Br. J. Pharmac. (1974), **51**, 259-268

INFLUENCE OF SKF-525A CONGENERS, STROPHANTHIDIN AND TISSUE-CULTURE MEDIA ON DESENSITIZATION IN FROG SKELETAL MUSCLE

D.A. TERRAR[1]

Department of Pharmacology, University College, London WC1E 6BT

1 Microelectrodes have been used to follow changes in membrane potential at end-plate regions of frog skeletal muscle fibres exposed to carbachol; the depolarizing drug was applied to narrow strips of muscle in a rapidly flowing solution containing relatively impermeant anions rather than chloride.

2 During prolonged applications of carbachol (10 to 20 μM), the depolarization caused by the drug showed a gradual decline which was attributed to desensitization.

3 Desensitization was little if at all affected by supplementing the external solution with factors present in tissue-culture media, or by treating the muscle with strophanthidin (25 μM).

4 The rate of repolarization in the presence of carbachol (10 to 20 μM) was greatly increased by the SKF-525A congeners pipenzolate bromide (10 μM) and adiphenine hydrochloride (1 μM). The desensitization-enhancing action of these compounds is discussed.

Introduction

If healthy tissue is exposed to a fixed concentration of agonist, it might be expected that its response will be constant and reproducible. However, this is found not to be so for many isolated tissues exposed to cholinergic agonists; in particular, the depolarization of the end-plate membrane of frog skeletal muscle elicited by these agonists declines even while the drug concentration is maintained (Fatt, 1950; Thesleff, 1955; Katz & Thesleff, 1957; Manthey, 1966; Magazanik, 1968; Magazanik & Vyskocil, 1970; Nastuk & Parsons, 1970; Rang & Ritter, 1970a). Two important additional observations are that the sensitivity does not remain low but recovers when the drug is washed away, and that the time course of this recovery is reproducible (Katz & Thesleff, 1957; Rang & Ritter, 1970a). It is though that during such prolonged applications of agonist, receptors may become 'desensitized' (Katz & Thesleff, 1957; Rang & Ritter, 1970a), in other words lose their effectiveness and that these desensitized receptors may revert to the functional state when the agonist is washed away.

However, receptor desensitization is not the only factor influencing the time course of the depolarization caused by agents such as carbachol. It has been shown (Jenkinson & Terrar, 1973) that the response can be greatly influenced by the movement of chloride ions even though the anion permeability is little if at all affected by the agonist. In view of the importance of chloride movements, it was clear that experiments to examine the onset of desensitization in isolated tissues should be made in conditions in which this factor is avoided (for example, by keeping the drug application brief, by voltage-clamping the membrane at the resting level, or by using chloride-free solutions as in the present work).

Since it has been reported that SKF-525A (2-diethylaminoethyl diphenylpropylacetate) and some structurally similar compounds increase desensitization as assessed from the decline in response to iontophoretically applied agonists (Magazanik, 1970; 1971) it seemed of interest to examine the effects of such substances on depolarizations caused by prolonged application of carbachol. Experiments were also done to test whether desensitization still occurs when the muscle is bathed in solution supplemented with factors present in tissue-culture media, and to examine the effect of strophanthidin.

Methods

Depolarizations were recorded from end-plate regions of frog *(Rana temporaria)* skeletal muscle fibres exposed to carbachol; conventional microelectrode techniques were used to record from single fibres in thin strips of muscle as

[1] Present address: Department of Pharmacology, The School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX.

described previously (Jenkinson & Terrar, 1973). Carbachol was applied in a rapidly flowing solution containing the relatively impermeant anions isethionate and methylsulphate rather than chloride (Jenkinson & Terrar, 1973). The records shown were obtained with a pen recorder (Devices M2).

All experiments were done at room temperature (20-23° C). The Ringer solution used in most experiments contained (mM): Na isethionate, 116; K methylsulphate, 2.0; $CaSO_4$, 2.4; Na_2HPO_4, 1.92; NaH_2PO_4, 0.48, and is referred to in the text as solution A. In a few instances, also mentioned in the text, a slightly different solution (B) having the following composition (mM) was used: NaCl, 15; Na isethionate, 100; K methylsulphate, 2.0; $CaSO_4$, 2.4; Na_2HPO_4, 1.92; NaH_2PO_4, 0.48. All solutions contained tetrodotoxin (100 nM).

It was considered that the decline in the depolarizations recorded from single fibres during prolonged applications of carbachol in chloride-free solution could provide a simple and rapid test system for a preliminary examination of the effects of several factors on the rate of desensitization. In most experiments, responses of individual fibres exposed to carbachol were recorded in a solution containing the compounds to be tested and compared to those of the same fibres in 'control' solution. Each response in the presence of the compound was bracketed with control responses, the interval between exposures to carbachol being 15 (or more) minutes. Since this is about five times greater than the half-time for recovery from desensitization in normal (Rang & Ritter, 1970a) as well as chloride-free solution (unpublished observations, D.A. Terrar) it would be expected that even if every receptor became inactive during a response, all but 3% would be functional after the recovery period. In every experiment the compound to be tested was first applied before exposure to carbachol, and remained until the carbachol was washed away Responses were rejected if the membrane potential did not return to within 3 mV of the resting level after exposure to carbachol.

