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The Influence of Amino-Reactive Substances on Contraction Threshold of Frog Skeletal Muscle

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Summary. The action of the amino-reactive substances pyridoxal phosphate, 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonic acid and 2,4,6-trinitrobenzene sulfonic acid on the contraction threshold, taken as parameter for the initiation of contraction, was investigated in fibers of the sartorius muscle of the frog. The contraction threshold was shifted by 1 to 11 mV to *more negative* potentials with 1 to 20 mM PDP. Similar shifts from 2 to 17 mV were produced by 0.66 to 20 mM SITS. The threshold shift was only partially reversible. The shift of the contraction threshold obtained with 2 mM SITS was nearly constant at different $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ from 1.5 to 50 mM with a tendency to increase at higher divalent cation concentration. TNBS had no effect on the contraction threshold.

The action of PDP and SITS on the contraction threshold was successfully described by the surface charge model used earlier to explain the effect of lanthanum, neuraminidase and ruthenium red on the contraction threshold (M. Dörrscheidt-Käfer, *Pfluegers Arch.* **380**:171–179, 181–187, 1979; *J. Membrane Biol.* **62**:95–103, 1981). Here it was assumed that PDP and SITS bind to *positive* fixed charges on the surface of the T-tubular wall. This results in a shift of the calculated surface potential to more negative values which is thought to account for the measured shift of the contraction threshold.

Key Words: Frog skeletal muscle · contraction threshold · amino-reactive substances

Introduction

In frog skeletal muscle the initiation of contraction investigated by various methods has been reported to depend on the ionic composition of the surrounding bath (Costantin, 1968; Kao & Stanfield, 1970; Dörrscheidt-Käfer, 1976, 1979*a*, 1981; Shlevin, 1979). Shlevin (1979) found by using voltage-clamp techniques that the charge movements within the triadic membrane of the T-tubulus, which are thought to be related to contraction in some way, exhibit a qualitatively similar dependence on $[Ca^{2+}]_o$ and pH_o as the contraction threshold obtained with a constant-current method (Dörrscheidt-Käfer, 1976).

The relationship between contraction initiation and the ion concentrations in the bath was ascribed to the interaction of the cations with negative surface charges fixed on the outer surface of the T-tubular wall at the triad, the probable site of coupling between excitation and contraction (Peachey, 1968). The quantitative description made use of the theory of the "diffuse double layer" (Grahame, 1947). Binding of the ions in the bathing medium to charges fixed on the membrane surface also was considered (Dörrscheidt-Käfer, 1976; *see also* Hille, Woodhull & Shapiro, 1975; Shlevin, 1979). For the model calculation it proved necessary to consider at least three groups of fixed charges with different dissociation constants to describe the pH dependence of the contraction threshold adequately (Dörrscheidt-Käfer, 1976). Two groups were taken to be negatively charged and one as positively charged. One of the negative groups has been analyzed to be the carboxyl group of sialic acid forming part of the surface coat of animal cells (Dörrscheidt-Käfer, 1979*a*, *b*).

The problem of the existence of free positive charges at the membrane surface, and of their possible role in biological functions is still of major interest (Bangham, 1972). By use of amino-reactive substances such as SITS[1] and TBNS, Knauf and Rothstein (1971) presented evidence that amino groups at the outer side of human erythrocyte membranes control anion permeability. In 1978, Vaughan and Fong reported the inhibitory action of SITS on chloride permeation in *Xenopus* skeletal muscle. This finding was the first direct evidence that free amino groups on the membrane of skeletal muscle fibers take part in the regulation of ion permeability.

Thus it was of interest whether amino groups also play a role in excitation-contraction coupling

[1] Abbreviations: SITS – 4-Acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonic acid; PDP – Pyridoxal phosphate; TNBS – 2,4,6-Trinitrobenzene sulfonic acid.

of the skeletal muscle of the frog. The experiments were performed with SITS, PDP and TNBS.

Preliminary reports have already been published (Dörrscheidt-Käfer, 1980*a, b*).

