the noise spectra. The values were in the range previously described

for ACh channels recorded following desensitization under whole-cell clamp in these cells (Clapham & Neher, 1984*a*). Interestingly, in three of the ten patches channels were observed which occurred less frequently than the main conductance level but had an amplitude which was clearly different from the main one in the same patch. Two patches possessed a subpopulation with larger than normal conductances: 43·5 pS compared with 39·5 pS, and 45·9 pS compared with 38·3 pS. One patch

TABLE 2. Kinetic properties of single ACh channels

	Control	+Clonidine (2 μM)	+Clonidine (5 μM)
Gap times (ms)			
g_1	0·060 ± 0·007 (57 ± 11)	0·051 (70)	—
g_2	1·09 ± 0·61 (8 ± 2)	0·157 (3)	—
g_3	25·6 ± 7·8 (8 ± 1)	186·1 (14)	—
g_4	386·6 ± 120·6 (26 ± 10)	1853·4 (13)	—
Apparent open time (ms)			
o_1	0·73 ± 0·16 (48 ± 4)	0·65 (76)	0·29 (85)
o_2	4·35 ± 0·64 (52 ± 4)	3·98 (24)	2·13 (15)
Burst length (ms)			
b_1	0·69 ± 0·17 (51 ± 9)	0·42 (63)	0·39 (88)
b_2	9·51 ± 0·84 (49 ± 9)	3·75 (37)	2·13 (12)
No. of apparent openings per burst			
$(o/b)_1$	1·15 ± 0·11 (61 ± 15)	1·00 (42)	—
$(o/b)_2$	2·46 ± 0·20 (39 ± 15)	1·42 (58)	—
Apparent opening frequency (s^{-1})			
$f_{o,app}$	6·57 ± 3·38	1·29	0·44
Apparent mean open time (ms)			
$m_{o,app}$	2·59 ± 0·33	1·46	0·57
Corrected mean open time (ms)			
$m_{o,corr}$	1·57 ± 0·19	0·43	—

The relative areas of each component are given in parentheses. The number of openings in the patch in the presence of 5 μM-clonidine were too few to allow the four-component gap distribution to be defined accurately. The t_c value used to define bursts for these data was calculated from control data in the same patch. Control data are from five cells.

possessed a subpopulation of small-amplitude events: 35 pS compared with 40·9 pS. This may reflect variable conductance states resembling those described for ACh channels in PC12 cells, a tumour cell line derived from chromaffin cells (Bormann & Matthaei, 1983) or it may simply mean that the amplitude of the unitary conductance channel varies from channel to channel (i.e. larger standard deviation). In this respect, it is of interest that while each of these three channel conductances was clearly distinguishable from the main level, in their respective patches, they all fell within two standard deviations of the mean value quoted above for the main conductance level in all ten patches.

Distribution of closed times. Under normal conditions the distributions of closed

times (gaps) between openings activated by 5 μM-ACh in outside-out patches were complex and were fitted by four exponential components (not shown). The four time constants in control conditions were 60 ± 7 μs, $1{\cdot}09 \pm 0{\cdot}61$ ms, $25{\cdot}6 \pm 7{\cdot}8$ ms and $386{\cdot}6 \pm 120{\cdot}6$ ms. The numbers of observations in the presence of clonidine were not usually sufficient for reliable comparison with the control values of the time constants. Furthermore, the value of the gaps represented by the two longer time