Three measurements were taken from each response: (1) the maximum rate of repolarization which occurred in the presence of carbachol, $(dV/dt)_{max}$ (mV/min); (2) the maximum depolarization, V_{max} (mV); and (3) the residual depolarization at the end of the period of exposure to carbachol, V_t (mV). Three indices were used as measures of desensitization, $V_{max} - V_t$, V_t/V_{max}, and $(dV/dt)_{max}$. It has to be recognized that these indices may vary with the maximum amplitude of the chosen control response; this difficulty could perhaps be avoided by using null methods in which 'dose-ratios' are estimated (see for example Rang & Ritter, 1970a). However, it was thought that the relatively simple procedure used here would be capable of detecting any substantial change in desensitization rate.

In a few preliminary experiments, a slightly different procedure was used to test whether or not some factors included in tissue-culture media influence desensitization. Muscles were dissected in solution supplemented with the factors to be tested. The preparation was then mounted in supplemented solution and responses to carbachol (20 μM for 2 min) were recorded from a series of fibres. The supplemented solution was then replaced by 'control' solution (a variant of Ringer, see later), and a second series of responses to carbachol recorded from fibres in the same region of the muscle. As above, the interval between applications of carbachol was at least 15 minutes. In these experiments supplemented and control solutions were recycled through a perfusion pump; the outlet from the bath was, however, diverted to waste while carbachol was applied, and for at least 5 min after the membrane potential had returned to its resting level at the end of each exposure.

Materials

The following drugs were used: carbachol (carbamoyl choline chloride, B.D.H.); tetrodotoxin (Sankyo); adiphenine hydrochloride (2-diethylaminoethyl diphenyl acetate hydrochloride, CIBA); pipenzolate bromide (N-ethyl-3-piperidyl benzilate methobromide, M.C.P. Pharmaceuticals, Alperton, Middx); SKF-525A (2-diethylaminoethyl diphenylpropylacetate hydrochloride, Smith Kline & French); oxyphenonium bromide (diethyl(2-hydroxyethyl)methylammonium α-phenylcyclohexane glycolate bromide, CIBA); strophanthidin (C.F. Boehringer & Soehne GmbH, Mannheim, Germany). Foetal calf serum was obtained from Wellcome, and the amino acids and vitamins from Bio-Cult.

Results

Desensitization in solution supplemented with constituents of tissue-culture media

Since there is some indirect evidence to suggest that desensitization is more rapid *in vitro* than *in vivo* (Zaimis, 1959, 1962; Maclagan, 1962), it could be argued that this is because the bathing solutions normally used with isolated muscles lacked an important factor present in the living animal. It is, of course, well known that isolated

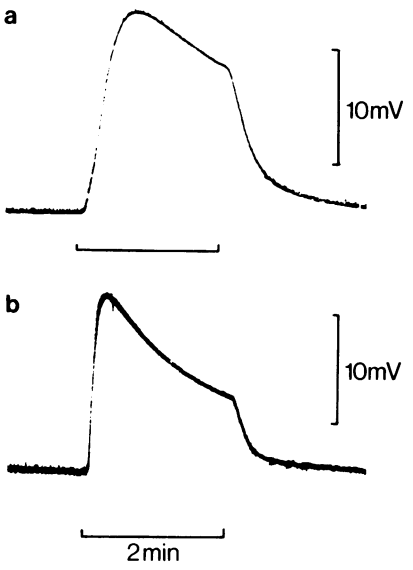

Fig. 1 The effect of carbachol (20 μM applied for 2 min) on the membrane potential at the end-plate region of two frog skeletal muscle fibres (resting potentials: (a) 89 mV; (b) 95 mV). In each case, the preparation was bathed in a continuously flowing solution supplemented with foetal calf serum and containing tetrodotoxin (100 nM) throughout.

cells survive for long periods only if they are kept in the supplemented media used in tissue culture, and it was thought possible that some of the factors included in such media might influence desensitization. Two particular supplements were tested: (1) foetal calf serum; and (2) the amino acids and vitamins included in amphibian tissue-culture medium described by Wolf & Quimby (1962, 1964).

Figure 1 shows depolarizations caused by carbachol (20 μM) applied in a solution containing foetal calf serum (20% by volume serum; 80% solution A, see methods section). Clearly the fibre repolarized while the carbachol concentration was maintained; this desensitization was seen in each of the twelve fibres examined. Responses were also recorded in 'control' solution B which contained chloride (15 mM) in an attempt to match the chloride content of the serum containing solution (since chloride entry could have partially masked desensitization). Values of the measures of desensitization $V_{max} - V_t$ and V_t/V_{max}, for responses in each solution were compared. It was concluded from two-sample t tests that none of the differences (which did not exceed 20%) was significant at the 5% level. Values were: (1) for supplemented solution, $V_{max} - V_t = 6.5$ (s.e. 0.8, $n = 12$), $V_t/V_{max} = 0.52$ (s.e. 0.05, $n = 12$); (2) for control solution, $V_{max} - V_t = 5.9$ (s.e. 1.1, $n = 12$), $V_t/V_{max} = 0.62$ (s.e. 0.07, $n = 12$).