Materials and Methods

Experiments were performed with the sartorius muscle of cold-adapted *Rana temporaria*. Dissected muscles were let to stand at room temperature for at least $1\frac{1}{2}$ hr. They were then transferred to a lucid Perspex® chamber of 4 ml volume and transilluminated by polarized light to improve visualization of contraction observed through a binocular microscope at 40-fold magnification. An individual fiber was impaled by two glass microcapillaries, one for stimulation and the other for potential recording. The bevelled electrodes had resistances of 15 to 25 MΩ and tip potentials of less than 5 mV. The fiber was stimulated with a 150-msec constant current pulse at 1 per second. To determine the threshold of contraction current strength was increased from subthreshold values to a superthreshold value. Current strength then was decreased until the observed local contraction of the fiber was about to disappear. The corresponding membrane potential was defined to be the *contraction threshold*. The procedure was repeated several times to ensure the contraction threshold to be constant. Potential and current traces were photographed and further evaluated. Up to 60 fibers of one muscle could be tested by this method.

Test solutions contained, in general (mM): 123 choline Cl, 2.5 KCl, 5 Tris (Tris(hydroxymethyl)aminomethane) and 1.5 or 5 $CaCl_2$ or $MgCl_2$. With 15 or 50 mM $CaCl_2$, or $MgCl_2$, choline Cl was reduced to 90 or zero mM, respectively, and the osmolarity was kept constant by addition of sucrose. The value of pH 9 was adjusted with NaOH. The addition of PDP (1 to 20 mM), SITS (0.66 to 20 mM), or TNBS (3 to 10 mM), as solid substances, resulted in a drastic shift of pH to acidic values, so that NaOH had to be used to readjust the pH to 9. With 10 and 20 mM SITS the amount of NaOH necessary to yield the appropriate pH often elicited action potentials because of the high Na-content, so tetrodotoxin (TTX, 10^{-6} M) had to be added as well.

In some experiments, after incubating the muscle with PDP, $NaBH_4$ or $NaCNBH_3$ was added with the idea of making the PDP effect irreversible by reduction of the Schiff base formed by PDP and a primary amino group (Cabantchik et al., 1975). In most cases this fixation treatment proved to be without effect.

The normal solution flow of 15 ml per min was arrested for drug application for 20 to 30 min, and the drug-containing solution (test solution with the appropriate divalent cation concentration, pH 9.0) was quickly exchanged for the respective drug-free test solution. The action of PDP and SITS being partly irreversible, only one drug concentration was tested on a muscle.

Contraction threshold values are given as measured potentials. Mean values were averaged from contraction threshold values of 8 to 15 fibers of one muscle unless otherwise stated. The shift in contraction threshold is the difference in the mean values of fibers of one muscle obtained in a given test solution ('control'), and in the same solution with a given drug concentration. Mean data are presented as the mean ± 1 SD. Where necessary, the significance was checked with the two-sided Student test, with significance level $P \leqq 1\%$.

All substances were reagent grade. PDP and SITS were obtained from SERVA Feinbiochemica (Heidelberg, F.R.G.), some charges of SITS from British Drug Houses (Poole, England), and TNBS from Sigma Chemical Company. Model calculations were done on a PDP12 computer (Digital Equipment Corp.). All experiments were performed at room temperature (22 to 24° C).

Results

Action of PDP and SITS on the Contraction Threshold

Figure 1 shows the results of two experiments with two amino-reactive substances: PDP (Fig. 1 *A*, 10 mM), and SITS (Fig. 1 *B*, 6.6 mM). The control solution in these and the following experiments was a test solution containing 5 mM Ca^{2+} and no

