Inhibition of Caffeine Rigor and Radiocalcium Movements by Local Anesthetics in Frog Sartorius Muscle

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ABSTRACT Local anesthetics have been found to act as competitive inhibitors of caffeine in frog sartorius muscle. They block caffeine-induced rigor and the attendant increase in Ca^{45} influx and efflux. Increased net uptake of sodium, loss of potassium, and concurrent increase in oxygen consumption are all effectively blocked by procaine. Evidence is presented that the inhibitory effect of the local anesthetics cannot be explained by the formation of molecular complexes with caffeine. Increased efflux of Ca^{45} produced by changing from zero calcium Ringer's to 0.1 mM or 1 mM calcium Ringer's is inhibited by procaine and tetracaine. EDTA-stimulated calcium efflux is not affected by either local anesthetic. Caffeine rigor develops in frog muscle depolarized with KCl or rendered electrically inexcitable by sodium lack. Both the rigor and the increased calcium fluxes are inhibited by local anesthetics in depolarized muscle.

INTRODUCTION

Pharmacological agents described as membrane "stabilizers" are presumed to block impulse transmission in nerve and muscle by reduction or prevention of alterations in membrane potential (1). Local anesthetics are among the most familiar of such stabilizers which inhibit the depolarizing effects of electrical stimulation, acetylcholine, potassium, and various chemical agents. They suppress any increase in the permeability to sodium and potassium (1). It has in fact been suggested that local anesthetics block transmission in nerve (2) and muscle (3) by a competitive antagonism to sodium. Another possible explanation of the action of membrane stabilizers concerns their ability to increase the surface pressure of monomolecular films of stearate

(4) and nerve lipids (5) which are presumed to have properties in common with cell membranes. On the basis of these studies it has been proposed that the local anesthetics may prevent the enlargement (by factors which tend to increase permeability) of pores or channels through which sodium and potassium move down their concentration gradients (1).

The inhibitory effects of local anesthetics on caffeine rigor (6–8) cannot be explained on the basis of these proposed mechanisms, for as will be shown in this paper, they block the action of caffeine in depolarized muscle. In addition, activation of contraction of muscle by caffeine is not dependent upon changes in membrane potential or the presence of extracellular sodium. It is obvious therefore that the inhibition of caffeine rigor by local anesthetics must occur *via* a mechanism which does not involve stabilization of the membrane potential or blockage of sodium influx.

Caffeine activation of frog sartorius muscle has been associated with increases in calcium flux (9). It was proposed that caffeine released calcium from membrane binding sites, the increased Ca^{2+} in turn being responsible for the ensuing contractile activity in accord with the current concepts regarding the essential role of Ca^{2+} in muscular contraction (10–15). In this report evidence is presented which indicates that local anesthetics are competitive inhibitors of caffeine and that, in agreement with the aforementioned view as to the mechanism of caffeine rigor, they suppress the caffeine-induced fluxes of calcium regardless of the level of the membrane potential. In addition exchange between extracellular Ca^{40} and muscle fiber–bound Ca^{45} in resting muscle is also suppressed by the local anesthetics.

METHODS

Paired sartorius muscles were dissected from pithed frogs (*R. pipiens*) and separated by splitting the pelvic bone without damaging the tendinous attachment. The pelvic bone was fixed firmly to a supporting rod and the distal tendon was attached to a transducer (Statham GI-4-250) lever by a metal chain to reduce mechanical compliance to a minimum. The isolated muscles were allowed to equilibrate with an oxygenated Ringer's solution for at least 1 hour prior to an experiment. Electrical stimulation was applied through Ag-AgCl electrodes by an AEL 104A stimulator. The resting tension in each experiment was adjusted to 2.5 gm. The Ringer's solution used throughout this study was composed of 116.8 mM NaCl, 2.5 mM KCl, 1.08 mM $CaCl_2$, and 11.9 mM $NaHCO_3$. When equilibrated with a gas mixture of 95 per cent O_2–5 per cent CO_2 the pH of the solution was 7.2. To make the results in different muscles comparable, caffeine responses (P_r) were expressed as a fraction of the tetanus tension (P_o) obtained in the same muscle.

Influx Experiments

The Ca^{45} uptake of muscles was determined as described by Bianchi (9). Spontaneous twitching, which produced erratic results in studies of Ca^{45} fluxes, was prevented

by addition of low concentrations of procaine: 0.07 mM procaine with 1 mM calcium and 0.37 mM in the absence of calcium. In every case the period of exposure to the radiocalcium Ringer's solution was 10 minutes, after which the muscles were washed for a period of 90 to 120 minutes with five or six 5 ml portions of non-radioactive Ringer's solution. After the washout period, which is intended to eliminate Ca^{45} from the extracellular space, the muscles were blotted on moist filter paper, weighed on a Roller-Smith balance, then dried at 105°C until a constant dry weight was obtained. The dried muscle was ashed at 500°C for 16 hours and dissolved in 0.1 N HCl. Aliquots of the HCl extracts were evaporated to dryness in planchettes and counted in a low background, gas flow, counter (Tracerlab). A correction, calculated from the "slow" component of the Ca^{45} efflux curves, was applied to the amount of Ca^{45} remaining in the muscle at the end of the washout period to compensate for the loss of radiocalcium from the intracellular compartment (9). The calcium uptake was calculated from the Ca^{45} space (milliliters per gram) multiplied by the concentration of the total calcium (μmole per milliliter) of the Ringer's solution. The influx was obtained by dividing the calcium uptake per gram by the estimated surface area of the fibers. The latter was assumed to be 300 cm^2/gm (16) to facilitate comparison with the data of Bianchi.

Efflux Experiments

Muscles were presoaked in Ca^{45} Ringer's for 2 to 4 hours and then rapidly rinsed to remove radioactive solution adhering to the surface. Ca^{45} efflux into "cold" Ringer's was followed over a period of 4 to 5 hours by washing out in 2 ml aliquots, at 5 or 10 minute intervals, and measuring the radioactivity in the effluents. Ca^{45} remaining in the muscle at the end of the washout period was determined as described above (see Influx experiments). Desaturation curves were plotted which describe the decline of tissue radioactivity, expressed as a percentage of the initial total tissue radioactivity) as a function of time. The total initial radioactivity equals the sum of the radioactivity in all the washes plus that remaining in the muscle at the end of the experiment. Ca^{45} efflux may also be depicted by rate coefficient curves which show the time course of the average per cent change in radioactivity per minute as given by the following expression: Radioactivity appearing in washout solution during a collection period ÷ (average radioactivity in the muscle during the collection period × duration of the collection period). The effect of caffeine and local anesthetics on Ca^{45} efflux has also been diagramatically expressed as the *relative* Ca^{45} efflux per collection period which is derived as follows: radioactivity washed out during any 10 minute collection period ÷ radioactivity washed out during a collection period chosen as the reference base (usually the collection period 40 minutes prior to the addition of any drug).