A second kind of supplemented solution was tested; in addition to the salts of solution A, this contained amino acids and vitamins present in the concentrations used by Wolf & Quimby (1962, 1964) in their amphibian tissue-culture medium. (The same proportions are used in 'Minimum Essential Medium' (Eagle, 1959).) All depolarizations caused by carbachol (20 μM) in this supplemented solution showed desensitization. Values of $V_{max} - V_t$ and V_t/V_{max} for responses in this solution were compared with those in 'control' solution A. The greatest difference from the control mean was 12%, and none of the differences were significant at the 5% level, as assessed from two sample t tests. Values were: (1) for supplemented solution $V_{max} - V_t = 5.3$ (s.e. 0.8, $n = 11$), $V_t/V_{max} = 0.66$ (s.e. 0.05, $n = 11$); (2) for control solution $V_{max} - V_t = 6.0$ (s.e. 1.3, $n = 9$), $V_t/V_{max} = 0.61$ (s.e. 0.07, $n = 9$).

The effect of strophanthidin

When a muscle fibre is exposed to carbachol for long periods there will be changes in the internal cation as well as anion concentrations: a fibre bathed in normal Ringer solution is expected to gain sodium, and to lose potassium. It is possible that these changes may modify the change in membrane potential in a fibre exposed to carbachol. However, the repolarization which occurs in the presence of carbachol cannot be attributed to depletion of potassium inside the fibre; this would cause depolarization rather than repolarization. A possibility which could account for the repolarization is that metabolically-linked ion movements, for example electrogenic sodium extrusion (see Thomas, 1972), might influence the membrane potential. If this were to be the case, the electrogenic action would be attenuated by the conductance increase during carbachol, and a substantial post-exposure hyperpolarization would be expected, as is the case in an autonomic ganglion (see e.g. Brown, Brownstein & Scholfield, 1972). Such a hyperpolarization was not seen in the present experiments. Another prediction of the electrogenic hypothesis which can be experimentally tested is that the time course of the repolarization would be expected to be modified by strophanthidin which, like ouabain has been found to reduce the active extrusion of sodium from muscle fibres of the frog sartorius (Horowicz, Taylor & Waggoner, 1970). The effect of two concentrations of strophanthidin (5 μM and 25 μM) was therefore examined; on the basis of the findings of Horowicz *et al.* (1970), these

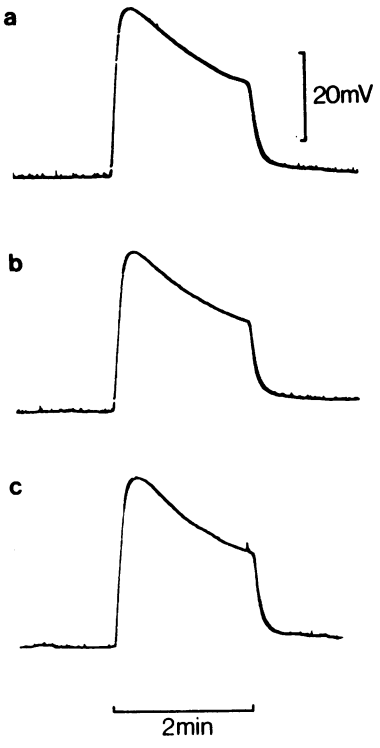

Fig. 2 Depolarizations recorded in sequence (interval >15 min) from one fibre exposed to carbachol (20 μM, during bar), applied either alone (a and c) or in the presence of strophanthidin (25 μM) added to the bathing fluid 3 min beforehand (b). Electrode was kept in place throughout. Resting potential −90 mV.

concentrations would be expected to inhibit 74% and 93% respectively of the strophanthidin-sensitive sodium efflux. Although it might be thought that the action of strophanthidin could be impaired in the modified solution of the present experiments, the drug has been found (unpublished observations of D. Gadsby and D. Ogden) to remain effective on frog skeletal muscle in a similar chloride-free solution (the principal anion again being isethionate).

Figure 2 shows depolarizations recorded in sequence from one fibre exposed to carbachol (20 μM), applied either alone or in the presence of strophanthidin (25 μM). It can be seen that strophanthidin had no great influence on the rate of repolarization in the presence of carbachol. However, close inspection of the combined results from several experiments showed a slight change in the presence of the drug; the mean paired differences from control values of $(dV/dt)_{max}$, $V_{max} - V_t$, and V_t/V_{max} were 8, 12, and 4%, respectively, of the control means (see Table 1). On the basis of paired t tests, it was concluded that the differences from control values of $(dV/dt)_{max}$ and $V_{max} - V_t$, but not of V_t/V_{max}, were significant at the 5% level. While this could mean that there was a small effect on desensitization, a likely alternative possibility is that the difference occurred because repolarization after carbachol was incomplete, by up to 3 mV. This incomplete recovery could, perhaps have resulted from loss of potassium ions inside the fibre, or alternatively from a local increase in the extracellular concentration of potassium. An incidental observation was that strophanthidin (25 μM) caused a slight depolarization (mean 1.9 mV, seven applications, maximum value observed, 2.5 mV), perhaps because of the reduced contribution of a small electrogenic component of the membrane potential which would be more obvious in chloride-free solution.