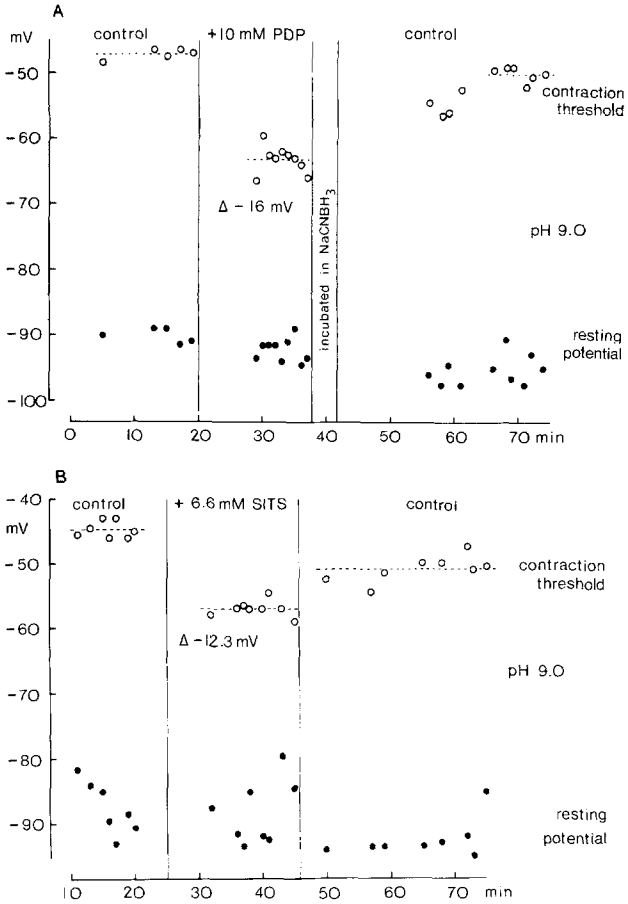

Fig. 1. *A*) Contraction threshold (open circles) and corresponding resting potentials (closed circles) of different fibers of one muscle in control solution (modified choline Ringer's solution with 5 mM Ca^{2+}) without and with 10 mM PDP. The fixation solution contained 100 mM $NaCNBH_3$ + NaOH as well. Mean values of contraction threshold are indicated by dashed lines. $\Delta - 16$ mV denotes the shift of the mean values to *more negative* potentials in the PDP-containing solution versus the control. pH 9.0 throughout. *B*) Values from a second muscle in control solution without and with 6.6 mM SITS at pH 9.0. In this case the mean shift of the contraction threshold to more negative potentials amounted to 12.3 mV

drugs. It may be seen that the contraction threshold was shifted by both agents to *more negative* potentials, the mean shift indicated by the difference of the mean threshold potentials in control solution and in the same control solution with the drug added (dashed lines in Fig. 1) being −16 mV with 10 mM PDP and −12.3 mV with 6.6 mM SITS. The effect of the drugs developing slowly the first contraction threshold value was read 5 min after drug application.

The fixation treatment with $NaCNBH_3$ (Fig. 1*A*) had almost no effect, as the contraction threshold returned to the control value within 30 min in PDP-free solution. With longer application the fixation could not be improved. On the other hand, the treatment with SITS resulted in a partly irreversible shift which varied considerably in different experiments (*see* Fig. 3), and which could not be abolished by longer periods in SITS-free solution (*cf.* Fig. 3).

The resting potential was only slightly affected by PDP and SITS. The most common effect was a shift to more negative potentials by 5 to 7 mV which could also be due to prolonged treatment with such a basic solution (*see below*). In Fig. 1, each pair of potentials (resting potential and contraction threshold) stems from a different fiber of one muscle, as it was not possible to record potentials of a fiber for more than 2 min in PDP or SITS.

It should be noted that experiments with PDP all were conducted at pH 9.0 since at lower pH PDP readily penetrates into the cell (Cabantchik et al., 1975; Rothstein, Cabantchik & Knauf, 1976). In one experiment at pH 6.5 10 mM PDP had no effect whatsoever on contraction threshold. For better comparison most experiments with SITS also were done at pH 9.0

To ensure that prolonged treatment of muscles at pH 9.0 had no consequence by itself on contraction threshold some specimens were kept as long as 2 hr in a test solution with 5 mM Ca^{2+} at pH 9.0. Every 5 min the contraction threshold and the resting potential of a different fiber was recorded. The contraction threshold appeared unchanged with −49 mV and the usual scatter of ±2 mV (Dörrscheidt-Käfer. 1979a), whereas the resting potential slowly progressed to more negative potential values.

Dose-Effect of PDP and SITS

Experiments like those in Fig. 1 were performed with a wide variation in PDP and SITS concentration to see whether the shift of the contraction threshold by these drugs is dose-dependent.

