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Succinyl derivatives of N-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid: their effects on the frog neuromuscular junction

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1 Succinic anhydride (SA) dissolved in Ringer solution buffered with N-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid (SA-TES solution) potentiates the depolarizing action of acetylcholine (ACh, 10–40 μM) on frog muscle and the tension induced by bath application of this agonist.

2 Applied from one side of a double-barrelled micropipette, SA-TES increases the amplitude of iontophoretically elicited ACh potentials.

3 The potentiation of the effects of ACh by SA-TES does not involve changes in either the activity of the ACh esterase or the input resistance of the muscle membrane.

4 For depolarizations of frog sartorius muscle, dose-response relationships obtained for ACh concentrations from 0.5 to 20 μM indicate that SA-TES increases the apparent affinity of ACh by a factor of 3.

5 SA-TES exerts an 'accelerating' effect on the responses elicited by bath-applied ACh; i.e., it increases the rate of depolarization when ACh is added to the bath and the rate of repolarization upon washing out. These effects are particularly marked in preparations treated with neostigmine (3 μM).

6 SA-TES does not potentiate the depolarizing action of agonists which do not contain an ester group. Moreover, the time course of the responses elicited by these compounds is not influenced by SA-TES.

7 SA-TES fails to influence significantly the effects of the neurally released transmitter. Only a 10% increase in the average amplitude of the endplate potentials was observed.

8 SA hydrolyzes in about 30 min at room temperature; however the SA-TES solution retains its activity for several weeks. Succinate is inactive, and so is SA in Ringer buffered with phosphate.

9 The SA-TES solution contains seven succinyl-TES derivatives, which were separated by ion-exchange chromatography and paper chromatography. At concentrations between 1 to 150 μM, these succinyl-TES derivatives affected the ACh-induced contraction of frog rectus abdominus muscle. The most abundant derivative potentiated the action of high doses of ACh, but was inhibitory at lower ones. The other derivatives were mostly inhibitory.

10 These results are discussed in terms of two hypotheses. One postulates the presence of a diffusion barrier formed by groups that bind ACh and are saturated by SA-TES. The other assumes that SA-TES acts directly on the ACh receptor exerting its potentiating effect through a cooperative mechanism.

Introduction

Certain small molecules containing ester or carboxylic anhydride groups increase the depolarizing action of acetylcholine (ACh) on frog muscle (Saji & del Castillo, 1975). One of such compounds, succinic

anhydride (SA), proved particularly suitable for pharmacological experiments because it did not affect the muscle membrane adversely. The actions of SA on frog muscle, mainly a potentiation of the ACh-induced depolarization were described by Escalona de Motta & del Castillo (1977) using frog Ringer solution buffered with N-tris (hydroxymethyl)methyl-2-aminoethane sulphonic acid (SA-TES solution). Here we show that, at the usual concentration of 1 mM SA in Ringer TES, this solution also potentiates ACh-induced muscle contraction. However, at lower SA-TES concentrations, it decreases the tension induced by ACh.

From the beginning of this work, a marked discrepancy was observed between the known chemical properties of SA and the experimental results. Thus, although SA in aqueous solution undergoes hydrolysis at a relatively high rate (Eberson, 1964), the pharmacological properties of SA dissolved in TES-buffered Ringer solution are maintained during long experimental periods. In addition, its hydrolysis product, succinic acid, is pharmacologically inactive at the concentrations used. Furthermore, we observed that SA exerts no significant pharmacological actions if dissolved in Ringer solution buffered with phosphate.

These observations suggested that the compound responsible for the pharmacological effects described by Escalona de Motta & del Castillo (1977) was not SA itself but a product or products formed by the interaction between SA and TES. Indeed, we have found that SA succinylates TES forming several active compounds which will be referred to provisionally as succinyl-TES derivatives. The activity of these compounds is dependent on the ACh concentration. The most abundant derivative potentiates ACh-induced contraction at high ACh dose, but strongly inhibits it at low ACh dose. The remaining derivatives were mostly inhibitory in the conditions used here.

Some of these results have been reported briefly (Ferchmin *et al.*, 1982; Escalona de Motta *et al.*, 1982; Eterović *et al.*, 1983). The present paper describes some of the effects of SA in TES-buffered Ringer solution as well as experiments designed to characterize the nature of the active compounds.

Methods

Biological preparations and saline solutions

Physiological experiments were performed on sartorius and rectus abdominus muscles and on sciatic-sartorius preparations obtained from small frogs (*Rana pipiens*). For intracellular recordings, sartorius muscle preparations were attached with insect pins passing through peripheral connective tissue to a layer of Sylgard plastic (Dow Corning) on small Petri dishes. In other experiments, surface potentials were recorded from these muscles using the liquid electrode technique described below.

Recti abdomini were placed in glass chambers perfused with aerated normal frog Ringer solution at room temperature. The lower end of each preparation was tied to a fixed support and the upper end was attached by a silk thread to a force transducer to measure the tension developed by the muscles in response to agonists. Muscle tension was recorded under partially isometric conditions as pen displacements on a chart recorder.

The normal frog Ringer solution employed had the following ionic composition (mM): 113 Na^+, 2.1 K^+, 1.87 Ca^{2+}; all as chloride salts; 5 mM TES was added to maintain the pH at 7.2 with NaOH. SA was dissolved in this saline solution to obtain final concentrations of 1 or 2 mM (SA-TES). Neostigmine (3 μM) or physostigmine (10 μM) were added in some experiments to block the muscle acetylcholinesterase (AChE).

Electrical techniques

Intracellular recordings of endplate potentials (e.p.ps), miniature e.p.ps (m.e.p.ps), and ACh-induced potentials was carried out with conventional 3 M KCl-filled glass micropipettes. Endplate currents (e.p.cs) and miniature e.p.cs (m.e.p.cs) were recorded with a two microelectrode voltage clamp system similar to that described by Connor & Stevens (1971). The potential difference across the endplate membrane was clamped routinely to − 100 mV. The input resistance of muscle fibres (R_{eff}) was measured by injecting hyperpolarizing current pulses through a microelectrode, while recording the resulting anelectrotonic potentials with a second one placed less than 100 μm away. R_{eff} was estimated directly from the V/I ratio.

The depolarizing action of ACh on the sartorius muscle was studied either by iontophoretic application or by adding ACh to the bath. Iontophoresis was carried out with double-barrelled microelectrodes; one barrel filled with 1 M ACh and the other with a 50 mM solution of SA in 10 mM NaCl, the pH adjusted to 6.0 with TES and NaOH. The drugs were ejected with positive (i.e. outward) current pulses.