The effects of SKF-525A congeners

The SKF-525A congeners which Magazanik (1970; 1971) found to increase desensitization as assessed from the decline in response to iontophoretically applied agonists included spasmolytin (adiphenine), mespenal, arpenal, tropacine, merpanit, and cypenam. Although some of these are muscarinic antagonists, atropine was found to be ineffective (Magazanik, 1971). Substances tested

Table 1 Effect of strophanthidin on depolarizations caused by carbachol (20 μM for 2 minutes)

	Control	*Strophanthidin*	*Paired differences*
V_{max} (mV)	31.6 ± 1.5(8)	28.5 ± 1.5(8)	−1.8 ± 0.9(8)
$(dV/dt)_{max}$ (mV min^{-1})	13.6 ± 0.7(8)	12.7 ± 0.5(8)	−1.1 ± 0.3(8)
$V_{max} - V_t$ (mV)	15.5 ± 0.7(8)	13.5 ± 0.6(8)	−1.8 ± 0.5(8)
V_t/V_{max}	0.50 ± 0.01(8)	0.52 ± 0.01(8)	+0.02 ± 0.01(8)

Figures in brackets are the number of observations. Measurements from six responses in strophanthidin (25 μM) have been pooled with those from two in 5 μM.

in the present study were adiphenine hydrochloride and pipenzolate bromide; a few preliminary experiments were made with SKF-525A and oxyphenonium bromide. Pipenzolate and oxyphenonium were not tested by Magazanik but are structurally similar to SKF-525A; however, both are quaternary nitrogen compounds.

(a) *Adiphenine hydrochloride.* Responses to carbachol were recorded in the presence of adiphenine hydrochloride (1 μM; applied 8 to 15 min beforehand). Records from such an experiment are illustrated in Figure 3. The main findings were that adiphenine reduced the initial response to carbachol, and seemed to increase the rate of desensitization.

Application of paired t tests to the results listed in Table 2 showed that:

(1) The increase in $(dV/dt)_{max}$ (to more than three times the control value) was significant at the 5% but not the 1% level. (95% confidence limits for the increase were +0.3 and +7.7 mV/min.)

(2) $V_{max} - V_t$: the difference, which was about 2% of the mean control value, was not significant at the 5% level.

(3) The reduction in V_t/V_{max} (to 73% of the mean control value) was significant at the 1% level.

For two responses in adiphenine (1 μM) the concentration of carbachol was increased to 40 μM to match control values of V_{max}. An example, bracketed by control responses to carbachol (10 μM) is illustrated in Figure 4. The maximum rate of repolarization was very greatly increased (to about 100 mV/min in each case compared with control values of 2.4, 2.3, and 2.0 mV/min), indicating a striking increase in desensitization. This suggestion was supported by the increase of V_{max} V_t and the decrease of V_t/V_{max} (see legend to Figure 4). Such an increase in desensitization when V_{max} was matched to control values would not have been expected if adiphenine had acted as a competitive antagonist of carbachol, and if neither drug had a preferential affinity for desensitized receptors (see discussion section). Had this been the case (as is thought to hold for tubocurarine), desensitization in the presence of antagonist would have been expected to be similar to that during a control response, provided that the values of V_{max} were matched. This point is illustrated in Fig. 5 which shows the response of the same fibre to carbachol (40 μM) in the presence of tubocurarine (1.3 μM giving a dose ratio of about four, close to that in the adiphenine

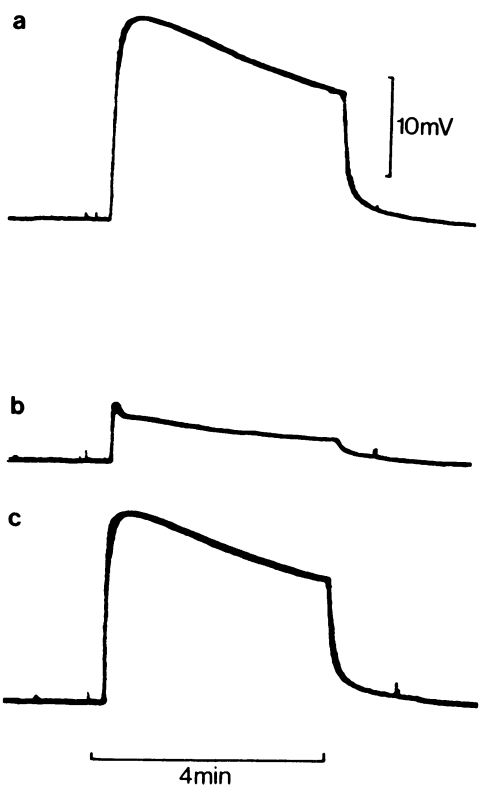

Fig. 3 Depolarizations recorded in sequence (interval >15 min) from one fibre exposed to carbachol 10 μM either alone (a and c) or in the presence of adiphenine 1 μM (b). Electrode was kept in place throughout. Resting potential −95 mV. (Note: reduction in V_{max} in this fibre was slightly greater than the mean paired difference.)

Table 2 Effect of adiphenine hydrochloride (1 μM) on depolarizations caused by carbachol (10 μM for 3 minutes).

	Control	*Adiphenine*	*Paired differences*
V_{max} (mV)	20.1 ± 2.4(9)	8.7 ± 1.6(7)	−10.4 ± 1.9(7)
$(dV/dt)_{max}$ (mV min^{-1})	1.9 ± 0.3(9)	5.9 ± 2.1(7)	+4.0 ± 1.9(7)
$V_{max} - V_t$ (mV)	4.2 ± 0.7(9)	3.8 ± 0.9(7)	−0.1 ± 0.9(7)
V_t/V_{max}	0.80 ± 0.03(9)	0.59 ± 0.06(7)	−0.22 ± 0.04(7)