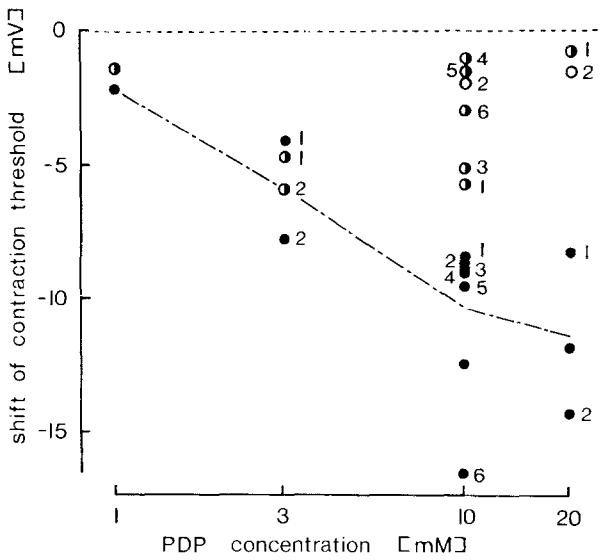

Fig. 2. Shift of the contraction threshold (mean values, *see* Materials and Methods) during treatment with PDP (closed circles) and after a washing period of 30 min in PDP-free solution with prior fixation (half-filled circles) and without fixation (open circles). PDP concentrations are given on the abscissa. Each symbol comes from a different muscle; numbers indicate values from the same muscle during and after PDP treatment, given separately for every PDP concentration. pH 9.0. The dashed line was drawn through the overall mean shift obtained at the respective PDP concentration

Results obtained with PDP are presented in Fig. 2. Here and in Fig. 3 only the mean shift of the contraction threshold is shown (closed circles). Each point comes from one muscle. With increasing PDP concentration the shift of the contraction threshold increased from −2 mV at 1 mM to −14 mV at 20 mM with a large variation at any given concentration. The dashed line was drawn through the overall mean shift at the respective PDP concentration. The remaining shift, after washing in PDP-free solution, is shown for preparations with prior fixation by half-filled circles, and for those without fixation by open circles. Values from one muscle during and after PDP treatment are indicated by identical numbers, separately for every concentration. There seems to be no consistent fixation effect.

Results of similar experiments with SITS are given in Fig. 3. The shift of contraction threshold was increased to −17 mV by increasing the SITS concentration to 20 mM (closed symbols in Fig. 3). Washing the muscles in SITS-free solution for 30 min resulted in a decrease of the shift with great scatter in the final value (open symbols in Fig. 3). Longer washing periods did not lead to a complete

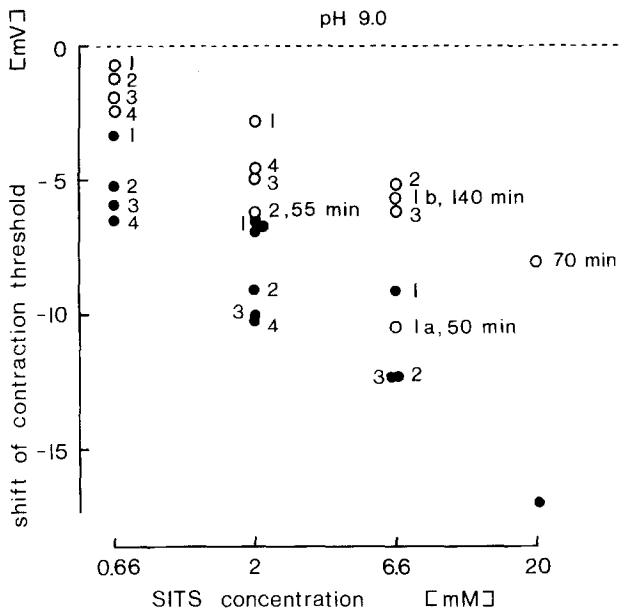

Fig. 3. Shift of the contraction threshold (mean values) during (closed symbols) and after (open symbols) treatment with different concentrations of SITS given on the abscissa. pH 9.0. Washing periods longer than 30 min are given next to the corresponding symbols