The depolarizing action of bath-applied ACh was measured recording the potential distribution on the muscle surface using a liquid electrode (Fatt, 1950). The muscle was fixed, pelvic end uppermost, in a 50 ml vertical chamber provided with two cotton wick electrodes connected by Ringer-agar bridges to calomel half-cells. One electrode made contact with the nerve-free pelvic end of the muscle, the other was

placed in the chamber below its tibial end. The chamber could be filled in about 10 s and emptied in 35 s. The liquid-air interphase acted as a moving electrode which scanned the potential of the muscle surface with reference to the nerve-free pelvic end. The potential difference was recorded at sensitivities of 0.5 to 5.0 mV cm^{-1} on a paper chart moving at 0.25 mm s^{-1}.

In normal Ringer the undamaged muscle surface was almost isopotential. In the presence of an agonist the lower two thirds of the muscle became negative with reference to the nerve-free pelvic end, showing two or more peaks which correspond to the regions of highest endplate density (see Figure 2). The amplitude of the highest peak was taken as a measure of the depolarization.

To obtain dose-response curves the muscle surface potential was scanned only once ('single scan' technique) immediately after the solution reached the pelvic end of the muscle (cf. Rang & Ritter, 1970). In other experiments we followed the amplitude of the response as a function of time (continuous scanning technique). After recording the baseline, the paper chart was set in motion as soon as the agonist solution reached the upper end of the muscle ($t = 0$). From then on the muscle was scanned about once per minute with agonist-containing solution. Although prolonged exposure to the agonist may lead to anion shifts and desensitization that distort the true time course of the response (Jenkinson & Terrar, 1973), this procedure was useful to detect changes in the rate of both depolarization and repolarization of the muscle.

To record endplate potentials (e.p.ps) in sciatic-sartorius preparations partially blocked with 3 μM (+)-tubocurarine (Tc), the same chamber was provided with two nerve stimulation electrodes. While stimulating with square pulses at a frequency of 0.1 Hz, the muscle surface was scanned slowly to determine the level of highest e.p.p. amplitude. This level was measured on a scale and easily re-established after changing solutions in the chamber.

Electromechanical uncoupling

In experiments involving motor nerve stimulation in the absence of Tc, the mechanical activity of the muscle was blocked by uncoupling the excitation-contraction mechanism with formamide (Fmd) (del Castillo & Escalona de Motta, 1978). To do so, the muscles were immersed in Ringer containing either 1.5 or 2.0 M Fmd. Contractility was tested by stimulating the sciatic nerve at intervals of 20 to 30 s. As soon as the twitching disappeared, the uncoupled preparation was brought back to normal Ringer solution.

Chemical techniques

Synthesis of succinyl-TES derivatives To 1 mmol of dry, powdered SA 0.05 mCi of radiolabelled SA ([^{14}C]-SA) in 1 ml toluene was added. After evaporating the toluene, the mixture of [^{14}C]-SA and unlabelled SA (sp.act. 50 μCi $mmol^{-1}$) was scraped from the tube and added stepwise to 500 ml of 10 mM TES pH 6.5. Throughout the process the solution was stirred and the pH was maintained at 6.5 by addition of NaOH. The resulting solution (SA-TES solution) was lyophylized and redissolved in a small volume of distilled water.

Ion-exchange chromatography SA-TES solution was absorbed on DEAE-cellulose packed into a 21 × 5 cm column equilibrated with distilled water and eluted as follows: (a) distilled water, 500 ml; (b) 10 mM ammonium acetate pH 5, 500 ml; (c) a concentration gradient from 100 mM to 200 mM ammonium acetate pH 5, 500 ml of each; (d) 200 mM ammonium acetate pH 5, 500 ml; (e) 500 mM ammonium acetate pH 5, 500 ml. The flow rate was 3 ml per min; fractions of 10 ml were collected. For radioactivity determination, one 0.5 ml aliquot was taken from each fraction, mixed with 2.5 ml Scintiverse and counted in a liquid scintillation counter. To detect unreacted TES and any other unlabelled substances, two 5 μl aliquots from each fraction were spotted onto Whatman 3MM chromatography paper; one was sprayed with bromophenol blue, the other with phenol red (see section on paper chromatography). The fractions containing TES or a radioactive peak were pooled, lyophilized and redissolved in a small volume of distilled water.

To eliminate ammonium acetate each pool was absorbed onto a 24 × 3 cm column of Dowex 50W, eluted with distilled water, lyophilized and redissolved in a small volume of distilled water.

Determination of ammonia The concentration of ammonium ions was determined using Sigma Kit No. 170-UV. In our system this method was very specific while the Nessler's reagent (Dawson *et al.*, 1969 p. 619) cross-reacted with TES.

Paper chromatography The procedure was similar to that described by Stepka (1957). For each peak, chromatograms were done at low loading to increase resolution and at high loading to detect minor impurities. Samples containing at least 500 c.p.m. in no more than 200 μl were spotted on pre-washed Whatman 3MM chromatography paper. Descending chromatograms were developed with *n*-butanol : acetic acid : water (100:22:50) for 4–8 h (15 to 25 cm). Spots of acids or bases were developed by spraying the chromatogram with the pH indicators

bromophenol blue or phenol red (0.044% in 95% ethanol); after bromophenol blue, the chromatogram was exposed to acetic acid fumes to lighten the background (Dawson *et al.*, 1969, p. 514). Bromophenol blue produced blue spots with TES and Na^+ or NH_4^+ ions, and a yellow spot with succinic acid. Phenol red differentiated TES (yellow) from Na^+ or NH_4^+ (red).

To determine the radioactive spots the chromatograms were cut into 1 cm wide strips. Each strip was placed in a minivial, eluted with 0.5 ml distilled water and counted in a liquid scintillation counter upon addition of 2.5 ml of Scintiverse.

Drugs and chemicals

The following drugs and chemicals were dissolved in the Ringer solution in amounts sufficient to give the final concentrations indicated: ACh, carbamylcholine (CCh), nicotine, neostigmine and Tc from Sigma; Fmd from Fisher; SA from Aldrich; tetramethylammonium and benzyltrimethyl ammonium from ICN Biochemicals; decamethonium from Burroughs-Wellcome.