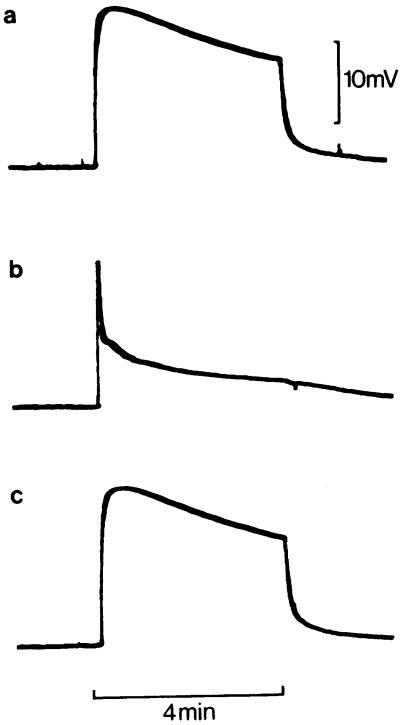

Fig. 4 Depolarizations recorded in sequence (interval >15 min) from one fibre exposed either to carbachol 10 μM alone (a and c) or (b) to carbachol 40 μM in the presence of adiphenine 1 μM. Same fibre as Figures 3 and 5. Electrode was kept in place throughout. Resting potential −95 mV.

The following values were measured from these and subsequent responses. Carbachol (10 μM) alone: V_{max} 20.0, 18.5, 21.0; $(dV/dt)_{max}$ 2.4, 2.3, 2.0; $V_{max} - V_t$ 6.0, 7.0, 5.0; V_t/V_{max} 0.70, 0.62, 0.76. Carbachol (40 μM) + adiphenine (1 μM): V_{max} 18.5, 16.5; $(dV/dt)_{max}$ 100, 100; $V_{max} - V_t$ 15.0, 13.5; V_t/V_{max} 0.19, 0.18.

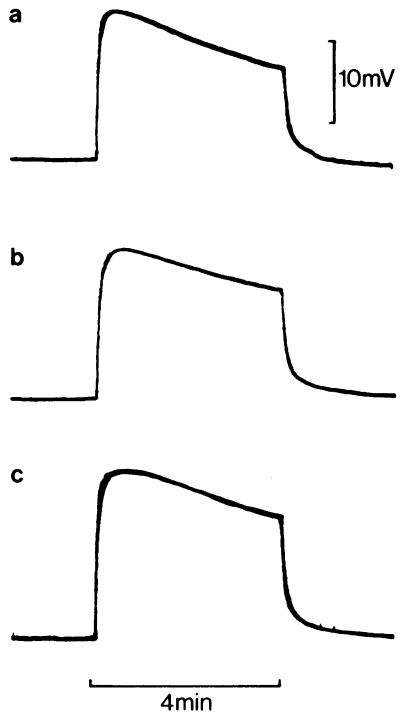

Fig. 5 Depolarizations recorded in sequence (interval >15 min) from one fibre exposed either to carbachol 10 μM alone (a and c) or (b) to carbachol 40 μM in the presence of tubocurarine 1.3 μM. Electrode was kept in place throughout. Resting potential −95 mV. Same fibre as Figures 3 and 4.

experiment). The three responses are similar (compare Figure 4).

(b) *Pipenzolate bromide.* Values of V_{max} and the three measures of desensitization are listed in Table 3. Examples of responses are illustrated in Figures 6 and 7.

Like adiphenine, pipenzolate (10 μM, pre-incubation time 5 to 83 min) decreased V_{max} and seemed to increase desensitization. Paired t tests showed that all the differences were significant at the 1% level. The percentage changes with respect to mean control values were: V_{max}, a 37% decrease; $(dV/dt)_{max}$, a 160% increase; $V_{max} - V_t$, a 29% increase; V_t/V_{max}, a 50% decrease. The effect was reversible.

(c) *SKF-525A and oxyphenonium.* A few observations were made of the effects of carbachol

Table 3 Effect of pipenzolate bromide (10 μM) on depolarizations caused by carbachol (10 μM for 3 minutes)

	Control	*Pipenzolate*	*Paired differences*
V_{max} (mV)	29.2 ± 2.0(9)	17.9 ± 2.5(7)	−10.8 ± 0.8(7)
$(dV/dt)_{max}$ (mV min^{-1})	5.0 ± 0.7(9)	12.7 ± 2.7(7)	+8.0 ± 2.0(7)
$V_{max} - V_t$ (mV)	9.7 ± 1.4(9)	12.1 ± 2.2(7)	+2.8 ± 0.8(7)
V_t/V_{max}	0.68 ± 0.03(9)	0.34 ± 0.03(7)	−0.34 ± 0.02(7)

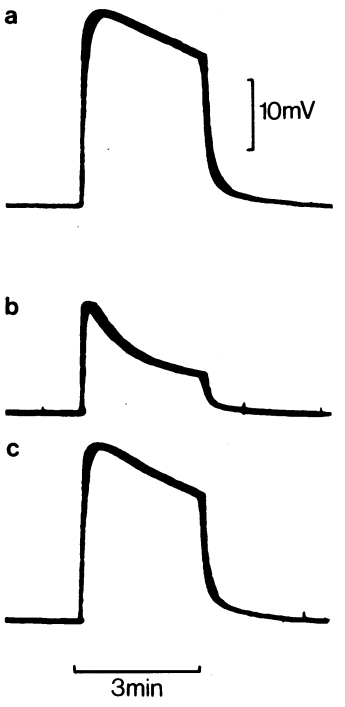

Fig. 6 Depolarizations recorded in sequence (interval >15 min) from one fibre exposed to carbachol 10 μM either alone (a and c) or in the presence of pipenzolate 10 μM (b). Electrode was kept in place throughout. Resting potential −97 mV.