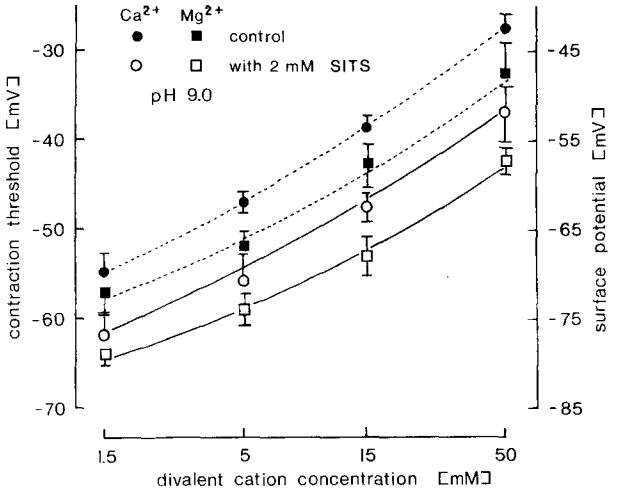

Fig. 4. Mean of contraction threshold ±SD of at least 10 fibers of several muscles in dependence on $[Ca^{2+}]_o$ (circles) and of $[Mg^{2+}]_o$ (squares; left-hand ordinate). The closed symbols are the controls, the values of the open symbols were obtained with 2 mM SITS added to every solution (*see* Materials and Methods for solutions). pH 9.0. The relationship between the surface potential (right-hand ordinate) and the concentration of $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ in the absence (dashed curves) and presence (smooth curves) were calculated by the surface charge model with parameters given in the Table (*see* text for further explanation)

return to the control values (*see* values after 2 mM and 55 min wash-out, after 6.6 mM and 140 min and after 20 mM and 70 min wash-out). Experiments performed at pH 5.5 and 7.2 yielded identical results, which is consistent with the idea that SITS is a nonpenetrating agent in contrast to PDP (Maddy, 1964).

The third amino-reactive substance, TNBS, tested at 3 and 10 mM, had no effect on the contraction threshold at pH 9.0.

Combined Effect of Divalent Cations and SITS on Contraction Threshold

The dependence of the contraction threshold on the concentration of calcium, $[Ca^{2+}]_o$, and of magnesium, $[Mg^{2+}]_o$, is shown in Fig. 4. The control values (closed symbols) show the familiar behavior: with increasing $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ (from 1.5 to 50 mM, pH 9.0) the contraction threshold was shifted to less negative potentials with Mg^{2+} being half as effective as Ca^{2+} (Dörrscheidt-Käfer, 1976, 1979*a, b*). The addition of 2 mM SITS to these solutions resulted in a shift of the contraction threshold to more negative values at all $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$, the shift ranging from −6.7 to −9.4 mV with a tendency to increase at higher divalent cation concentration (open symbols in Fig. 4).

Discussion

The results obtained with the amino-group-specific substances PDP and SITS support the idea that amino groups play a significant role in the regulation of the initiation of contraction.

In erythrocytes, the anion transport system can be inhibited by SITS and PDP as well as by TNBS (Knauf & Rothstein, 1971; Cabantchik et al., 1975; Barzilay, Ship & Cabantchik, 1979), but PDP and TNBS were reported to gradually penetrate into the cells (Knauf & Rothstein, 1971; but *see* Foulks & Perry, 1979). In the present experiments to avoid complications, the penetration of PDP, which forms a Schiff-base with primary amino groups, was abolished by increasing the pH to 9.0. The case of TNBS seems to be more complex (Knauf & Rothstein, 1971). The lack of effect of PDP at pH 6.5 and of TNBS at pH 9.0 on contraction threshold is most easily explained by their ability to penetrate into the fibers.

It should be noted that in all instances the reaction was slow, taking up to 5 min for the full effect. Furthermore, relatively high concentrations of PDP and SITS were necessary to produce a maximal effect. This is in favor of a physical reaction of these drugs with sites on the membrane, a reaction which seems partially irreversible. At the highest concentrations used (20 mM), the mechani-

cal stability of the membrane became very poor resulting in spontaneous contractures of fibers not injured by impalement. Another aspect is the great variability in the shift, with any one concentration (*see* Figs. 2 and 3). These features can be explained assuming the reaction site of PDP or SITS to lie below the negatively charged surface coat so that the anions PDP and SITS are impeded from easy access to these sites.