Labelled succinic anhydride, [1,4-^{14}C]SA, specific activity 111 mCi mmol^{-1}, was obtained from New England Nuclear. Succinic acid, physostigmine, DEAE-cellulose (diethyl aminoethyl cellulose, medium mesh, capacity 0.88 mEq g^{-1}), and the reagents for determination of ammonia (Kit No. 170), were from Sigma. Dowex 50W-X8, ionic form H^+, 20–50 mesh, was from J.T. Baker Chemical, Co. The remaining chemicals and materials (acetic acid glacial, *n*-butanol, bromophenol blue, phenol red, am-

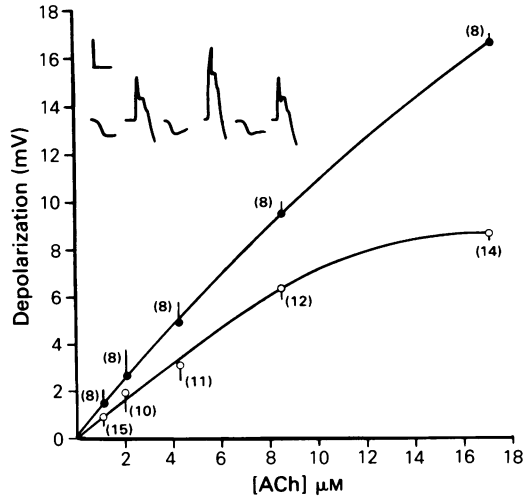

Figure 2 Depolarization of frog sartorius muscles by acetylcholine (ACh) measured using the 'single scan' technique. Points represent the average amplitude of the depolarization (in mV) elicited by 1 to 17 μM ACh, in normal Ringer solution (open circles) and in 1 mM SA-TES Ringer solution (solid circles). Number of measurements are in parentheses. Vertical lines indicate s.d. for each point. Inset shows three 'single scan' records obtained from a muscle exposed to 10 μM ACh: the first in normal Ringer, the second in presence of 1 mM SA-TES Ringer and the third after return to normal Ringer solution. The baseline record of the muscle surface potential is shown before each test scan. Inset calibrations are: vertical, 1 mV; horizontal, 30 s.

Figure 1 Potentiating action of SA-TES on amplitude of iontophoretic acetylcholine (ACh) potentials. A double-barrelled micropipette filled with ACh and SA-TES was used (see Methods). Upper trace in each record shows the potential across the endplate membrane. Lower trace monitors the current flowing through the pipette. ACh pulses of equal amplitude were delivered in (a) and (b). In (b) the amplitude of the resulting ACh-potential elicited while a positive current was made to flow through the SA-TES barrel was greatly enhanced. A steady depolarization also occurred probably due to the enhancement of a small continuous depolarization produced by ACh leaking between the applied pulses.

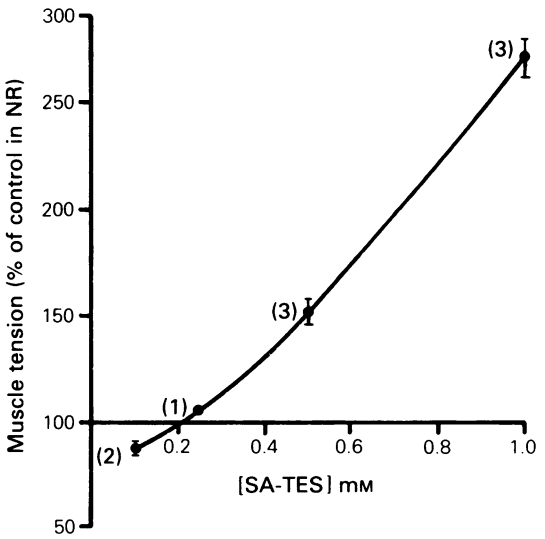

Figure 3 Effect of SA-TES dilutions on the tension developed by a frog rectus abdominis muscle in response to 0.8 μM acetylcholine (ACh). The 1 mM SA-TES solution, prepared as described in Methods, was diluted with normal Ringer solution to give the final SA concentrations indicated. Muscle tension was measured as described in Methods and is expressed as a percentage of the control tension elicited by ACh in normal Ringer. Physostigmine (10 μM) was present throughout the experiments. Number of measurements are in parentheses. Vertical lines indicate s.d.

monium hydroxide, Scintiverse (Universal Liquid Scintillation Cocktail) and Whatman 3MM chromatography paper) were obtained from Fisher Chemical Co.

Results

SA-TES potentiates the action of externally applied choline esters

(a) *Iontophoretic ACh-potentials* Working with twin pipettes filled with SA-TES and ACh it was observed that small amounts of SA-TES markedly potentiated the depolarizing action of ACh. If a positive (outward) current, was made to flow through the SA-TES barrel while delivering constant ACh pulses through the other barrel, the amplitude of the resulting ACh potentials was seen to increase, as shown in Figure 1.

(b) *Bath-applied ACh* The effect of ACh on the surface potential of sartorius muscles was potentiated in the presence of 1 mM SA-TES. The effect of this concentration of SA-TES on the responses elicited by ACh is shown in Figure 2. The depolarization induced by the highest ACh concentration used increased approximately by a factor of 2 in SA-TES.

The SA-TES solution also potentiated the tension developed by rectus abdominus muscles in response to ACh. This effect was dependent on the concentration of SA-TES. As the SA-TES solution was diluted with normal Ringer, the potentiation decreased. At the smallest concentration tested, a small inhibition was observed (Figure 3).

Table 1 Average amplitude of frog motor end-plate potentials (mV) in SA-TES solutions

	SA (mM)	*Control*	*Experimental*
e.p.ps	0.1	14.1 ± 0.5 (51)	13.0 ± 0.1 (21)
	0.5	13.3 ± 0.3 (28)	13.9 ± 0.5 (49)
	1.0	12.8 ± 0.2 (19)	14.1 ± 0.4 (45)
m.e.p.ps	1.0	0.6 ± 0.1 (518)	0.6 ± 0.1 (700)

(c) *Nerve-released ACh* In contrast to the effects of SA-TES on the action of externally applied ACh, its influence on neurally released ACh was minimal. SA 30 μM in TES buffered saline, which exerted a definite potentiation on the depolarizing action of bath-applied ACh, had no appreciable effect on the amplitude of the e.p.ps. Increasing the SA concentration to 1 mM resulted only in a slight increase in the average size of e.p.ps while no changes were observed on the amplitude of m.e.p.ps (Table 1). Neither the amplitude of the m.e.p.cs (Table 2), nor the half-decay times of m.e.p.cs or of single e.p.c. (Table 3) were affected.

SA-TES changes the time course of the responses elicited by acetylcholine

In addition to its potentiating action on the ACh-induced depolarizations, SA-TES increased both the

Table 2 Average amplitude of miniature end-plate currents in the presence and absence of 1 mM SA in TES buffered saline

	Normal Ringer	*SA*
Exp. 1	6.50 ± 1.15 nA (257)	5.80 ± 1.27 nA (85)
Exp. 2	5.02 ± 0.89 nA (101)	4.99 ± 1.13 nA (157)

Values expressed are averages ± s.d.; numbers of measurements in parentheses.

Table 3 Average half-decay times of miniature endplate currents and single endplate currents in the presence and absence of 1 mM SA in TES buffered saline

	Normal Ringer	*SA-TES* (1 mM)
m.e.p.cs	2.91 ± 0.70 ms* (28)	3.34 ± 0.80 ms* (47)
	3.25 ± 0.35 ms* (10)	3.91 ± 0.40 ms** (10)
e.p.cs	2.18 ± 0.40 ms*** (12)	2.23 ± 0.35 ms*** (28)

*m.e.p.cs were measured by extracellular focal recording.
**recorded with voltage clamp in a muscle uncoupled with formamide.
***recorded with voltage clamp in a muscle partially blocked with (+)-tubocurarine.
Values expressed are average ± s.d.; numbers of measurements in parentheses.