Metal Ion Analysis

Calcium, sodium, and potassium were determined in 0.1 N HCl extracts of ashed muscle by flame photometric analysis using a Beckman model B spectrophotometer with flame attachment. Standard solutions contained calcium, sodium, and potassium

in the same relative proportions as found in muscle. The specific activity of muscle calcium is expressed as counts per minute per micromole of tissue calcium.

Oxygen Consumption

Oxygen consumption of whole frog sartorius muscles was determined by conventional Warburg manometric techniques. The effects of caffeine and procaine were evaluated by tipping these agents into the flask from a side well after a 30 minute period for the determination of the resting respiratory activity.

Caffeine-Local Anesthetic Complexes

Formation of molecular complexes between caffeine and the local anesthetics was studied by measuring the partition of caffeine between water and an organic phase (17),—a mixture of benzene:ligroin (30:70). The degree of complex formation was determined, from the amount of caffeine found in the organic phase, as follows: concentration of caffeine in complex with procaine (or other drug) = total initial caffeine concentration in water − (caffeine in the organic phase + free caffeine in the water phase). The caffeine content of the organic phase was determined as described below. The free caffeine in the water phase is equal to the caffeine concentration of the organic phase times the partition coefficient for caffeine in a water/benzene:ligroin system. The validity of the method depends upon the insolubility of the local anesthetic-caffeine complexes in the organic phase so that only free caffeine in the water phase can enter the organic phase. This has been shown to be true for $(caffeine)_2$-procaine complexes (17) and was assumed to be true, for the purpose of making the calculations presented in this paper, for the other local anesthetics. Should this assumption be false, in the case of tetracaine and butacaine, the degree of complex formation would be *underestimated*. In the presence of procaine HCl it was possible to measure caffeine by ultraviolet absorption at 277 mμ because of the insolubility of the former in the organic phase. However, carbon^{14}-labeled caffeine was usually employed and determined by its radioactivity, because the solubility of tetracaine HCl and butacaine SO_4 in the organic phase was sufficient to produce considerable absorption in this region of the ultraviolet.

RESULTS

Effects of Local Anesthetics on Caffeine Rigor Fig. 1 is a plot, obtained by the method of least squares, of the reciprocal of the caffeine response, $(P_r/P_o)^{-1}$, *vs.* the reciprocal of the molar concentration of caffeine in the Ringer's solution. The relationship is linear over the concentration range indicated. The local anesthetics, procaine and tetracaine, added with the caffeine, increased the slopes of the lines without significantly changing the intercept. This is the effect to be expected from a competitive type of inhibitor (18). The slope of the caffeine regression line is 0.0062 (±0.0006 SE) whereas in the presence of 1.83 mM procaine HCl the slope is 0.0154 (±0.0033 SE) and increases to 0.0334 (±0.0035 SE) with 3.67 mM procaine. Tetracaine HCl at 3.67 mM increases the slope to 0.157 (±0.01 SE). The intercepts for

caffeine alone, and in the presence of 1.83 mM and 3.67 mM procaine HCl, and 3.67 mM tetracaine HCl are 1.32, 1.17, 1.44, and 1.06 respectively. By analysis of variance (*F*-test) it was determined that the probability of such chance differences between the intercept values is considerably greater than 5 per cent so that a common intercept is indicated.

As the caffeine concentration is decreased below the values plotted in Fig. 1, the tension falls much more steeply so that a sharp break in the slope occurs.

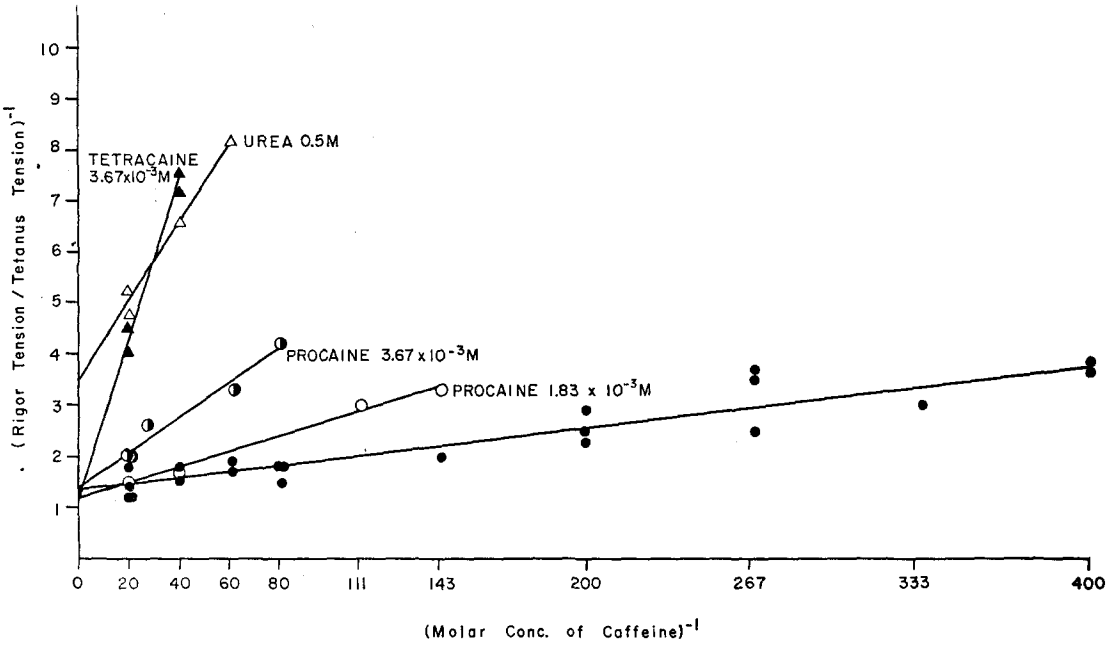

FIGURE 1. Plot of the reciprocal of the caffeine response, $(P_r/P_o)^{-1}$, *vs.* the reciprocal of the molar concentration of caffeine in the Ringer's solution bathing the muscle. The addition of local anesthetics, procaine (○, ◑) or tetracaine (▲), increased the slope of the lines as compared to caffeine alone (●) without significant change in the intercept. Urea (△) increased both slope and intercept.

For example, the lowest caffeine concentration at which a response is shown is 2.5 mM (reciprocal value = 400) which gives a $(P_r/P_o)^{-1}$ of about 3.5. At slightly lower concentrations, 2.25 mM (reciprocal = 444) and 2.0 mM (reciprocal = 500), the $(P_r/P_o)^{-1}$ values increased to 23 and 65 respectively. Thus the line becomes nearly vertical. The same phenomenon is observed in the presence of local anesthetics, but the concentration range where caffeine suddenly becomes much less effective is shifted to higher values. The sudden upward shift in slope occurs at the extreme right end of each line plotted in Fig. 1. This remarkable change in slope appears to be connected with a

qualitative change in the response to caffeine from reversible contractures (at low concentrations) to irreversible rigor.