The shift of the contraction threshold produced by PDP and SITS at pH 9.0 always was to *more negative* potentials. This effect is the same as that produced by decreasing $[Ca^{2+}]_o$ or increasing pH. Thus the *addition* of PDP and SITS affected the contraction threshold in the same way as the *removal* of cations.

The latter effect has been described by assuming an influence of divalent cations and pH on the negative surface potential of the outer surface of the T-tubular wall in two ways: by increasing net negative charge density because of less binding to negative charges, and by less screening of these same charges. Both actions result in a shift of the surface potential to more negative values (Dörrscheidt-Käfer, 1976, 1979*a, b*, 1981). The shift of the contraction threshold due to the addition of the impermeant substances PDP and SITS may be accounted for in the same way. Known to be amino-reactive they may decrease the density of a positively charged group which would result in an increase of net negative charge density, thus shifting the surface potential to more negative values. Furthermore, being anions they could contribute negative charges to the overall charge density.

Calculations were done with the surface charge model used previously comprising two negatively charged groups and one positively charged group with a net charge density of $-5.9\times10^{-3}/\text{Å}^2$ (Dörrscheidt-Käfer, 1979*a, b*, 1981). Parameters were essentially the same. The positive charge density, σ_3, had to be slightly increased to afford a satisfactory description of the measurement, and therefore the charge density of one of the negative groups was increased by the same amount to keep the net charge density constant (Table). It was assumed that PDP and SITS bind exclusively to σ_3 with apparant dissociation constants K_{PDP} and K_{SITS}. The effect of Ca^{2+} and Mg^{2+} as well as the action of pH is as earlier described (Dörrscheidt-Käfer, 1979*a*).

The equation relating the free charge density σ_f to the ion concentrations of Ca^{2+}, Mg^{2+}, H^+ and $SITS^{2-}$ at the surface of the membrane now reads

Table. Parameters of the surface charge model

	Charge density	K_{Hi} (M)*	K_{Ca} (m)*	K_{Mg} (M)*	K_{SITS} (M)*	K_{PDP} (M)*
σ_1	$-3.1\cdot10^{-3}/\text{Å}^2$	$1.78\cdot10^{-3}$	4.5	4.5	∞	∞
σ_2	$-4.8\cdot10^{-3}/\text{Å}^2$	$7.1\cdot10^{-5}$	4.5	∞	∞	∞
σ_3	$+2.0\cdot10^{-3}/\text{Å}^2$	$1.0\cdot10^{-9}$	∞	∞	$5\cdot10^{-6}$	$3\cdot10^{-4}$

* Apparent dissociation constants for hydrogen ions (K_{Hi}), calcium (K_{Ca}), magnesium (K_{Mg}), SITS (K_{SITS}), and PDP (K_{PDP}). Net charge density amounts to $-5.9\times10^{-3}/\text{Å}^2$. The surface potential calculated for the control solution with 5 mM Ca^{2+}, pH 9.0, was -62 mV.

$$\sigma_f=\frac{\sigma_1}{1+\frac{[H^+]_s}{K_{H1}}+\frac{[Ca^{2+}]_s}{K_{Ca}}+\frac{[Mg^{2+}]_s}{K_{Mg}}}+\frac{\sigma_2}{1+\frac{[H^+]_s}{K_{H2}}+\frac{[Ca^{2+}]_s}{K_{Ca}}}+\frac{\sigma_3\left(1-\frac{[SITS^{2-}]_s}{K_{SITS}}\right)}{1+\frac{K_{H3}}{[H^+]_s}+\frac{[SITS^{2-}]_s}{K_{SITS}}} \tag{1}$$

with σ_1, σ_2 charge density of the two negatively charged groups, σ_3 charge density of the positively charged group, and K_i the respective dissociation constants of the i-th ion. The ion concentrations at the membrane surface, C_{is}, were calculated with the Boltzmann relation

$$C_{is}=C_i\exp-z_i\,\psi_o\,F/RT \tag{2}$$

where C_i denotes the concentration of the i-th ion in the bath, z_i its valency, ψ_o the surface potential. F, R and T have their usual meaning. The screening ability of the ions was described by the Grahame equation (Grahame, 1947) with

$$\sigma_f=\frac{1}{k}\left\{\sum_{i=1}^{n}C_i\exp(-z_i\psi_o F/RT)-1\right\}^{1/2}. \tag{3}$$

The compound constant k comprises the dielectric constant ε, permittivity of free space ε_o, Avogadro's number N, and F, R, T. For 22 °C: $k=272$ and $RT/F=25.3$ mV.