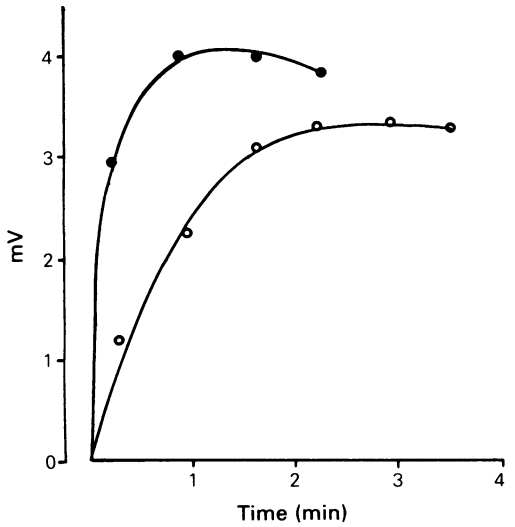

Figure 4 Influence of SA-TES on the depolarizations induced by acetylcholine (ACh) on a neostigmine-treated sartorius muscle, recorded by the continuous scanning technique. The muscle was exposed to neostigmine 3 μM for 30 min before and throughout the experiment. Lower curve (○) shows the time course of the response to 10 μM ACh. Upper curve (●) shows the response to the same agonist concentration in the presence of 1 mM SA-TES. Notice faster rate of depolarization, increased amplitude of the response and faster rate of desensitization.

rates of depolarization when ACh was introduced into the bath, and the rate of repolarization when it was washed out. These effects were particularly marked in neostigmine-treated muscles.

Figure 4 shows a typical experiment in which SA-TES accelerated both the onset of the depolarization elicited by ACh (10 μM) and its rate of decay by desensitization. In four such experiments the average rise time of the depolarization was 3.66 ± 0.76 min (s.d.) in control conditions. This was reduced to 2.18 ± 0.5 min (s.d.) in the presence of SA-TES. Figure 5 shows that SA-TES also increased the rate of repolarization of the muscle when ACh was removed from the bath. The half-time of repolarization observed in the continuous presence of SA-TES was approximately 70 s as compared to 240 s in the control series.

SA-TES was seen to exert similar though less pronounced effects on the rates of depolarization and repolarization in muscles not exposed to neostigmine and in preparations where CCh was used as the agonist (see Figure 6).

SA-TES action in muscle is specific for choline esters

Using the 'single scan' technique (see Methods) we were unable to demonstrate a potentiating effect of SA-TES on the depolarizations elicited by tetramethylammonium, nicotine, benzyltrimethylammonium and decamethonium, all of which lack an ester group. After addition of 1 mM SA to the TES buffered Ringer, the depolarizations elicited by these agonists were either equal or slightly smaller than the controls (Figure 7). This suggests that the potentiating effect of SA-TES on the responses to ACh and CCh is related to the presence of an ester (O-CO-) group in these molecules. In contrast to the results obtained using ACh (with and without neostigmine) and CCh, the time course of the responses induced by these other agonists did not change appreciably in the presence of SA-TES.

SA-TES does not inhibit acetylcholinesterase nor alter R_{eff}

The potentiation of the depolarizing response to ACh observed in muscles exposed to 3 μM neostigmine for 30 min (see Figure 5) suggests that inhibition of AChE activity is not the mode of action of SA-TES. In addition, SA-TES does not affect the R_{eff} of the muscle membrane. In normal Ringer the average R_{eff} measured for 20 identified muscle fibres was $4.2 \times 10^5 \pm 1.5 \times 10^5 \Omega$ (s.d.) while in the SA-TES solution the same fibres had an average R_{eff} of $4.1 \times 10^5 \pm 1.6 \times 10^5 \Omega$ (s.d.), indicating that an increase in membrane resistance is not involved in the observed potentiation of the responses to ACh.

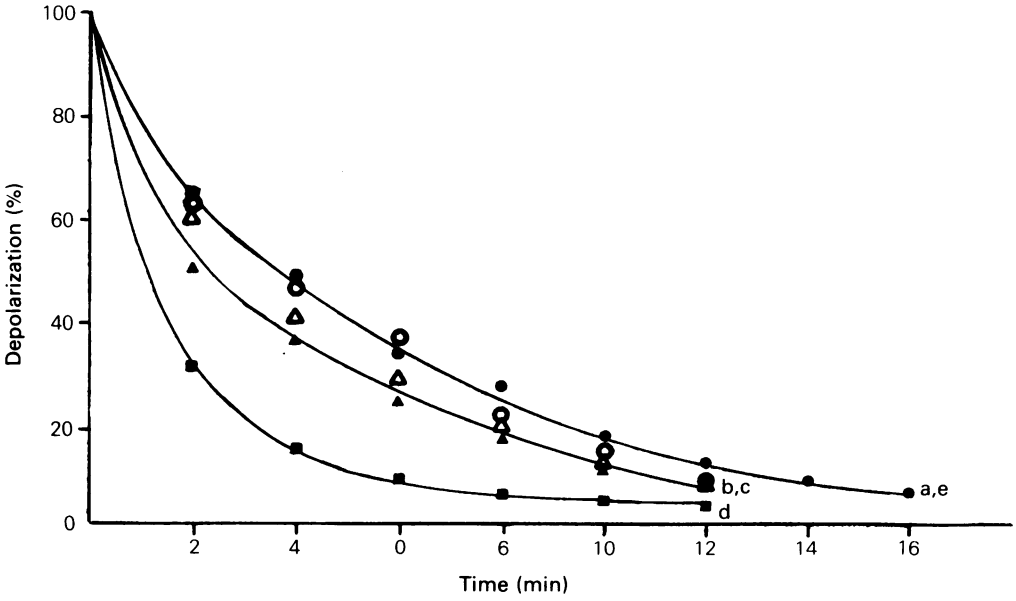

Figure 5 Time course of repolarization of a neostigmine-treated sartorius when acetylcholine (ACh) solution was washed out. Data were obtained from an experiment similar to that illustrated in Figure 4. The muscle was pre-incubated in neostigmine 3 μM for 30 min, all the solutions used contained the same concentration of this inhibitor. After recording the baseline, 10 μM ACh was introduced into the chamber, a single scan was made 50 s later and the ACh solution was washed out immediately. Afterwards, scans were performed every 2 min, washing the muscle twice between scans. The time course of the repolarizations in series (a) and (e) was drawn as a single curve. The same was done for series (b) and (c). Series (a) and (e) were obtained in normal Ringer at the beginning and at the end of the experiment (open and solid circles respectively). In series (b) (solid triangles) 1 mM SA-TES was present in the ACh solution but the muscle was washed with normal Ringer. In series (c) (open triangles) only the Ringer used to wash the muscle contained 1 mM SA-TES. Finally, in series (d) (solid squares) the muscle was continuously exposed to 1 mM SA-TES. Muscle repolarization is expressed as percentage remaining of the maximal depolarization induced by ACh.