If it is assumed that caffeine reacts with a specific substance, or "receptor," in the cell, an apparent dissociation constant (K_d) for the drug-receptor complex can be calculated from the equation:[1]

$$(P_r/P_o)^{-1} = (P_r/P_o)^{-1}_{\max} + \frac{K_d}{(P_r/P_o)_{\max}} \cdot \frac{1}{(D)}$$

where $(P_r/P_o)_{\max}$ is the theoretical maximum response obtainable with infinite concentration of the drug and (D) is the drug concentration. From the data of Fig. 1 K_d was found to equal 4.7 mM for caffeine. This constant is only a first approximation of the true dissociation constant because it is not known whether the assumptions implicit in the derivation of the equation hold for this example of drug-muscle interaction.

In a manner analogous to the treatment of kinetic data of enzyme-substrate-inhibitor reactions, it is possible to determine an apparent dissociation constant for the inhibitor-receptor complex, assuming caffeine and the local anesthetics are competing for the same active site. The K_I, or inhibitor-receptor dissociation constant, may be calculated from the expression:

$$\text{Slope of line in presence of inhibitor} = \frac{K_d}{(P_r/P_o)_{\max}}\left(1 + \frac{I}{K_I}\right)$$

where (I) represents the inhibitor concentration (procaine or tetracaine). The K_I for procaine HCl at 1.83 mM and 3.67 mM was found to be 1.2 mM and 0.84 mM respectively. The K_I for tetracaine HCl was 0.15 mM.[2] The ratio of K_I procaine/K_I tetracaine is therefore about 7, indicating a relative potency for tetracaine seven times that of procaine under the prevailing conditions. On other batches of frogs somewhat higher relative potencies for tetracaine were observed.

Urea, which has been characterized as an "interaction inhibitor" because it causes dissociation of actomyosin into actin and myosin and decreases the high ATPase activity of actomyosin to the lower level characteristic of L-myosin, inhibits caffeine rigor (23). Fig. 1 illustrates that both the intercept (3.45) and the slope (0.078) are significantly increased. An increase of slope and intercept is indicative of a non-competitive type of inhibition. But the

[1] This equation is analogous to the Lineweaver-Burk equation used in studies of enzyme kinetics. For discussion of the applicability of equations of this type to the study of drug-tissue interactions see references 19–22.

[2] It should be noted that the apparent K_I values were not obtained under equilibrium conditions as the drug was added simultaneously with caffeine. Presoaking muscles with a local anesthetic for 30 minutes prior to addition of caffeine gave lower values for K_I as expected.

slope and intercept should increase by the same factor $(1 + I/K_I)$ whereas in this instance the slope increased about five times more than the intercept. The urea effect is therefore more complex than described by the usual equations for non-competitive inhibition.

TABLE I

CALCIUM INFLUX IN FROG SARTORIUS MUSCLE

Experiment	No. of muscles	Ca^{45} influx
		($\mu\mu$mole/cm² sec. ± SE)
10 min. in Ca^{45} Ringer's	19	0.083 ± 0.008
10 min. in Ca^{45} Ringer's + 5 mM caffeine	16	0.269 ± 0.021
10 min. in Ca^{45} Ringer's + 5 mM caffeine + 3.67 mM procaine HCl	16	0.107 ± 0.009
10 min. in Ca^{45} 0.123 M KCl + 3.67 mM tetracaine + 5 mM caffeine (after 30 min. in 0.123 M KCl)	6	0.110 ± 0.011
10 min. in Ca^{45} 0.123 M KCl + 5 mM caffeine (after 30 min. in 0.123 M KCl)	6	0.340 ± 0.098

TABLE II

CALCIUM INFLUX IN FROG SARTORIUS MUSCLE

No. of pairs of muscles	Experimental conditions	Calcium influx Experimental–Control
		$\mu\mu$mole/cm² sec. (± SE)
7	Exp. 10 min. in Ca^{45} Ringer's + 5 mM caffeine Cont. 10 min. in Ca^{45} Ringer's	0.145 ± 0.020
11	Exp. 10 min. in Ca^{45} Ringer's + 5 mM caffeine + 3.67 mM procaine HCl Cont. 10 min. in Ca^{45} Ringer's	0.022 ± 0.029
9	Exp. 10 min. in Ca^{45} Ringer's + 5 mM caffeine Cont. 10 min. in Ca^{5} Ringer's + 5 mM caffeine + 3.67 mM procaine HCl	0.125 ± 0.031
6	Exp. 10 min. in Ca^{45}–0.123 M KCl + 1.0 mM $CaCl_2$ + 5 mM caffeine Cont. 10 min. in Ca^{45}–0.123 M KCl + 1.0 mM $CaCl_2$ + 5 mM caffeine + 3.67 mM tetracaine HCl	0.229 ± 0.09

Local Anesthetic Effects on Caffeine-Induced Ca Influx and Efflux

A. INFLUX The data in Tables I and II show that 3.67 mM (1 mg/ml) procaine almost completely suppressed the increased calcium influx elicited by 5 mM caffeine. The influx rate in resting muscle was 0.083 $\mu\mu$mole/cm² sec., a value which is in good agreement with that reported by Bianchi (0.094

$\mu\mu$mole/cm^2 sec.). Caffeine produced a more than threefold increase in the influx to 0.269 $\mu\mu$mole/cm^2 sec. In the presence of procaine, caffeine increased Ca^{45} influx by only about 20 per cent. Procaine was effective in blocking calcium influx whether added 10 minutes prior to, or simultaneously with, the caffeine. In Table II the same results are expressed as the differences between pairs of muscles from the same animal. There was an increase of 0.145 $\mu\mu$mole/cm^2 sec. in the Ca^{45} influx of caffeine-treated muscles

TABLE III

No. of muscles	Experimental conditions	Calcium influx	Calcium specific activity*	Concentration			Water content
				Ca	Na	K	
		$\mu\mu$mole/cm^2 sec.	*CPM/μmole*	*μmole/gm‡*			*per cent total muscle weight*
8	Freshly dissected	—	—	1.42 (±0.07)	27 (±4)	80 (±2)	— —
8	10 min. in Ca^{45} Ringer's + 2 hr. washout§	0.062 (±0.014)	2,228 (±453)	1.89 (±0.14)	52 (±3)	73 (±2)	80 (±1.2)
9	10 min. in Ca^{45} Ringer's + 5 mM caffeine + 2 hr. washout§	0.262 (±0.040)	13,290 (±3488)	1.86 (±0.18)	93 (±12)	44 (±7)	82 (±0.7)
9	10 min. in Ca^{45} Ringer's + 5 mM caffeine + 3.67 mM procaine HCl + 2 hr. washout§	0.096 (±0.017)	3,649 (±748)	1.80 (±0.09)	48 (±3)	75 (±2)	79 (±0.7)

Figures in parentheses are standard errors of the means.
* Specific activity of calcium in the bath was 500,000 CPM/μmole.
‡ Total content for wet tissue, not corrected for extracellular content.
§ Washout solution in every experiment was non-radioactive Ringer's solution.

compared to controls without caffeine or procaine. Nearly the same increase, 0.125 $\mu\mu$mole/cm^2 sec., was noted when muscles exposed to caffeine alone were compared to their mates treated with procaine plus caffeine. Muscles exposed to caffeine plus procaine exhibited only a 0.022 $\mu\mu$mole/cm^2 sec. increase over their mates in normal Ringer's.