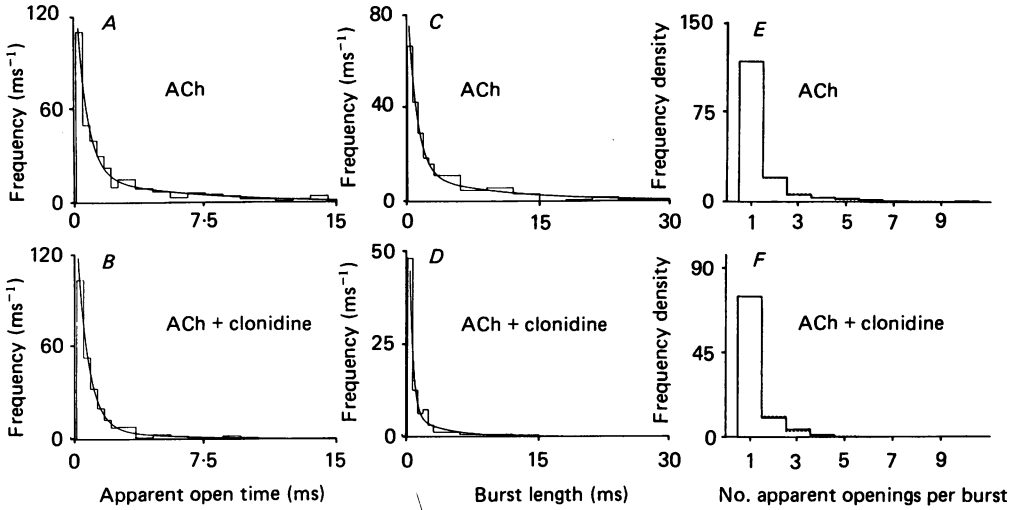

Fig. 10. *A* and *B*, examples of the distributions of all apparent open times of ACh channels in outside-out patches. *A*, 5 μM-ACh alone, distribution fitted with two exponential components, $\tau_f = 0{\cdot}61$ ms and $\tau_s = 5{\cdot}49$ ms ($V_m = -90$ mV). *B*, 5 μM-ACh and 2 μM-clonidine, fitted with two exponentials, $\tau_f = 0{\cdot}65$ ms and $\tau_s = 3{\cdot}98$ ms ($V_m = -110$ mV). *C* and *D*, examples of the burst length distributions for ACh channels in outside-out patches. *C*, 5 μM-ACh alone (critical gap length = 1·05 ms, see text for details), fitted with two exponentials, $\tau_f = 1{\cdot}02$ ms and $\tau_s = 10{\cdot}3$ ms ($V_m = -90$ mV). *D*, 5 μM-ACh and 2 μM-clonidine (critical gap length = 0·896 ms) fitted with two exponentials, $\tau_f = 0{\cdot}42$ ms and $\tau_s = 3{\cdot}75$ ms ($V_m = -110$ mV). *E* and *F*, examples of the distribution of the number of apparent openings per burst for ACh channels in outside-out patches. *E*, 5 μM-ACh alone (critical gap length = 0·93 ms), fitted with two components with means of 1·13 and 2·85, ($V_m = -110$ mV). *F*, 5 μM-ACh and 2 μM-clonidine (critical gap length = 0·896 ms), fitted with two components with means of 1·00 and 1·42. For all histograms the resolution was set at 90 μs for open time and 60 μs for shut times.

constants will depend on the number of channels in a given patch, which is unknown and will vary from patch to patch. However, the distributions can be used to define bursts of openings. The gaps represented by the two shorter time constants were defined as gaps within bursts; they are similar to the short gaps and intermediate gaps seen for nicotinic receptors at the frog end-plate (Colquhoun & Sakmann, 1985), and in BC3H1 cells (Sine & Steinbach, 1986). The two longer time constants were defined as gaps between bursts.

Apparent open times, burst lengths and number of openings per burst. Open-time histograms are difficult to interpret when short gaps exist, because many gaps between openings will be below the resolution of the recording system and many

erroneously long openings will therefore be recorded. This means that the values (Table 2 and Fig. 10*A* and *B*) for apparent open time are difficult to interpret with confidence, although 2 μM-clonidine did appear to reduce the apparent mean open time from 2·59 to 1·46 ms. The corrected mean open time (see Methods) was reduced from 1·57 to 0·43 ms by 2 μM-clonidine (see Table 2). The burst length of the ACh channels is a more useful parameter for comparison as it overcomes the problem by defining a critical gap length (t_c), below which all gaps between openings are regarded as gaps within a burst. The two shortest time constants equivalent to short and intermediate gaps have been defined as gaps within a burst. The 23-fold difference between the 2nd and 3rd time constants is not large enough to avoid completely the possibility of some misclassifications, so the critical gap length was calculated as previously described (Colquhoun & Sakmann, 1985) to make the proportion of misclassified long intervals equal to the proportion of short intervals. Burst length distributions are summarized in Table 2 and an example is illustrated in Fig. 10*C* and *D*. The burst length distributions resemble the apparent open-time distributions in being fitted by two exponential components, one with a relatively short time constant. The time constant of the long bursts produced by ACh in normal medium is similar to τ_{noise} obtained in control ACh noise experiments (9·5 ms for single channels compared with 11·0 ms for noise). Clonidine reduced both time constants of burst length. For example in 5 μM-clonidine the shorter time constant was reduced from 0·69 to 0·39 ms, and the longer time constant was reduced from 9·51 to 2·13 ms. Furthermore, the relative areas of the two components for records from a given patch were altered (see Table 2), such that a larger proportion of the bursts could be described by the shorter time constant.