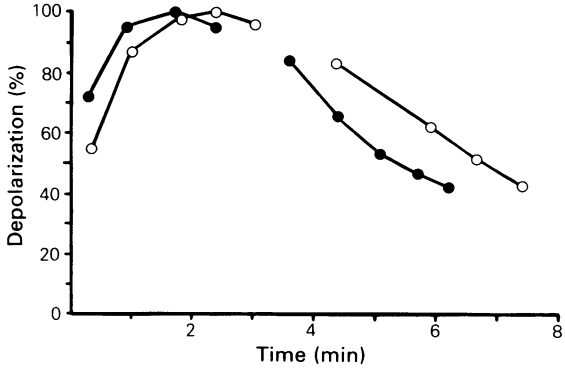

Figure 6 Time course of the depolarization elicited by carbachol 20 μM on a sartorius muscle. Recorded by the continuous scanning technique in normal Ringer (○) and in 1 mM SA-TES (●). Depolarization is expressed as a percentage of maximal response. Interruption in the graphs indicates period of washing with normal Ringer. Curves show rate of muscle repolarization.

SA-TES action is probably mediated by succinyl-TES derivatives

As already mentioned, although SA in solution undergoes hydrolysis at a relatively high rate, we have observed that the SA-TES solution maintains its potentiating activity during long experimental periods. Neither lyophylization nor storage at − 15°C for three weeks decreased the activity of this solution. Moreover, addition of 1 mM succinate to a TES buffered saline had no influence on the depolarizing action of ACh on sartorius muscles. Similarly, addition of 1 mM SA to a Ringer solution buffered with phosphate did not result in a potentiation of the muscle responses to ACh (Table 4).

Since SA is a potent succinylating agent (Klapper & Klotz, 1972) we tried to determine if succinylated derivatives of TES were being formed in the SA-TES solution. The following results show that several succinyl-TES derivatives are indeed synthesized under our experimental conditions and that these compounds are pharmacologically active.

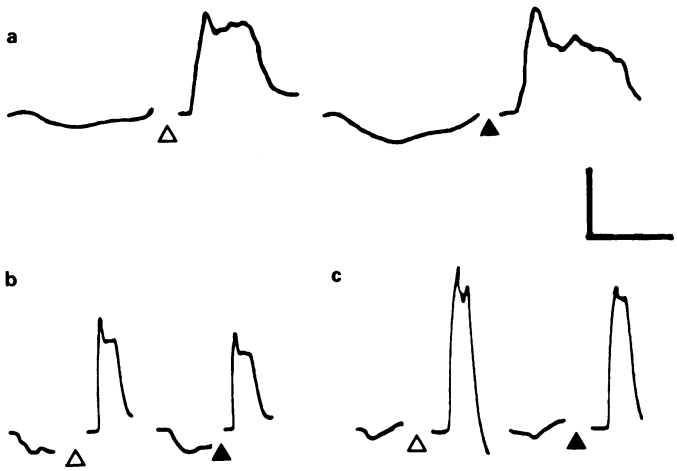

Figure 7 Depolarizations evoked in three different muscles by: (a) nicotine 10 μM; (b) methyltrimethylammonium 50 μM and (c) benzyltrimethylammonium 40 μM. Recorded with the 'single scan' method. The first and third record in each series are baselines recorded in normal Ringer. Open triangles indicate addition of agonist to the bath in normal Ringer, filled triangles in the presence of 1 mM SA-TES. Calibrations: Vertical, (a) 0.5 mV, (b) and (c) 1 mV; Horizontal, (a) 0.5 min; (b) and (c) 1 min.

(a) *Isolation of succinyl-TES derivatives by ion exchange chromatography* Figure 8 illustrates the solution pattern of a [^{14}C]-SA-TES solution from a DEAE-cellulose column. Eight peaks were separated. Peak 1 contained TES and Na^+, and no radioactivity; it was detected in fractions 25 to 33 with bromophenol blue, which produced blue spots, and phenol red which produced red spots (not shown). All the remaining peaks contained radiolabelled compounds. Total recovery of radioac-

Table 4 Muscle response to acetylcholine (ACh) in presence of succinic anhydride (SA) in buffer TES, sodium succinate in buffer TES, or SA in buffer phosphate

(a) *Depolarization of sartorius muscle by 10 μM ACh*

	mV	*% difference*
ACh/TES	1.3	30.8%
ACh + SA/TES	1.7	
ACh/TES	1.0	
ACh + succinate/TES	1.0	0.0%

(b) *Contraction of rectus abdominus muscle*

	7 μM ACh		*14 μM ACh*	
	g	*% diff*	*g*	*% diff*
ACh/TES	0.94	26%	1.38	41%
ACh + SA/TES	1.18		1.95	
ACh/Phosphate	0.93	1%	1.68	2%
ACh + SA/Phosphate	0.94		1.72	
ACh/TES	0.84	43%	1.71	14%
ACh + SA/TES	1.20		1.95	

The depolarizing responses were recorded with the continuous scanning technique. Muscle tension was measured as explained in 'Biological preparations and saline solutions'. Frog Ringer solution was buffered with 5 mM TES pH 7.3 or 1 mM phosphate buffer pH 7.3. AChE was not inhibited in these experiments. The concentration of SA and sodium succinate was 1 mM.