The dependence of the surface potential ψ_o on the ion concentration in the bath may be obtained by equating Eqs. (1) and (3). Changes of the surface potential thus calculated are thought to reflect the measured changes of the contraction threshold. To justify this one assumption is necessary. The internal surface potential and the electric field within the membrane, which depends on the mem-

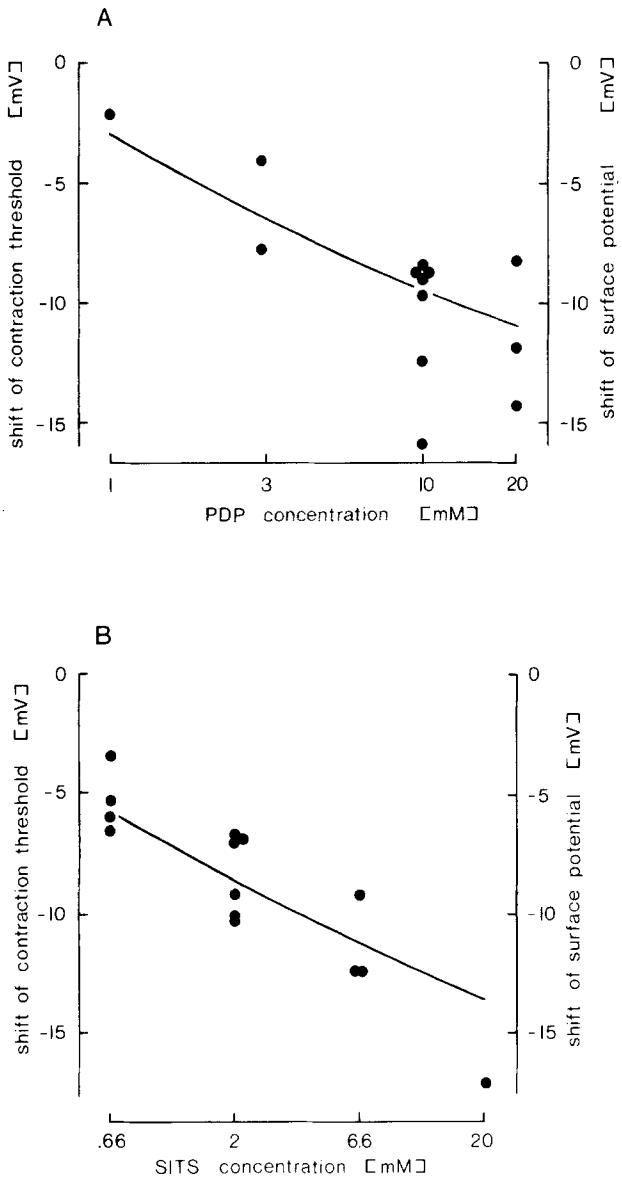

Fig. 5. Shift of the concentration threshold *A)* versus PDP concentration and *B)* versus SITS concentration. Closed circles are the same as in Figs. 2 and 3. The smooth curve shows the shift of the surface potential as calculated by the surface charge model with parameters given in the Table

brane potential and the external and the internal surface potentials, have to be constant at the contraction threshold under the experimental conditions used.

The surface potential was calculated as a function of the concentration of PDP^- and $SITS^{2-}$ with the parameters presented in the Table (Fig. 5*A* & *B*, continuous curves), and as a function of $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ without and with 2 mM SITS (Fig. 4, dashed and continuous curves). The measured mean shifts of the contraction threshold are nearly congruent with the calculated curves (Fig. 5*A* and *B*). The effect of SITS at different $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ also is successfully described by the surface charge theory (Fig. 4).

Thus it seems plausible to argue that PDP and SITS affected the contraction threshold by binding to amino groups on the surface of the T-tubular wall hereby altering electrical properties by the same mechanism as divalent cations and pH, but with the opposite effect because of their reaction with *positive* fixed charges.

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