The number of apparent openings per burst (Table 2, Fig. 10*E* and *F*) also appeared to be reduced by clonidine. However, these observations are again rather difficult to interpret fully for the same reasons described above for the apparent open-time histograms. It is clear from Fig. 10*E* and *F* that many bursts consist of single openings, and when the distribution is fitted with two components one component of the fit has a value close to unity. This implies that bursts can be split into two types, one of which consists of only one opening. This, in turn, could explain why the burst length distributions (and probably the apparent open-time distributions as well) are fitted by two exponentials.

Opening frequency. From Table 2 it appears that clonidine reduces the frequency of apparent openings of ACh channels in chromaffin cells. The opening frequency will depend on the number of channels in a patch, which will undoubtedly vary from patch to patch. For example, in one particular experiment, ACh-activated channels were recorded from a single patch in normal medium and following application of 5 μM-clonidine. The frequency of apparent openings produced by 5 μM-ACh was reduced from 4·57 to 0·44 s^{-1} by clonidine. The number of available channels is expected to be the same before and after clonidine. Outside-out patches from these cells show 'run-down' of channel activity but not to this extent (about 10-fold) in this length of time (in this experiment about 2 min). It therefore seems reasonable to conclude that clonidine is reducing the opening frequency of ACh channels.

DISCUSSION

The nicotinic ACh channels in bovine chromaffin cells have previously been studied with noise analysis (Fenwick *et al.* 1982) and recordings of single-channel currents in whole-cell voltage clamp (Clapham & Neher, 1984*a*, *b*).

Our value of 39 pS, for single channels examined in outside-out patches, is similar to the earlier estimates of 44 pS (Fenwick *et al.* 1982) and 33 pS (for an assumed equilibrium potential of 0 mV; Clapham & Neher, 1984*a*). However, our estimate from noise analysis (24 pS) is lower; a similar phenomenon has been widely described by others. In the chromaffin cells this may result from the existence of conductance levels which are smaller than the main level, and possibly from the fast kinetic components of channel activity which will be filtered during analysis of noise causing the 'time-averaged' single-channel conductance to be underestimated. Nevertheless, channel conductances estimated from noise were similar in all cells, as were the single-channel conductances measured directly in excised patches. Furthermore, the unitary conductance of these channels was unaltered by clonidine.

Site of action of clonidine

The onset of action of clonidine was fast, the drug being equally effective whether applied before ACh or simultaneously with it. In addition, clonidine was found to act only from the outside of the cell and only then when applied to the same area of membrane as the ACh; so clonidine had to be present at a site close to a particular receptor channel complex in order to block it. The effect does not appear to be mediated by an α-adrenoceptor since adrenaline in concentrations up to 60 μM had no effect on the ACh response (cf. Powis, 1985; Powis & Baker, 1986). Clonidine had no apparent effect on voltage-activated inward and outward currents (S. G. Cull-Candy & A. Mathie, unpublished observations) or on GABA-activated Cl^- channels, suggesting that its action is specific to ACh channels. Taken together, these results suggest that clonidine acts directly and selectively on the nicotinic-receptor-channel protein of these cells.

Clonidine action on the nicotinic receptor channel

The spectrum of the ACh noise could be fitted by a single Lorentzian component with a time constant of about 11 ms. This value is similar to that obtained from single-channel records for the slow component of the burst length distribution (about 10 ms). It is, however, shorter than the value of 27 ms obtained from the mean duration of ACh channel openings (neglecting short interruptions) (Fenwick *et al.* 1982) or the mean open time value of 35 ms obtained for single channels seen in whole-cell recordings (Clapham & Neher, 1984*a*, *b*). It has usually been found that the time constant of the control single Lorentzian represents a burst of channel openings in response to a single receptor activation. Clonidine altered the ACh noise spectrum so that it was best fitted by two Lorentzian components, one faster and one slower than the control noise, implying that clonidine alters the gating properties of the channel.