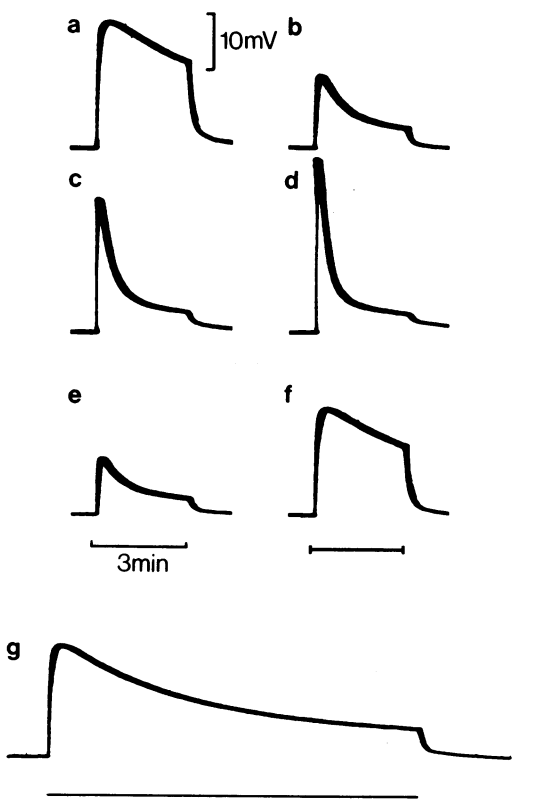

Fig. 7 Depolarizations recorded in sequence (interval > 15 min) from one fibre. Carbachol 10 μM was applied alone in (a and f). Records (b) to (e) in the presence of pipenzolate 10 μM: carbachol concentrations were 10 μM (b and e), 20 μM (c), and 30 μM (d). Electrode was kept in place throughout. Resting potential −97 mV. Same fibre as Figure 6.

(g) Depolarization caused by a 12 min application of carbachol 10 μM in the same fibre.

(10 to 20 μM for 3 min) in the presence of SKF-525A and oxyphenonium. The tentative conclusion was that these drugs also increase desensitization. In the presence of SKF-525A (0.45 μM), $(dV/dt)_{max}$ was increased to 7.8 (range 4.1 to 15.0, three observations) from a control value of 1.8 (range 1.2 to 2.3, three observations), the mean paired difference being +6.0 (range +2.3 to 13.2). In the presence of oxyphenonium (10 μM), $(dV/dt)_{max}$ was increased to 10.8 (range 7.4 to 15.0, three observations) from a control value of 5.9 (range 4.0 to 8.4, three observations), the mean paired difference being +5.0 (range +3.0 to 7.1). The effects were slowly reversible.

Discussion

The main finding is that although the time course of depolarization caused by prolonged application of carbachol is affected little if at all by supplementing the external solution with factors present in tissue-culture media, or by treating the muscle with strophanthidin, it can be markedly modified in the presence of compounds with structures similar to SKF-525A. Although the effect is striking, it has not been analysed quantitatively. One complication is that desensitization may become so rapid that V_{max} becomes smaller because a substantial number of receptors are already desensitized before the final concentration of carbachol is reached in the region of receptors. In addition, the compounds may also exert a curare-like action. Nevertheless, it seems clear (particularly from the results obtained with pipenzolate) that desensitization was markedly increased, confirming Magazanik's conclusions.

What mechanisms could be involved? As a starting point, it should be recognized that

although the rapid decline in carbachol depolarization which occurs in the presence of pipenzolate has been described as 'desensitization', it is uncertain whether the mechanism is similar to that which operates in normal solution. Two possibilities are that: (1) pipenzolate may enhance receptor desensitization; and (2) pipenzolate may affect stages which intervene between receptor occupation and the subsequent conductance change. These alternatives may perhaps be distinguished experimentally by examining the interaction of pipenzolate both with agonists which are thought to cause faster receptor desensitization than carbachol (e.g. phenyl trimethyl ammonium (Rang & Ritter, 1970a)), and with toxins which seem to bind to normal but not to desensitized receptors (Miledi & Potter, 1971; Lester, 1972). Another approach to this problem is to determine whether the rate of recovery of sensitivity after carbachol with pipenzolate differs from that after carbachol alone.

Although at present the available evidence cannot be used to distinguish these alternatives, the applicability of some simple models can be considered. Rang & Ritter (1970a) discussed several models of receptor desensitization and favoured the cyclic model proposed by Katz & Thesleff (1957; see also del Castillo & Katz, 1957). They have considered the effect of an antagonist on the rate constants for onset of, and recovery from, desensitization as predicted from four models involving non-interacting receptors. Following their analysis, it seems that if pipenzolate competed with carbachol for normal receptors in a curare-like fashion and had no preferential affinity for desensitized receptors, the rate and extent of desensitization would be expected to be *reduced* unless the concentration of carbachol was increased to match V_{max}. This expectation contrasts with the observation of an increase in $(dV/dt)_{max}$ measured from responses to the control concentration of carbachol; also contrast Fig. 5 with Figures 4 and 7.