In some experiments, in addition to Ca^{45} influx, the muscle content of calcium, sodium, and potassium was determined. Muscles soaked in Ringer's for 2.5 to 3 hours (the duration of exposure to the perfusion solution during an experiment) accumulated calcium and sodium and lost some potassium (Table III) when compared to those analyzed directly after dissection. Cosmos and Harris (24) previously noted the same phenomenon. In muscles equilibrated with Ringer's solution the Ca^{45} influx was 0.062 $\mu\mu$mole/cm^2 sec. whereas caffeine (5 mM) treated muscles showed a higher Ca^{45} influx of 0.262 $\mu\mu$mole/cm^2 sec. No significant change in net calcium concentration

between these two groups of muscles was observed, but there was an 80 per cent increase (41 μmole/gm) in sodium and a 40 per cent (29 μmole/gm) decrease in potassium content. The specific activity of the muscle calcium (determined at the end of the 2 hour washout period) was increased nearly sixfold over resting conditions. When 3.67 mM procaine was added along with caffeine the Ca^{45} influx and specific activity of the tissue calcium increased by only 50 per cent and there was no net change in sodium or potassium. The results in Table III show that caffeine treatment resulted in an increase in specific activity of muscle calcium without significant change in the net calcium content. This should not be interpreted to mean that the increase in specific activity was necessarily the result of exchange rather than net uptake. All the Ca^{45} entered the muscle during the first 10 minute period; however, changes in net calcium content could take place during the 2 hour washout period as is evident by comparison of analyses of freshly dissected muscle with those equilibrated in Ringer's for at least 2 hours. Any change in calcium concentration during this period would obscure what had occurred during the short period of exposure to the solution containing isotope and caffeine. To evaluate properly the relative contributions of self-exchange and net uptake to the total calcium influx it would be necessary to know what changes, if any, in the calcium concentration of the muscles occurred during the 10 minute exposure to caffeine and Ca^{45}.

B. EFFLUX Stimulation of Ca^{45} efflux by caffeine is shown in Figs. 2 to 4. Figs. 2 and 4 are plots of desaturation curves and rate coefficients, respectively, of individual experiments. Fig. 3 summarizes the effects of caffeine, with and without tetracaine or procaine, on the *relative* Ca^{45} efflux. An average (seven experiments) 2.5-fold increase in calcium efflux occurs during the first 10 minute exposure to caffeine. Thereafter the calcium efflux decreases very rapidly at first but even after 30 minutes is still above normal. In the presence of a local anesthetic, added simultaneously, the average (seven muscles, mates of the muscles treated with caffeine alone) calcium efflux was no more than 10 to 20 per cent above normal. The relative efflux decreases from the reference value of 1.0, 40 minutes prior to addition of caffeine, to an average value of 0.72 for both sets of muscles. During the first 10 minutes of exposure to the drug the values for relative Ca^{45} efflux for six caffeine-treated muscles ranged from 0.73 to 3.23 (average 1.76); one muscle gave a value of 9.59. The range in muscles treated with caffeine plus local anesthetic was 0.34 to 0.95 (average 0.73). In every pair of muscles the local anesthetic decreased the Ca^{45} efflux.

By contrast, increased Ca^{45} influx and efflux persisted in the presence of 1.0 M urea, which completely blocked caffeine rigor. Caffeine (5 mM) increased the calcium influx, in several experiments, by only 50 per cent,

which would imply that urea may partially inhibit calcium influx. However, urea alone increased the *resting* calcium influx by about two- to threefold. It is possible that the high passive influx obscures any effect by caffeine. The high concentrations of urea (0.5 to 1.0 M) required to inhibit caffeine rigor had rather deleterious effects on frog muscle; *e.g.*, marked swelling

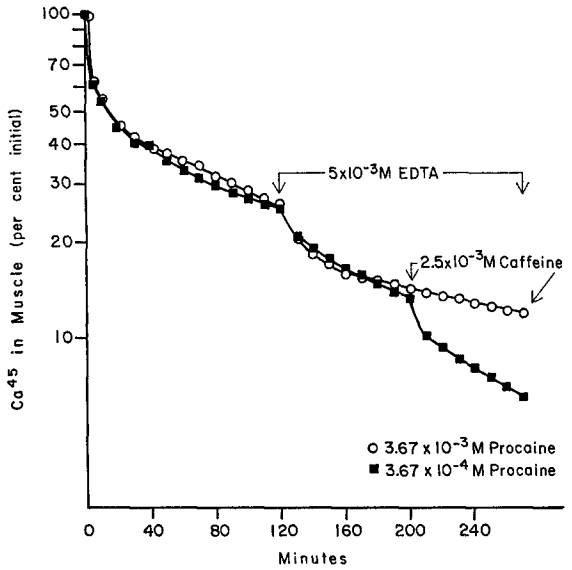

FIGURE 2. Time course of the decline of tissue Ca^{45} in paired muscles previously soaked in Ringer's solutions containing Ca^{45}. Muscles were washed with cold Ringer's starting at zero time. From 120 minutes until the conclusion of the experiment 5×10^{-3} M EDTA calcium-free Ringer's was used as the wash solution. Caffeine, at 2.5 mM, was added to both muscles at 200 minutes. From 110 minutes until the conclusion of the experiment the wash solution for one muscle (■) contained 0.367 mM procaine HCl to prevent spontaneous twitching, whereas that for the other muscle (o) contained 3.67 mM procaine HCl. Procaine was without effect on the EDTA-stimulated Ca^{45} efflux, whereas at 3.67 mM it blocked the caffeine-induced calcium efflux.

and opacity resulted when the urea was washed out with normal Ringer's. For this reason the influence of urea on caffeine rigor, and the associated ion movements, was not studied extensively. It is evident though that the mechanism of rigor inhibition by urea is quite different from that of the local anesthetics.