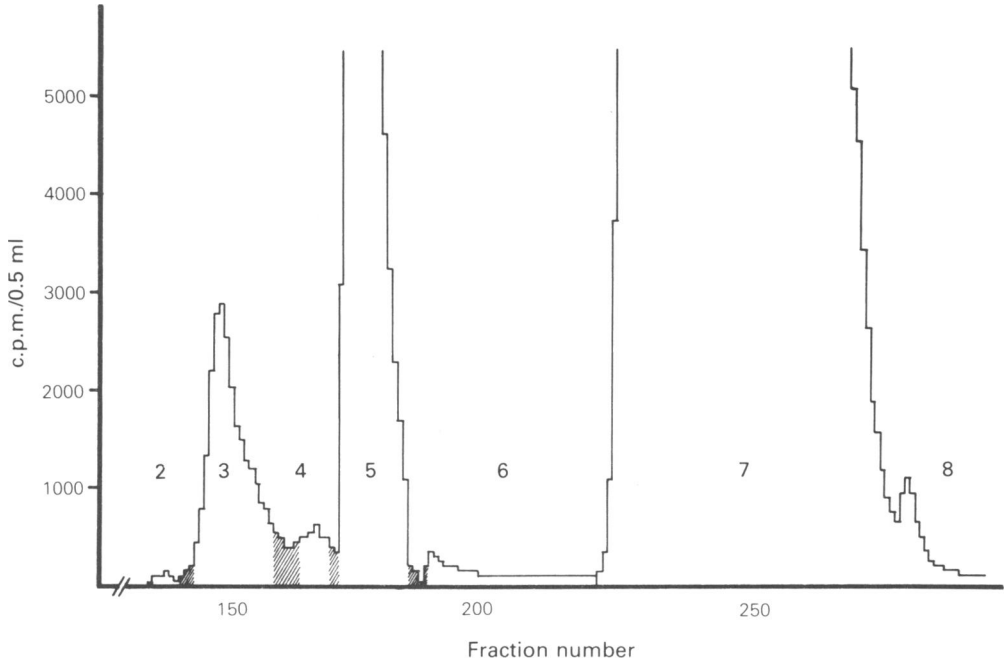

Figure 8 Chromatography of the SA-TES solution on DEAE-cellulose. Fractions belonging to a peak were pooled as indicated; dashed areas were discarded to decrease cross-contamination between peaks.

tivity from the column was over 97% (Table 5). The bulk of radioactivity was eluted in peak 7, which was shown by paper chromatography to be succinic acid (see below). The remaining 15% of [^{14}C]-SA was incorporated into derivatives, six of which were separated by the DEAE-cellulose column. Their amounts varied from 0.25% for peak 2 to 11% for peak 5. The amount of [^{14}C]-SA incorporated as well as the ratios between the derivatives formed depended critically on the pH of the SA-TES solution during the succinylation. When the pH was maintained at 7.5, instead of 6.5, the amount of radioactivity incorporated increased to 30% with almost exclusive formation of peak 5.

To concentrate the fractions each peak was lyophilized. This procedure also eliminated most of the acetic acid but not the ammonium acetate originated by the elution buffers. Since ammonia could interfere with physiological assays it was removed by passing each peak through a column of Dowex 50W, a cation exchanger. The recoveries from these columns were above 75% except for peak 4 (10%). A second lyophilization step eliminated more acetic acid; the recoveries were above 87% for all the peaks. Each peak was subsequently redissolved in 5 to 50 ml of distilled water. The concentration of ammonium ion at this stage was less than 100 μM for all peaks.

Table 5 Fractionation of SA-TES solution on DEAE-cellulose

Peak No.	*c.p.m. recovered in each peak*	*% recovery of radioactivity in each peak*	*Estimated concentration in the original SA-TES solution* (μM)
1	0	0.00	5000
2	10,700	0.025	0.26
3	469,300	1.11	11.4
4	64,550	0.15	1.5
5	4,721,300	11.16	114.7
6	62,150	0.15	1.5
7	35,678,550	84.31	866.8
8	151,680	0.36	3.7
Total	41,159,610	97.27	

Table 6 Paper chromatography of peaks eluted from DEAE-cellulose column

Sample	R_F[a] *Mean*	*Range*	*% radioactivity*[b] *in the major spot*	*Colour* *Bromophenol blue*	*Phenol red*
Standards					
TES	0.24	(0.19–0.27)		Blue	Yellow
Na^+ or NH_4^+	0.35	(0.30–0.37)		Blue	Red
Succinic acid	0.76	(0.73–0.78)		Yellow	Yellow
Peak No.					
1	0.25	(0.24–0.26)[c]		Blue	Yellow
	0.34	(0.33–0.34)[d]		Blue	Red
2	0.60	(0.59–0.60)	100		
3	0.62	(0.61–0.62)	73		
4	0.92	(0.92–0.92)	93		
5	0.36	(0.33–0.38)	97		
6	0.30	(0.27–0.32)	73		
7	0.78	(0.76–0.81)[e]	100	Yellow	Yellow
8	0.34	(0.33–0.34)[f]	33		
	0.55	(0.54–0.56)[g]	66		

Only the major spots are shown. A major spot is one containing more than 20% of radioactivity recovered in that chromatogram.
[a] R_F is the distance from origin to the middle of the strip with highest radioactivity for that spot, over the distance from origin to the solvent front.
[b] 100% is the total radioactivity recovered from that chromatogram.
[c] TES; [d] Na^+; [e] succinic acid; [f] 8a; [g] 8b.

(b) *Identification of succinyl-TES derivatives by paper chromatography* The results obtained from paper chromatograms of peak 1 to 8 are summarized in Table 6. By comparing the R_F values of standards with those of peaks 1 and 7 we inferred that these peaks contained TES and Na^+, and succinic acid respectively (see legend to Table 6). Colour reactions with bromophenol blue and phenol red confirmed this assumption. For the remaining peaks (2 to 6 and 8) the radioactive spots were not associated with colours, probably because the amounts used were not sufficient. The R_F values of the remaining peaks differed from both TES and succinic acid and covered a range from 0.30 (peak 6) to 0.92 (peak 4). As for the purity of the succinyl-TES derivatives, peaks 2, 4 and 5 contained more than 90% of radioactivity in one spot, and peaks 3 and 6 more than 70%. Peak 8 contained two radiolabelled compounds; 8a and 8b. Thus, a total of seven succinyl-TES derivatives were identified.

Figure 9 illustrates two main characteristics of these compounds: (1) *Cross-contamination.* For those peaks with widely different R_F values, such as 3, 4 and 5 on one hand and 6, 7 and 8 on the other, it is obvious that cross-contamination with neighbouring peaks cannot be serious. For peaks with similar R_F values (2 and 3, 5 and 6) no such a conclusion can be drawn. (2) *Hydrolysis.* The shaded rectangles in Figure 9 indicate the range of R_F values for succinic acid. For all peaks except peak 7, very little radioactivity is seen in this region, indicating that little hydrolysis has occurred at this stage. However, after repeated freezing and thawing, all peaks except peak 5, contained a considerable proportion of succinic acid.

Ammonium ion arising from the elution buffers for DEAE-cellulose column was clearly visible in these chromatograms, but not so in chromatograms done on samples eluted from Dowex 50W column (data not shown).