The possibility remains that clonidine may have a secondary competitive action on the receptor. However, the effects of clonidine on the noise spectrum suggest that at least part of its action occurs subsequent to binding of ACh to the receptor. A

simple mechanism which would account for clonidine producing two Lorentzian components is to suggest that it introduces a third kinetic state – a blocked state (Armstrong, 1971; Adams, 1976), i.e.

$$A_2R \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2R^* \underset{k_{-B}}{\overset{k_{+B}}{\rightleftharpoons}} A_2R^*B, \tag{6}$$

where A represents the ACh molecule, R the closed receptor channel, R* the open receptor channel and B the blocker clonidine. β, α, k_{+B} and k_{-B} are the forward and backward microscopic rate constants for transitions between states. This sequential scheme specifies that there is a single blocked state, recovery from which can occur only via the open state. Such a scheme allows certain predictions to be made about the time constants obtained from noise when the antagonist concentration is varied. Firstly the fast time constant from noise should get briefer and the slow time constant longer as the antagonist concentration is increased (see Neher & Steinbach, 1978). Secondly, and related to this, the sum of the reciprocals of the fast and slow time constants (τ_f^{-1} and τ_s^{-1}) minus the reciprocal of the control time constant (τ_{con}^{-1}) should increase linearly with antagonist concentrations (Colquhoun & Sheridan, 1981; Ogden, Siegelbaum & Colquhoun, 1981). As shown in Table 1 (and Fig. 6), there appears to be no obvious relation between the slow time constant and antagonist concentration although the fast time constant does decrease with increasing antagonist concentration. Furthermore, while $\tau_f^{-1}+\tau_s^{-1}-\tau_{con}^{-1}$ increases with antagonist concentration, the relationship is non-linear. A further consequence of a sequential reaction is that the total length of time the channel stays open during a single activation, a burst, should be independent of the antagonist concentration (Neher & Steinbach, 1978; Colquhoun & Hawkes, 1982, 1983). The burst length will increase with antagonist concentration due to the increased time spent in the blocked state, while the lengths of time spent in the open state within a single burst will add up to the same length of time as that seen in control conditions. Our single-channel data suggest that with clonidine the total length of time the channel spends in the open state is considerably reduced.

A simple three-state sequential blocking model does not, therefore, adequately describe the action of clonidine on the ACh channel in chromaffin cells. Instead, clonidine appears to fall into the same category as an increasingly large number of other drugs which block receptor-gated ion channels. While the actions of a number of channel blockers can be described by a sequential scheme, e.g. tubocurarine (Manalis, 1977; Ascher, Marty & Neild, 1978; Katz & Miledi, 1978; Colquhoun, Dreyer & Sheridan, 1979; Ascher, Large & Rang, 1979; Rang, 1982), gallamine (Katz & Miledi, 1978; Colquhoun & Sheridan, 1981) and some barbiturate local anaesthetics (Adams, 1976), other drugs such as procaine (Katz & Miledi, 1975; Adams, 1977), substance P (Clapham & Neher, 1984*a*; Role, 1984), trifluoperazine (Clapham & Neher, 1984*b*), benzocaine (Ogden *et al.* 1981), pentobarbitone (Gage & McKinnon, 1985) and even the classical ganglion blocker hexamethonium (Gurney & Rang, 1984) appear to have more complex blocking actions.

Interestingly, QX222, a lignocaine derivative, originally thought to have a simple 'sequential-type' block (Ruff, 1977, 1982; Neher & Steinbach, 1978), was found to have a more complex action when given in higher concentrations (Neher, 1983).

Complex channel block

The most obvious alternatives to a simple sequential mechanism are either the addition of a long-lived blocked state (e.g. Gurney & Rang, 1984) or the suggestion of a cyclical mechanism in which blocked channels can return to the resting state without having to return through the open state. Such mechanisms are more difficult to correlate with any particular physical model. One can propose that the blocking drug acts at more than one site inside the channel to give two or more blocked states or else it is possible that the drug acts at sites close to the protein to alter its kinetics allosterically rather than to simply plug the channel. In support of this idea, it was shown that the effect of clonidine is relatively insensitive to changes in membrane potential. This might at first sight seem surprising since at pH 7·2 the ratio of ionized to non-ionized clonidine is about 15 to 1. The non-ionized form of clonidine is extremely lipophilic. The lack of voltage dependence suggests that it may be the uncharged drug molecules which block the channel. This could conceivably result from the drug passing through the membrane lipid to a specific site of action close to the channel. In this respect it is worth noting that while a number of uncharged drugs have previously been shown to block nicotinic ion channels, their action when examined in detail usually seems to be more complex than simple open-channel block (e.g. Ogden *et al.* 1981).