Effect of Procaine on Caffeine-Induced Stimulation of Muscle Respiration

Experiments carried out in Warburg manometers with whole frog sartorius muscles in phosphate-buffered Ringer's revealed that procaine (3.67 mM) completely suppressed the four- to sixfold increase in oxygen consumption

induced by caffeine. These experiments will not be discussed in detail as the same observation has recently been made independently by Novotny *et al.* (25).[3]

Caffeine Rigor and Ca^{45} Fluxes in Depolarized Muscle

Axelsson and Thesleff (26) had observed that caffeine activated depolarized muscle. Local anesthetics inhibit the action of caffeine in depolarized muscle. Fig. 5 *a* shows a typical caffeine response in a frog sartorius muscle depolarized

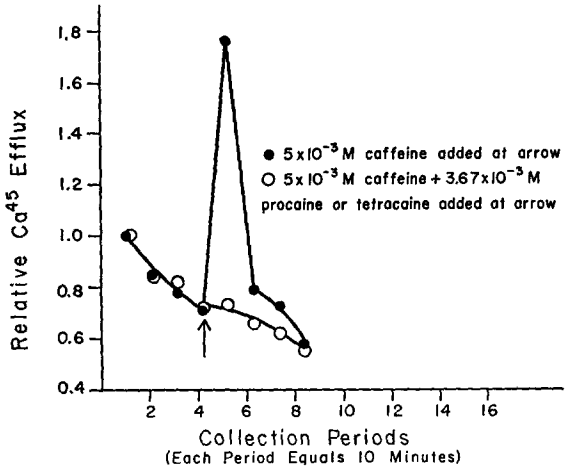

FIGURE 3. Average values for relative Ca^{45} efflux in seven pairs of frog sartorius muscles washed out with Ringer's solution at 10 minute intervals. After 30 minutes the amount of Ca^{45} appearing in the effluent fell to about 0.7 of the initial value for all muscles. Addition of 5 mM caffeine to one member of each pair of muscles increased Ca^{45} efflux about 2.5-fold. In the presence of procaine or tetracaine, caffeine was practically without effect on the Ca^{45} efflux.

with 0.123 M KCl 30 minutes prior to addition of 25 mM caffeine. The P_r/P_o value for this experiment was 0.53. In Fig. 5 *b* the response of the mate of the muscle in *a* was reduced by tetracaine to $P_r/P_o = 0.15$. Measurement of resting potentials by the sucrose-gap method, in other muscles treated in the same way, showed that no repolarization resulted from the exposure to tetracaine. Although caffeine rigor could be evoked in depolarized muscle, sensitivity to the alkaloid appeared to be somewhat lower. Occasionally little or no response was obtained at low caffeine (5 mM) concentrations. This phenomenon was reflected in studies of Ca^{45} fluxes, at the same caffeine

[3] Novotny *et al.* also reported an inhibition of the extra oxygen consumption due to 20 mM KCl using 10^{-4} M physostigmine salicylate or 10^{-3} M procaine HCl. In our laboratory physostigmine salicylate and methapyrilene HCl were found to effectively inhibit caffeine rigor. Studies on Ca^{45} fluxes have not yet been carried out.

concentration, where in several experiments the increase in efflux or influx was quite small or could not be detected at all. Table I illustrates the inhibitory effect of tetracaine on calcium influx in the experiments in which caffeine was effective in the other muscle of the pair. In five other pairs of

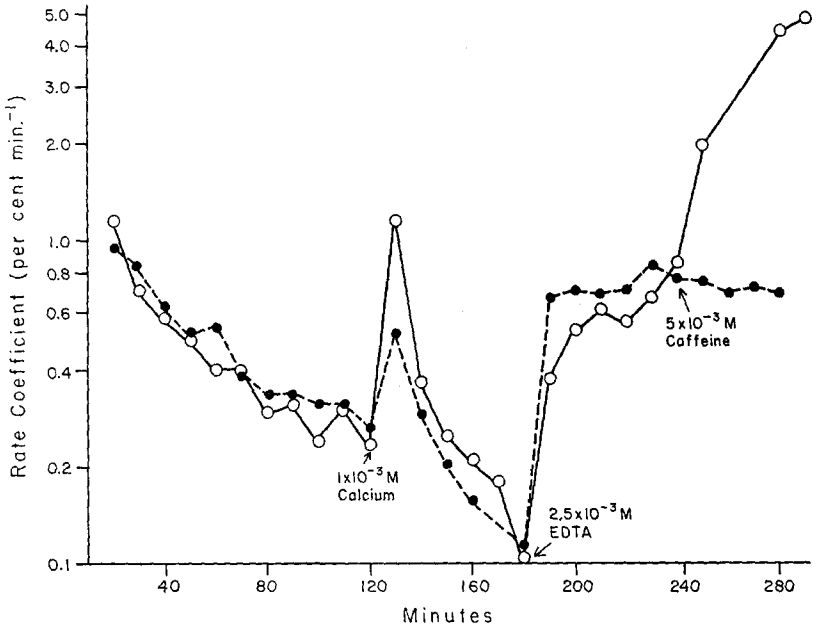

FIGURE 4. Time course of the changes in "rate coefficients" for Ca^{45} release from paired sartorius muscles, previously exposed to Ca^{45} Ringer's for 3 hours. Muscles were washed with calcium-free Ringer's containing 0.367 mM procaine HCl in one case (○) and 3.67 mM tetracaine HCl in the other (●). At 120 minutes 1.0 mM $CaCl_2$ was added to both muscles. Ca^{45} efflux was substantially smaller in the tetracaine-treated muscle. At 180 minutes addition of 2.5 mM EDTA increased Ca^{45} efflux in both muscles. The rate coefficient was initially higher in the tetracaine-treated muscle but later became nearly the same for both muscles. Caffeine, 5 mM added at 240 minutes markedly increased Ca^{45} efflux in the presence of EDTA in one muscle, an effect which was blocked by the presence of tetracaine in the other muscle.

muscles, not shown in the table, caffeine (5 mM) increased Ca^{45} influx three- to fourfold over control muscles in isotonic KCl. Similarly, caffeine increased calcium efflux in seven out of nine experiments, and this effect was blocked, in the other muscle of the pair, by procaine or tetracaine. The somewhat lower calcium efflux and rigor response to caffeine in KCl solution, compared to Ringer's, may be due to the fact that, as first described by Shanes and Bianchi (27), some of the membrane-bound calcium was released when the muscles were depolarized.

Effect of Sodium Lack on Caffeine Rigor

To determine whether sodium was required for caffeine-induced rigor, muscles were soaked in isotonic sucrose solution or in Ringer's solution containing an equivalent amount of choline chloride in place of NaCl. After 1