(c) *Pharmacological actions of succinyl-TES derivatives* As mentioned previously, the SA-TES solution increases the strength of muscle contraction induced by ACh. In Figure 10, we compare this activity of the SA-TES solution with the activity of its isolated components, peaks 1 to 8. The components were tested at concentrations similar to those which they had in the original SA-TES solution. Peak 1, which contained TES and Na, and peak 7, which contained succinic acid, had no effect on muscle contraction and are not shown in the figure. The most abundant succinyl-TES derivative, peak 5, potentiated ACh action at intermediate or high ACh concentrations, but was strongly inhibitory at low ACh concentrations. This compound was inactive at concentrations of 100 μM or less. The remaining derivatives were

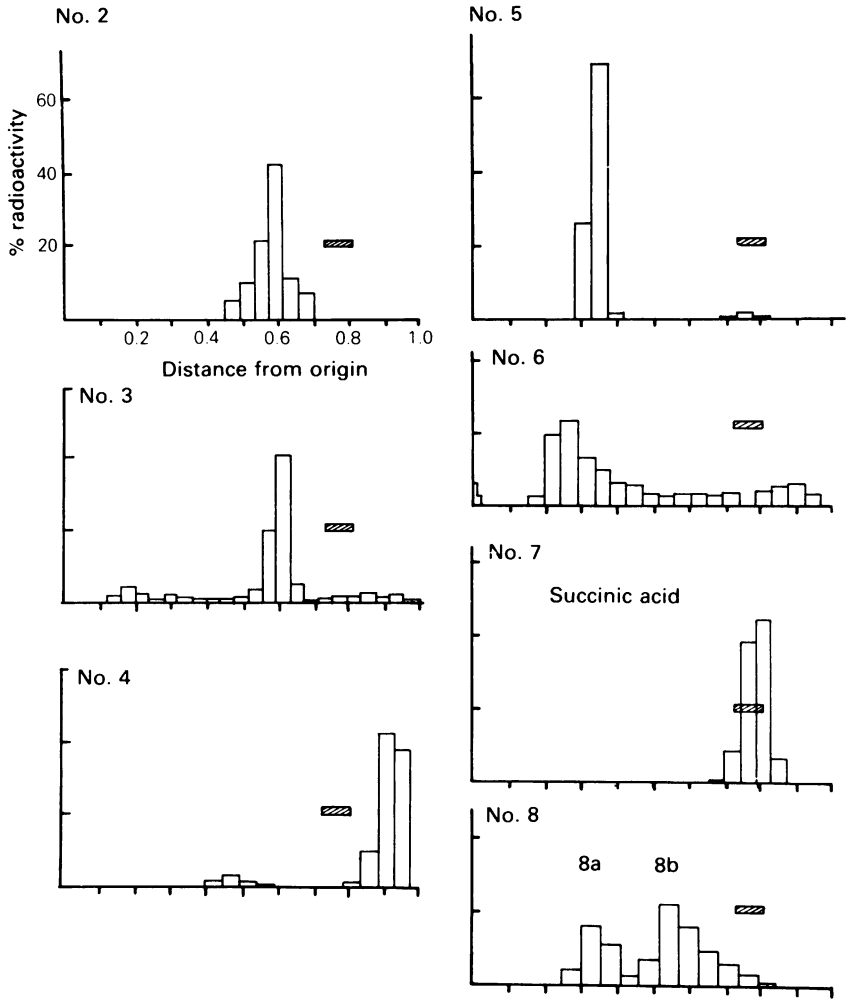

Figure 9 Example of results from paper chromatograms for peaks 2 to 8. The distance from the origin to the solvent front was normalized to 1; the amount of radioactivity recovered from each chromatogram was taken as 100%. The dashed rectangle indicates the range where the succinic acid was localized.

active at concentrations between 1 and 15 μM. Their action was mostly inhibitory, following a general tendency toward less inhibition or activation at higher ACh concentrations.

Discussion

Effects of the SA-TES solution

The SA-TES solution exerts interesting actions on the chemical sensitivity of the endplate membrane. It potentiates the strength of ACh-induced muscle contraction. It increases the amplitude of ACh-potentials elicited by iontophoresis and the depolarizing action of bath-applied ACh. It has the unusual property of increasing the rates of both the depolarization when ACh is added to the bath and the repolarization when this agonist is washed out. These effects are particularly obvious in neostigmine-treated preparations.

In addition, the presence of SA-TES suppresses the more-than-linear summation of small doses of electrophoretically applied ACh (Escalona de Motta & del Castillo, 1977). However, SA-TES exerts only a slight influence on the effects of nerve-released ACh. The average amplitude of the e.p.ps is only increased by 10% and the size of the m.e.p.ps re-

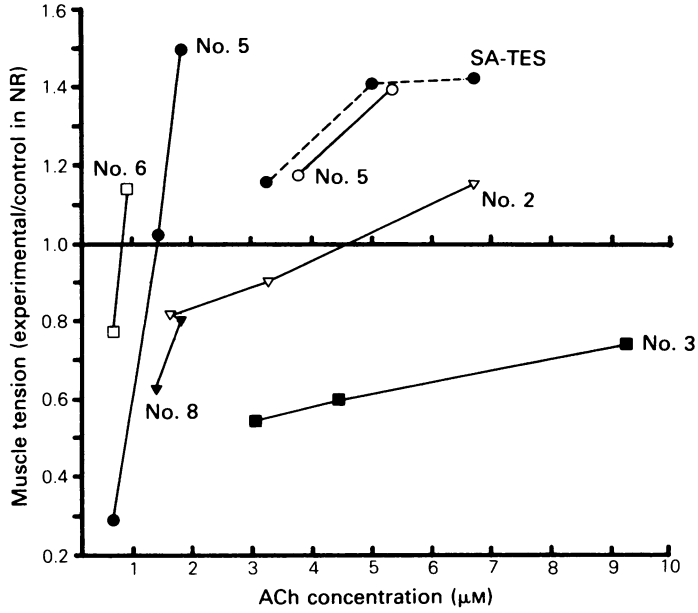

Figure 10 Effect of SA-TES solution and its isolated components on acetylcholine (ACh)-induced muscle contraction. Tension developed by frog rectus abdominus muscles in response to bath-applied ACh was measured as described in Methods. Control values in normal Ringer (NR) were obtained by averaging 2–4 contractions obtained before application of SA-TES or its components. Eight muscles were used. The preparation of the SA-TES solution and its separation into peaks 1–8 is also described in Methods. Each peak was dissolved in normal Ringer pH 7.2, which also contained physostigmine 10^{-5} M. The concentrations of the succinyl-TES derivatives (Peaks 2–6 and 8) and that of succinic acid (Peak 7) were calculated from the specific activity of [^{14}C]-SA and the amount of radioactivity associated with each compound. In all cases it was assumed that the compounds were monosuccinylated, thus the molar concentration of compounds containing more than one succinyl residue were overestimated. The concentrations of succinyl-TES derivatives were: No. 2, 1 μM (▽); No. 3, 15 μM (■); No. 5, 150 μM (●) and 350 μM (○); No. 6, 12 μM (□); No. 8, 3 μM (▼).

mains unaltered. Furthermore, SA-TES does not change the half-decay times of the m.e.p.cs and of single e.p.cs. SA-TES exerts similar effects on the actions of CCh, yet it fails to potentiate the depolarizations induced by non-esterase agonists and does not accelerate their time course.