A further possibility may be that the blocking drug enhances the phenomenon of desensitization induced by the natural agonist (see Clapham & Neher, 1984*a*). The simplest kinetic scheme, at present, for describing desensitization of nicotinic ACh channels is cyclical involving two desensitized states (e.g. Feltz & Trautmann, 1982). Such a scheme is the same as that proposed for a cyclical blocking mechanism with two blocked states. The possibility of a link between the two phenomena (Clapham & Neher, 1984*a*), has led to the suggestion that substance P may possibly reduce the ACh current in bovine chromaffin cells by enhancing desensitization. It would be difficult to prove such an effect until either the physical mechanism of desensitization is better understood, or a method is found for inhibiting or reducing the desensitization of ACh receptors.

The single-channel data might be expected to help differentiate between the three possibilities described above. Both the overall mean open time and the mean burst length are reduced by clonidine. This suggests that clonidine pushes the channel into a blocked state from which it can close directly. However, if a very long-lived blocked state exists (i.e. the blocker has a very slow dissociation constant from a particular state) then it becomes extremely difficult to distinguish experimentally between a sequential four-state model and a cyclical model (Ogden & Colquhoun, 1985), especially since the effect is not voltage dependent. The definition of burst length, for example, in the presence of the blocker may become difficult. Nevertheless, quite a convincing correlation can be seen between the burst length from single-channel data and the fast time constant of the noise, when the clonidine concentration was less than 10 μM.

The slow time constant induced in the ACh noise spectrum by clonidine presents more of a problem. Bursts of this length were not observed in single-channel records. It could imply that there is a second population of ACh channels which is affected

differently by clonidine. The existence of a second population of ACh channels in chromaffin cells has been suggested before (Clapham & Neher, 1984*a*). These channels are suggested to be resistant to desensitization and may help explain the difference between single-channel results and noise. However, our noise data in the absence of clonidine suggest only a single population of ACh receptors are present in these cells.

The action of clonidine on nicotinic ACh receptors seems, therefore, to involve a complex blocking mechanism which may perhaps be related to agonist-induced desensitization. A more detailed kinetic study, to distinguish between these possibilities, is desirable but might seem inappropriate at present since the physical mechanisms related to desensitization and complex channel block are unknown and because such studies may not give a clear answer (Clapham & Neher, 1984*a*; Ogden & Colquhoun, 1985). Recently, we have described a preparation of isolated rat sympathetic neurones possessing nicotinic ACh receptors which are suitable for patch-clamp recording (Cull-Candy, Magnus & Mathie, 1986; Cull-Candy & Mathie, 1986). The density of receptors in these cells is sufficiently high to allow single ACh channels to be regularly observed in cell-attached and outside-out patches, which is not the case for cultured bovine chromaffin cells. Preliminary results suggest that the nicotinic response in these cells is also blocked by clonidine (S. G. Cull-Candy & A. Mathie, unpublished experiments). Thus these cells may be better for any future detailed investigation of clonidine effects.

Finally, it is worth considering briefly whether this effect may become important when clonidine is used clinically. An adult human, given a normal dose of clonidine, will have a concentration of 5–6 nM in their bloodstream over a 24 h period. It is known, however, that certain glands such as the spleen and submaxillary glands accumulate the drug at 40–60 times plasma concentration (Cho, Curry & Jacobsen, 1969). If the adrenal glands behave similarly, they could accumulate a concentration of 0·3–0·4 μM if the drug is used chronically. Since 2 μM-clonidine has a clear effect on ACh channels over a short time scale it is quite conceivable that 0·3–0·4 μM-clonidine present for extended time periods may affect these channels (see also Powis & Baker, 1986) and consequently may influence nicotinic transmission in these cells, and in sympathetic ganglia.

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