Another possibility is that pipenzolate 'uncouples' receptors from further steps in the response mechanism. In this case, agonist would still be expected to combine with the same number of receptors as in the absence of pipenzolate, but its effect would be reduced. Thus, the same proportion of receptors would become desensitized unless the carbachol concentration was increased. If receptors did become uncoupled in this fashion a change in the number of desensitized receptors could not be inferred from changes in $V_{max} - V_t$ or V_t/V_{max} unless the carbachol dose-response relationship both in the presence and absence of pipenzolate were established. Nevertheless, the possibility of 'uncoupling' without an effect on receptors could not explain the observed increase in $(dV/dt)_{max}$ which occurred in the presence of pipenzolate and adiphenine (and probably of SKF-525A and oxyphenonium though fewer experiments were performed) even when the concentration of carbachol was not increased to match V_{max}.

It therefore seems that a more complex model than the two already discussed is required to explain the apparent antagonistic and desensitization-enhancing properties of such compounds. One possibility is that these substances may behave as metaphilic antagonists (i.e. show preferential affinity for desensitized receptors; Rang & Ritter, 1969; 1970b) on carbachol receptors. If this were the case, and if the cyclic model accurately represents desensitization, a decrease in the rate constant for approach to an equilibrium number of desensitized receptors would be expected (because of a slower rate of reversion of inactive receptors to the functional state while desensitization develops, see the expression for rate constants derived by Rang & Ritter, 1970); this would be expected even if the agonist concentration was increased to match V_{max}. This could be difficult to establish. However, a rough estimate of the rate of onset of desensitization can be made from the response to a maintained concentration of agonist. In the response illustrated in Fig. 7g, the amplitude fell in 360 s to that of the response to an earlier application of half this concentration of carbachol; furthermore, the half-time of repolarization in the presence of carbachol was 270 seconds. In contrast, the time for half-repolarization measured from a response to the same concentration of carbachol in the presence of pipenzolate (10 μM) was 60 s, and half-times measured from responses to higher concentrations of carbachol were smaller still (see Figure 7a to f). If the approximately exponential time-course of repolarization accurately reflected the time-course of receptor inactivation, it could be concluded that pipenzolate had increased the rate constant for approach to equilibrium in contrast to the decrease expected of a metaphilic antagonist. However, quantitative experiments would be needed to establish this point, and it must be mentioned that the response of chick muscle to repeated test doses of carbachol declined more rapidly in the presence of metaphilic antagonists (Rang & Ritter, 1969).

Another way to approach the problem might be to examine the binding of these compounds to tissues or preparations containing cholinergic receptors. Some results of binding studies carried out on detergent-dispersed, receptor-like proteins from *Torpedo* electroplax are of interest here:

SKF-525A, at 100 μM (a concentration 200 times greater than that used in the present study), in contrast to curare (5 μM), was found not to influence the binding of ^{131}I-labelled α-bungarotoxin (Franklin & Potter, 1972). It must, of course, be recognized that these toxin-binding sites may differ from carbachol receptors in frog skeletal muscle. Nevertheless, these results are consistent with Magazanik's suggestion that SKF-525A congeners enhance desensitization not by combining with the same sites as carbachol but rather by influencing another aspect of the response mechanism For example, one possibility consistent with the present observations is that pipenzolate may block conductance 'channels' once they have been activated by carbachol; the rapid decline of carbachol depolarization might then reflect the rate of binding of pipenzolate to channel-blocking sites.

All the compounds found to be active are esters of acetic acid derivatives and nitrogen-containing alcohols. In each, the α-carbon of the acid is linked to large hydrophobic groups, either two phenyls or one phenyl and a cyclohexane; the nitrogen is separated from the carbonyl carbon by two other carbon atoms. Since their structures have some resemblance to that of carbachol it is conceivable (despite the evidence from *Torpedo* tissue) that they combine with similar sites. Indeed, there is evidence that SKF-525A and adiphenine inhibit cholinesterase in human and mouse plasma (Netter, 1959), and an anticholinesterase effect of SKF-525A (in addition to its inhibitory action) was also thought to account for the finding that end-plate potentials in frog muscle were unaffected by SKF-525A (100 μM) in the absence of another anticholinesterase, but were reduced by SKF-525A (20 μM) in its presence (Magazanik, 1970). However, another possibility is that the site of action of SKF-525A (which is also known to be a microsomal enzyme inhibitor) and adiphenine is intracellular (see Vyskocil & Magazanik, 1972). Nevertheless, the present results show that quaternary nitrogen compounds also reduced V_{max} and increased desensitization, although concentrations some 10 to 20 times higher than of the tertiary compounds were needed.

These preliminary results suggest that it would be well worth exploring the structure-activity relationship of such compounds in greater detail. Another approach which could be informative is to compare the effects of these compounds with the action of certain xylocaine congeners which also influence the operation of nicotinic receptors, possibly by combining with agonist-receptor complexes (Steinbach, 1968).

I thank Dr D.H. Jenkinson for his advice and encouragement throughout the course of the work, Mrs J.E. Haylett for expert technical assistance, and Mr F. Ballhatchet for construction and maintenance of apparatus. An M.R.C. Scholarship provided support for the study.

References

BROWN, D.A., BROWNSTEIN, M.J. & SCHOLFIELD, C.N. (1972). Origin of the after-hyperpolarization that follows removal of depolarizing agents from the isolated superior cervical ganglion of the rat. *Br. J. Pharmac.*, **44**, 651-671.

DEL CASTILLO, J. & KATZ, B. (1957). A comparison of acetylcholine and stable depolarizing agents. *Proc. Roy. Soc. B.*, **146**, 362-368.