These actions can be discussed in terms of two hypotheses:

(1) *Saturating effect of SA-TES on a diffusion barrier* The marked effects of SA-TES on the time course of the onset and decay of the depolarizations induced by ACh and CCh led us to assume that the principal action of SA-TES would be to enhance the rate of diffusion of choline esters in both directions, between the receptors and the bulk of the bath solution (Escalona de Motta & del Castillo, 1977). As the effects of SA-TES are fully reversible, it seemed unlikely that this compound would remove a structural diffusional barrier. It was more plausible to assume that SA-TES accelerates ACh diffusion by preventing the transmitter from binding to sites that under normal conditions interact with it and hinder its movement.

This hypothesis explains most of the observed effects, namely: (a) The potentiating action of SA-TES on electrophoretic ACh potentials and on the effects of bath-applied ACh and CCh. (b) The specificity of the effects of SA-TES for choline esters; as the only common chemical feature of ACh and SA-TES is the carbonyl group, molecules that lack carbonyl groups would not bind to the barrier, and (c) the suppression by SA-TES of the more-than-linear summation of small ACh-potentials (Escalona de Motta & del Castillo, 1977). As one possible explanation of the latter effect, Katz & Thesleff (1957) had proposed the existence at the myoneural junction of groups with affinity for ACh but with no depolarizing power. According to this hypothesis SA-TES would occupy those sites blocking the summation effect.

From our observations, two major objections could be raised against the 'diffusion hypothesis'. The first was based on the view that a change in the rate of

diffusion should influence only the time needed for a concentration to reach equilibrium but not its steady state value. Therefore, SA-TES should be expected to potentiate the depolarization recorded with the 'single scan' technique but not the absolute values recorded in 'continuous scanning' experiments, as shown in Figure 5. This objection is based on the assumption that the amplitude of a pharmacological response is determined solely by the drug concentration at equilibrium. Yet, such amplitude is also a function of the rate at which the agonist concentration at the receptor increases. Thus, the response to the highest drug concentration in a cumulative dose-response curve (Ariens *et al.*, 1964) is smaller than the response elicited by the same concentration when applied alone.

The second objection was the lack of influence of SA-TES on the effects of neurally released ACh. If the groups that bind both ACh and SA-TES were close to the receptors, SA-TES would be expected to decrease the half-decay times of both e.p.cs and m.e.p.cs, as low doses of Tc do, by decreasing the number of receptors available for binding (Katz & Miledi, 1973).

However, the lack of effect of SA-TES on these currents may only indicate that the diffusional barrier saturated by SA-TES is not located in the synaptic space but at some distance away from it. An electrophysiological 'blind space' would thus exist, as only the ACh concentration at the receptor level can be detected. When ACh is added to the bath, the entire space within the barrier will be filled with a homogeneous concentration of the transmitter. In contrast, the physiological release of ACh will build up highly localized focal concentrations which will promptly dissipate by diffusion into the 'blind space'.

Thus, changes in the effectiveness of the barrier will be reflected in the time course of the synaptic currents only in neostigmine-treated preparations. In these, when the transmitter is released at a high rate it may build up a significant concentration in the space within the barrier. This idea is supported by the observation that SA-TES significantly decreases the half-decay times of e.p.cs elicited by stimulation of the motor nerve at 200 Hz in the presence of neostigmine (Escalona de Motta & del Castillo, 1977, Figure 2).

(2) *A cooperative effect of SA-TES on the endplate receptors* An alternative hypothesis is to assume that SA-TES binds to the ACh receptors increasing their sensitivity to the transmitter. This could happen either by increasing the affinity for ACh or the probability of channel opening.

The lack of action of SA-TES on the neurally evoked response could be explained by arguing that the high focal concentrations of ACh built up by the release of synaptic vesicles overcome the action of the allosteric modulator (cf. Hartzell *et al.*, 1975).

The allosteric hypothesis cannot explain the chemical specificity of SA-TES for choline esters, unless additional *ad hoc* assumptions are made (e.g. that SA-TES affects the esterasic subsite only). Nor does this hypothesis explain faster repolarization rates found in the presence of SA-TES.

Both hypotheses are not mutually exclusive. It is conceivable that in addition to saturating the groups that form a diffusion barrier, SA-TES may exert an allosteric effect on the AChR.

The succinyl-TES derivatives

In the second part of the present study we have demonstrated that the addition of SA to Ringer solution buffered with TES produces a mixture of succinyl-TES derivatives and succinic acid. Since succinic acid was inactive in physiological experiments as was SA when added to Ringer-phosphate without TES, we conclude that the succinyl-TES derivatives are responsible for all the pharmacological effects of the SA-TES solution. Assay of muscle contraction showed indeed that these compounds have a strong pharmacological effect, but minor derivatives were

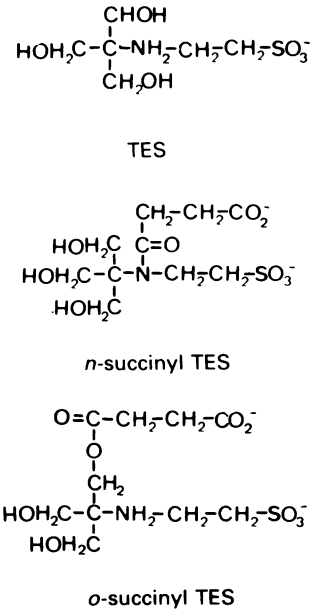

Figure 11 Chemical formulae of possible succinyl-TES derivatives. The two monosuccinylated compounds are shown. In addition, two disuccinyl, two trisuccinyl and one tetra-succinyl derivative could be formed. This amounts to seven possible derivatives.

mostly inhibitory, while the most abundant derivative potentiated at high ACh doses and inhibited at low ones (Figure 10). Inhibition by whole SA-TES solution was observed only at low concentrations of that solution (Figure 3). It could be argued that in the total SA-TES solution (1 mM SA), the potentiating action of the main derivative (peak 5) overcomes the inhibitory action of the less abundant compounds. When the SA-TES solution is diluted, peak 5 falls below its rather high threshold concentration (approximately 100 μM) while the concerted activity of the more active, inhibitory derivatives is now expressed. In any case, the activity of the SA-TES solution seems to be the result of a rather complex interaction of several succinyl-TES derivatives and ACh.

As for the molecular structure of the seven compounds found in this study, they could correspond to the seven possible succinyl-derivatives of TES (Figure 11). From what is known about succinylations in general, the primary target of SA in the molecule of TES is the amine group (Klapper & Klotz, 1972). For optimum succinylation of amine groups the pH has to be maintained above 7. Because peak 5 is practically the only derivative formed at pH 7.5 and its synthesis decreases drastically at lower pH, we tentatively propose that peak 5 is the N-succinyl-TES. Such assignment would also explain the relative resistance to spontaneous hydrolysis of peak 5, an amide, as compared to the ester groups of other derivatives. On the other hand, succinylation of hydroxyl groups occur under suitable conditions (Klotz & Stryker, 1959; Riordan & Vallee, 1964) such as those used during the preparation of the SA-TES solution for physiological experiments.

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