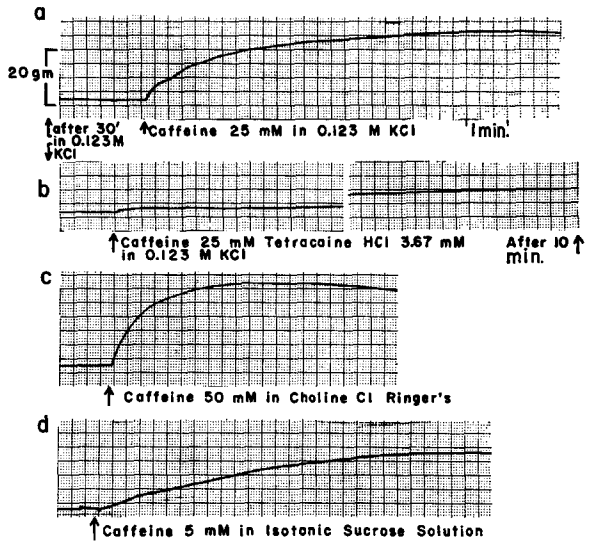

FIGURE 5. Caffeine rigor in frog sartorius muscle depolarized with KCl or rendered electrically inexcitable due to sodium lack. (*a*) Caffeine (25 mM) produced a tension of 24.5 gm in a muscle which had given a tetanus tension of 46 gm prior to depolarization with 0.123 M KCl. (*b*) The mate of the muscle in (*a*) produced a tension of 8.0 gm in the presence of 25 mM caffeine and 3.67 mM tetracaine. The tetanus tension in this muscle prior to depolarization with 0.123 M KCl was 52.5 gm. There was a 10 minute interval between the two parts of the record. (*c*) Response to 5.0 mM caffeine after soaking in choline chloride Ringer's solution for 1 hour. The response to electrical stimulation fell to 8 per cent of normal. The response to caffeine was equal to that obtained in muscles in normal sodium Ringer's. (*d*) Response to 5 mM caffeine in a muscle soaked in isotonic sucrose solution for 30 minutes, at which point the muscle was electrically inexcitable. The response to caffeine was equal to that obtained in muscles in normal sodium Ringer's.

hour in choline chloride Ringer's, the response to electrical stimulation was only 8 per cent of that in sodium Ringer's, whereas another muscle, 30 minutes in sucrose solution, was electrically inexcitable. Caffeine was capable of inducing rigor equal in magnitude to that obtained in normal Ringer's in either case (Fig. 5 *c*, *d*).

Effects of Ca^{40} and EDTA on Ca^{45} Efflux

In view of the results described above it was of interest to determine whether local anesthetics influence calcium fluxes in general. No influence on passive

Ca^{45} efflux was observed. Other experimental conditions known to alter calcium movements have been studied, such as the Ca^{40}-Ca^{45} self-exchange reaction (27) and the effect of EDTA (9).

After a period of washing out a muscle, previously loaded with Ca^{45}, with Ca^{2+}-free Ringer's the addition of 1.0 mM Ca^{40} (non-radioactive) initiated a large, transient, increase in Ca^{45} efflux. This effect has been attributed apparently to the rapid exchange of Ca^{40} for Ca^{45} in easily accessible surface

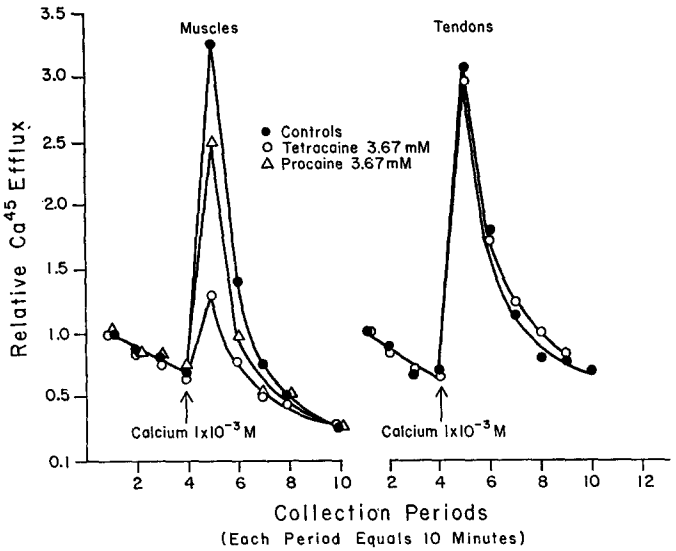

FIGURE 6. Efflux of Ca^{45} from muscle and Achilles tendon upon addition of 1 mM calcium to the Ringer's solution. Up to the time when calcium was added the muscles and tendons were washed, at 10 minute intervals, with calcium-free Ringer's. Addition of 1.0 mM calcium increased Ca^{45} efflux from both muscle (five experiments) and tendon (seven experiments) (●) by about fivefold. Procaine (△) decreased the Ca^{45} efflux about 25 per cent in two muscles, whereas tetracaine (○) decreased the efflux by about 60 per cent in three muscles. In seven tendons tetracaine (○) was without effect on the Ca^{45} efflux.

sites on the muscle fibers and in the connective tissue (27). Similarly, addition of 2.5 to 5 mM EDTA to the perfusion solution, as first shown by Bianchi (9), greatly increased the efflux of Ca^{45} from muscle. Experiments were performed in which either procaine or tetracaine was added to the solution bathing the muscle 2 hours prior to the addition of EDTA or switching from calcium-free to 1 mM calcium Ringer's. The local anesthetics were also present in the solutions containing calcium or EDTA. The results are depicted in Figs. 2, 4, and 6. Fig. 6 illustrates the relative calcium efflux as described under Methods. The collection period 40 minutes prior to the addition of Ca^{40} serves as the reference point. The amount of muscle Ca^{45} washed out during this collection period is set equal to 1.0. The Ca^{45} washed

out during succeeding 10 minute collection periods is then calculated as some fraction of the reference amount. It is evident that there is a progressive decrease in the amount of radiocalcium being washed out of the muscles during the succeeding collection periods. Upon addition of Ca^{40} the Ca^{45} appearing in the bath suddenly increases and then falls off to normal levels during the next 10 to 20 minutes. Procaine HCl (3.67 mM) decreased the self-exchange by about 25 per cent in two experiments, whereas tetracaine HCl brought about a 60 per cent inhibition in three muscles. In a fourth muscle tetracaine produced a similar degree of inhibition of the self-exchange when 0.1 mM Ca^{40}, rather than 1.0 mM, was added. In each muscle, compared to its mate which served as a control, procaine and tetracaine decreased the amount of self-exchange. In five control muscles the relative Ca^{45} efflux increased 2.65 to 4.3 times the value at the reference point. In the procaine-treated muscles the values were 2.25 to 2.77 times the base rate, and tetracaine gave values from 0.99 to 1.56. In tendon, tetracaine had no influence on the Ca^{40}-Ca^{45} self-exchange. The increase in radiocalcium efflux in seven untreated tendons ranged from 2.05 to 3.71 times that at the reference point. In tetracaine-treated tendons the range was 1.96 to 3.92 for seven experiments. Three of the seven tetracaine-treated tendons actually had a slightly higher Ca^{45} efflux than their mates. It is evident therefore that the self-exchange reaction that is effected in whole muscle most likely takes place in the muscle fibers themselves and not in the connective tissue elements.

Neither procaine nor tetracaine inhibited the increased Ca^{45} efflux induced by 2.5 to 5 mM EDTA (Figs. 2 and 4). An experiment which shows, in sequence, the influence of tetracaine on the Ca self-exchange reaction, EDTA, and caffeine-stimulated calcium effluxes is shown in Fig. 4. Here the rate coefficient, expressed as per cent $min.^{-1}$, is plotted *versus* time. Addition of 1.0 mM calcium increased the efflux fivefold. In the presence of 3.67 mM tetracaine HCl the rate increased only 2.5 times. EDTA (2.5 mM) subsequently increased the efflux about ninefold even in the presence of tetracaine. During the first 10 minute collection period after EDTA addition, the Ca^{45} efflux was about twice as great in the tetracaine-treated muscle as in the control. This was probably a result of the fact that less Ca^{45} had previously been exchanged for Ca^{40}, due to tetracaine, but was now readily removed by EDTA. By the third collection period the efflux rates were practically equal. When 5 mM caffeine was added, no increase in calcium efflux was observed in the tetracaine-treated muscle, whereas it increased fivefold in the muscle without tetracaine.