EAGLE, H. (1959). Amino acid metabolism in mammalian cell cultures. *Science*, **130**, 432.

FATT, P. (1950). The electromotive action of acetylcholine at the motor end-plate. *J. Physiol., Lond.*, **111**, 408-422.

FRANKLIN, G.I. & POTTER, L.T. (1972). Studies of the binding of α-bungarotoxin to membrane-bound and detergent-dispersed acetylcholine receptors from *Torpedo* electric tissue. *FEBS Letters*, **28**, 101-106.

HOROWICZ, P., TAYLOR, J.W. & WAGGONER, D.M. (1970). Fractionation of sodium efflux in frog sartorius muscles by strophanthidin and removal of external sodium. *J. gen. Physiol.*, **55**, 401-425.

JENKINSON, D.H. & TERRAR, D.A. (1973). Influence of chloride ions on changes in membrane potential during prolonged application of carbachol to frog skeletal muscle. *Br. J. Pharmac.*, **47**, 363-376.

KATZ, B. & THESLEFF, S. (1957). A study of the 'desensitization' produced by acetylcholine at the motor end-plate. *J. Physiol., Lond.*, **138**, 63-80.

LESTER, H.A. (1972). Vulnerability of desensitized or curare-treated acetylcholine receptors to irreversible blockade by cobra toxin. *Molec. Pharmac.*, **8**, 632-644.

MACLAGAN, J. (1962). A comparison of the responses of the tenuissimus muscle to neuromuscular blocking drugs *in vivo* and *in vitro*. *Br. J. Pharmac. Chemother.*, **18**, 204-216.

MAGAZANIK, L.G. (1968). On the mechanism of the desensitization of muscle postsynaptic membrane. *Biofysica*, **13**, 199-203.

MAGAZANIK, L.G. (1970). Mechanism of the influence of the diethylaminoethyl ester of diphenylpropylacetic acid (SKF-525A) on neuromuscular synapses. *Byull. Eksp. Biol. Med.*, **69**, (3), 10-14. See also *Chem. Abs.*, **72**, 130719p.

MAGAZANIK, L.G. (1971). On mechanism of antiacetylcholine effects of some mononitrogen anticholinergics in the neuromuscular synapse. *Farmakol. Toksikol.*, **34**(3), 292-297.

MAGAZANIK, L.G. & VYSKOČIL, F. (1970). Dependence of acetylcholine desensitization on the

membrane potential of frog muscle fibre and on the ionic changes in the medium. *J. Physiol., Lond.*, **210**, 507-518.

MANTHEY, A.A. (1966). The effect of calcium on the desensitization of membrane receptors at the neuromuscular junction. *J. gen. Physiol.*, **49**, 963-976.

MILEDI, R. & POTTER, L.T. (1971). Acetylcholine receptors in muscle fibres. *Nature, Lond.*, **233**, 599-603.

NASTUK, W.L. & PARSONS, R.L. (1970). Factors in the inactivation of postjunctional membrane receptors of frog skeletal muscle. *J. gen. Physiol.*, **56**, 218-249.

NETTER, K.J. (1959). Inhibition of procaine hydrolysis by the diethylaminoethanol esters of diphenylpropylacetic acid and diphenylacetic acid (Trasentin). *Arch. exp. Path. Pharmac.*, **235**, 498-512. See also *Chem. Abs.*, **53**, 14334h.

RANG, H.P. & RITTER, J.M. (1969). A new kind of drug antagonism: evidence that agonists cause a molecular change in acetylcholine receptors. *Molec. Pharmac.*, **5**, 394-411.

RANG, H.P. & RITTER, J.M. (1970a). On the mechanism of desensitization at cholinergic receptors. *Molec. Pharmac.*, **6**, 357-382.

RANG, H.P. & RITTER, J.M. (1970b). The relationship between desensitization and the metaphilic effect at cholinergic receptors. *Molec. Pharmac.*, **6**, 383-390.

STEINBACH, A.B. (1968). Alteration by xylocaine (lidocaine) and its derivatives of the time course of the end plate potential. *J. gen. Physiol.*, **52**, 144-161.

THESLEFF, S. (1955). Neuromuscular block caused by acetylcholine. *Nature, Lond.*, **175**, 594-595.

THOMAS, R.C. (1972). Electrogenic pump in nerve and muscle cells. *Physiol. Rev.*, **52**, 563-594.

VYSKOČIL, F. & MAGAZANIK, L.G. (1972). The desensitization of postjunctional muscle membrane after intracellular application of membrane stabilizers and snake venom polypeptides. *Brain Research*, **48**, 417-419.

WOLF, K. & QUIMBY, M.C. (1962). Progress report on efforts to establish a line of frog cells. *Excerpta med. Amst.*, (sect. 1), **16**, 823-824.

WOLF, K. & QUIMBY, M.C. (1964). Amphibian cell culture: permanent cell line from the bullfrog (*Rana catesbeiana*). *Science*, **144**, 1578.

ZAIMIS, E. (1959). In: *Curare and Curare-like Agents.* Ed. Bovet, D., Bovet-Nitti, F. & Marini-Bettolo, G.B., p. 191. Amsterdam: Elsevier.

ZAIMIS, E. (1962). In: *Curare and Curare-like Agents.* Ed. De Reuck, A.V.S., p. 75. London: Churchill.

(Revised November 26, 1973)