Relationship of Caffeine Rigor Inhibition by Local Anesthetics to the Tendency to Form Molecular Complexes of Caffeine and Local Anesthetics

It had previously been suggested (28) that the ability of procaine to alter the distribution coefficient of caffeine was the basis for the inhibitory effect

of the former on caffeine rigor in muscle. Physico-chemical evidence (17, 29) has been obtained which indeed demonstrates that caffeine and procaine form molecular complexes of the type (caffeine)$_2$–procaine, which are insoluble in benzene. However, decrease in free caffeine by complex formation cannot explain the blocking action of procaine and related drugs. The following evidence supports this view.

Direct determinations have been made by Lachman *et al.* (17) and in this laboratory, of the amount of caffeine reacting with the local anesthetics as described in Methods. Comparison of the degree of inhibition of rigor

TABLE IV

	Local anesthetic concentration	Total caffeine concentration	Caffeine in complex	Free Caffeine	Observed P_T/P_o	Expected* P_T/P_o
	mM	mM	mM	mM		
Procaine	3.67	50	2.8‡	47.2	0.47	0.70
	14.7	30	5.0§	25.0	0.51	0.64
	51.4	50	21.5§	28.5	0.14	0.65
	25	25	8.3‡	16.7	0.36	0.59
Tetracaine	3.67	50	5.2‡	44.8	0.21	0.68
	25	25	12.2‡	12.8	0.02	0.56
	3.67	25	—	—	0.14	—
	3.67	30	—	—	0.10	—

* Calculated from the equation for the regression line in Fig. 1; $P_o/P_T = 1.32 + \frac{0.0062}{D}$, where 0.0062 is the slope with standard error of ±0.0006.

‡ Determined directly as described in Methods.

§ From the data of Lachman *et al.* (17).

produced by a local anesthetic was made with the degree of inhibition which was to be expected solely on the basis of the decrease in free caffeine concentration in solution. Table IV summarizes several of these experiments and indicates conclusively that the local anesthetics produce a far greater inhibition of rigor than can be expected from their ability to reduce the free caffeine concentration. For example, in one experiment at 50 mM caffeine $P_T/P_o = 0.70$, whereas in the presence of 51 mM procaine HCl, P_T/P_o was reduced to 0.136, a decrease of 0.564. At these concentrations of caffeine and procaine the amount of caffeine in complex with procaine is 22 mM (from the data of Lachman *et al.*), leaving 28 mM free caffeine in solution. A decrease in caffeine concentration from 50 to 28 mM would be expected to produce only a 0.05 fall in P_T/P_o as calculated from the caffeine regression line equation of Fig. 1. At 14.7 mM procaine HCl plus 30 mM caffeine the free caffeine concentration was 25 mM. At 51.4 mM procaine HCl plus 50 mM

caffeine the free caffeine concentration was slightly higher at 28.5 mM. In spite of the higher free caffeine concentration of the second solution, it gave less than one-third the P_r/P_o when tested on a pair of muscles from the same animal. Obviously the difference in free *procaine* concentration, 12.2 mM *versus* 40.7 mM, must be responsible for this effect. Experiments with tetracaine lead to the same conclusion. At caffeine and tetracaine concentrations of 25 mM and 3.67 mM, respectively, P_r/P_o was found to be 0.14. Seven times as much tetracaine (25 mM) would leave enough free caffeine to give an expected response of 0.56 (Table IV). Even if all the tetracaine (3.67 mM) existed as a $(\text{caffeine})_2$–tetracaine complex, enough free caffeine would exist in solution (17.7 mM) to give an expected P_r/P_o of 0.6.

Another experiment conclusively indicates that local anesthetic action is not due to complex formation with caffeine. Muscles of about 100 mg wet weight were soaked in 100 ml of 3.67 mM tetracaine HCl for 30 minutes, after which the muscles were washed several times within 2 to 3 minutes with 100 ml portions of Ringer's solution to remove tetracaine from the muscle chamber and the surface of the muscles. Addition of 100 ml Ringer's containing 5 mM caffeine (total of 100 mg; or 500 μmoles, of caffeine in the bath) produced no contraction. The only tetracaine present at this time was that within the muscle fibers and the extracellular space after the washout. Calculation of the amount of tetracaine present (assuming as much as a 50-fold accumulation of local anesthetic in the tissue solids based on Skou's data in ox nerve) indicates that no more than 1 mg (3.67 μmoles) could have been in a muscle. This is less than 1 per cent of the total amount of caffeine present in the bath and could hardly account for suppression of rigor by decreasing the free caffeine concentration.

DISCUSSION

Several possibilities may be suggested as explanations for the inhibition of caffeine rigor by local anesthetics (L.A.): L.A. may (*a*) decrease the permeability of the muscle fibers to caffeine, (*b*) react chemically with caffeine to reduce the concentration of the free drug in solution, (*c*) prevent inactivation of "relaxing factor" by caffeine, (*d*) prevent release of calcium from binding sites in the muscle fibers.

Regarding the first possibility, Bianchi (30) has found that procaine (up to 7.34 mM) does not affect caffeine uptake or release from muscle. The second hypothesis must be discarded because it has been shown that the effectiveness of the local anesthetics at blocking caffeine rigor is substantially greater than can be expected on the basis of the reduction in free caffeine concentration due to complex formation with the local anesthetics.

It is also unlikely that protection of relaxing factor by local anesthetics is responsible for inhibition of rigor. Nagai and Uchida (31) reported that

caffeine inhibited relaxing factor activity in glycerinated muscle fibers, an effect which they were unable to prevent with procaine. Activation of intact living muscle by caffeine *is* inhibited by local anesthetics and therefore cannot be primarily due to "factor" inactivation. An increase in sarcoplasmic Ca^{2+} activity, induced by caffeine, is a more likely explanation for the initial stimulatory action of the drug. Protection against caffeine rigor by local anesthetics would therefore reside in their ability to prevent the initial increase in Ca^{2+} activity. This is borne out by the fact that caffeine rigor can be prevented by local anesthetics but cannot be reversed once it has been induced. The factors responsible for the development of an irreversible rigor are not known. Possibly inactivation of the relaxing factor system may result secondarily from a combination of circumstances, such as an increased sarcoplasmic Ca^{2+} concentration and a decrease in high energy phosphate compounds.

The ability of local anesthetics to suppress the Ca^{40}-Ca^{45} self-exchange suggests a common mechanism with regard to this phenomenon and the inhibition of caffeine-induced Ca^{2+} fluxes. One possibility is that the local anesthetics may block Ca^{2+} transport across the plasma membrane thereby preventing access to calcium-binding sites deeper within the muscle fibers (*i.e.* endoplasmic reticulum). This would, in effect, prevent exchange of extracellular Ca^{40} with intracellular Ca^{45}. Similarly the caffeine effect could be considered as primarily due to an increase of membrane Ca^{2+} permeability. Thus the increased Ca^{45} efflux in caffeine-treated muscles could reflect an increased exchange with the extracellular Ca^{40}. However, in the presence of very low extracellular Ca^{2+} due to the addition of EDTA, caffeine still increases Ca^{45} efflux (Figs. 2 and 4). This cannot be the result of exchange with the extracellular Ca^{2+}, but is probably due to release of calcium from substances to which it is bound in the cell, with the increased Ca^{2+} efflux reflecting the higher sarcoplasmic Ca^{2+} concentration. The fact that caffeine induces rigor in muscles soaked in 5 mM EDTA for sufficient time (30 minutes) to reduce the extracellular Ca^{2+} to a very low level (response to electrical stimulation was blocked at this time) indicates that the contractile activity is not induced by increased movement of extracellular Ca^{2+} into the muscle fibers, but more likely is the result of an action of caffeine upon calcium bound within the fibers. Local anesthetic action may be due to protection of the binding sites against the action of caffeine. Similarly local anesthetics may block the Ca^{40}-Ca^{45} self-exchange reaction by inhibiting dissociation of Ca^{45} from the cellular binding sites.

Additional evidence concerning the influence of local anesthetics on calcium mobility comes from experiments performed on smooth muscle. Mammalian smooth muscle depolarized by isotonic K_2SO_4 solution can be induced to contract with acetylcholine (33). Ca^{2+} is required for this response

(33) and indeed Ca^{2+} itself can evoke graded contractions of the depolarized rat uterus (34). Procaine and tetracaine (3.67 mM) prevent contractions produced by Ca^{2+} alone, or in conjunction with acetyl-β-methylcholine or oxytocin (35). Full relaxation could also be induced by adding the local anesthetic drug subsequent to Ca^{2+} or the other stimulating agents. Epinephrine, as previously reported (32), also has the same effect as local anesthetics on contraction of the rat uterus. The efflux of Ca^{45} from depolarized rat uterus has been studied in our laboratory. Addition of 0.3 to 1.0 mM Ca^{40} to calcium-free K_2SO_4 solution produced a large increase in the rate of Ca^{45} escape from the muscle, similar to the Ca^{40}-Ca^{45} self-exchange reaction in frog sartorius. Local anesthetics markedly inhibited the Ca^{45} efflux, whereas epinephrine was without effect (35). Therefore inhibition of contraction in depolarized rat uterus, by local anesthetics, may be due to reduction of Ca^{2+} permeability or inhibition of calcium dissociation from cellular binding sites.

Action of Local Anesthetics on Membrane Excitability

The experiments described in this paper do not directly concern the propagation of impulses in either muscle or nerve. On the other hand, the influence of local anesthetics on cellular calcium mobility is such that its relationship to their membrane stabilizing action should be discussed. A number of investigators have observed that inhibition of excitation in nerve and muscle by local anesthetics is characterized by a prevention of the increased sodium conductance responsible for generation of the action potential, as determined by the voltage-clamping technique (36, 37) or analysis of changes in the appearance of the intracellularly recorded action potential (2, 3). Depression of potassium permeability has also been demonstrated, indirectly, by inhibition of muscle swelling (38) and depolarization (38, 39) in high K^+ solutions, and analysis of conductance changes in voltage-clamped nerve fibers (36, 37). Other "stabilizers" such as certain antihistaminics and physostigmine act similarly (38, 40). Support for the electrophysiological findings comes from study of the fluxes of radioactive ions; Na^+ influx and K^+ outflux appear to be inhibited by cocaine, whereas Na^+ outflux and active K^+ influx were unaffected (1, 41, 42). The observation that local anesthetic block of nerve (2, 43) and muscle (3) excitation is reversed by increasing the extracellular Na^+ concentration, whereas it is potentiated in nerve (with antihistaminics and physostigmine as well) by low Na^+ (44) adds further support to the hypothesis that these drugs act by competitive antagonism to the sodium ion.

What then is the relevance, if any, of the observed effects of local anesthetics on Ca^{2+} movements in muscle to the mechanism by which they block membrane excitation? Several lines of evidence suggest the distinct possibility of a direct relationship. Changes in permeability with membrane potential

have been attributed to changes in calcium content of the membrane (1, 45, 46). It is well known that both sodium and potassium permeability are significantly influenced by the calcium content of the medium. Decreasing extracellular Ca^{2+} increases Na^+ and K^+ conductance of muscle and nerve as determined by the voltage clamp method (47, 48) or measurement of radioactive ion fluxes (49). A fivefold decrease in Ca^{2+} was equivalent to a 10 to 15 mv depolarization in its effect on Na^+ and K^+ conductance (47). Reduction of Ca^{2+} in the medium increased the loss of K^+ from striated muscle (50) and vertebrate nerve (51). Increasing Ca^{2+} decreases Na^+ and K^+ permeability in squid axon (47). A rapid increase in Ca^{2+} (80 mM) was found to repolarize the electrically stimulated toad nerve membrane (52), an action which was attributed to replacement of monovalent ions bound to negative sites in the membrane. Direct evidence for a competition between Na^+ and Ca^{2+} for tissue binding sites has been obtained in cardiac (53, 54) and striated (55) muscle.

These observations, as well as others not referred to, have led to proposals by many workers concerning the role of calcium in the regulation of membrane permeability and excitability which are essentially similar in their general features (1, 52, 56–60). Displacement of bound membrane calcium has been suggested as a prerequisite for increased Na^+ and K^+ permeability during excitation. Cations have been viewed as competing with each other for anionic sites in the membrane, divalent ions (Ca^{2+}, Mg^{2+}) being preferentially bound in the resting state. Excitation presumably results in displacement of the divalent ions[4] with subsequent exchange for the monovalent ions, rapid increase in Na^+ permeability, and generation of the spike. Local anesthetics appear to act by reducing the availability of sites for Na^+ entry during depolarization (36, 37, 66). However, an alternative explanation for local anesthetic action is possible, consistent with the data in this paper, and other unpublished data obtained in this laboratory (35): by primarily inhibiting release of calcium from the sites to which it is bound in the membrane, local anesthetics may prevent the *secondary* changes in Na^+ and K^+ permeability thereby preventing propagation of the wave of excitation along the cell membrane.

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[4] Increased Ca^{45} entry accompanying excitation, without a corresponding increase in efflux, has been noted in squid axons (61, 62) whereas an increase in efflux as well has been reported in frog nerve (63) and muscle (64). Also prevention of KCl depolarization of muscle by local anesthetics was associated with inhibition of Ca^{45} efflux (65).

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