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CHARACTERIZATION OF "DEPOLARIZATION"-INDUCED CALCIUM RELEASE FROM SARCOPLASMIC RETICULUM IN VITRO WITH THE USE OF MEMBRANE POTENTIAL PROBE[1]

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SUMMARY In the triad, the complex of transverse (T) tubule and sarcoplasmic reticulum (SR) Ca^{2+} release is induced from SR by mediation of the T-tubule. We report here evidence that this Ca^{2+} release is produced by depolarization of the T-tubule moiety. Thus, we found that the amount of [^{14}C]SCN^- taken up by T-tubules and triads (but not that by SR) increased upon incubation with (K, Na) gluconate, Mg ATP, indicating that the T-tubule was polarized making the lumenal side (equivalent to the extracellular side of an intact muscle fiber) more positive. Upon mixing with choline chloride, the procedure to induce Ca^{2+} release, [^{14}C]SCN^- uptake decreased, indicating that the T-tubule became depolarized. Activation of the T-tubule polarization by Na^+ and prevention of it by digoxin [inhibitor of the (Na^+, K^+) pump], respectively, led to activation and inhibition of choline chloride-induced SR Ca^{2+} release.

How transient changes in the T-tubule membrane potential lead to Ca^{2+} release from SR is one of the most important unresolved questions in muscle physiology (1, 2). Since ionic replacement of (K, Na) gluconate of triad-containing vesicles with choline chloride led to a rapid Ca^{2+} release from SR by mediation of the attached T-tubule (3), this system has been considered as a suitable model for studying the above question at a molecular level. However, it has remained unsolved whether the T-tubule membrane is depolarized when Ca^{2+} release is triggered as in the case of physiological e-c coupling. We report here evidence,

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The abbreviations used: e-c coupling, excitation-contraction coupling; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; MES, 2-(N-morpholino)ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SR, sarcoplasmic reticulum; T-tubule, transverse tubule.

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with the use of the potential-dependent movement of the membrane-permeable tracer [^{14}C]SCN^-, that the SR Ca^{2+} release is mediated in fact by depolarization of the T-tubule membrane.

EXPERIMENTAL PROCEDURES

Preparations: The triad-containing microsomal fraction was prepared from rabbit leg and back muscles by differential centrifugation as described previously (5). After the final centrifugation, the sedimented fraction was homogenized in a solution containing 0.3 M sucrose, 0.15 M K gluconate, proteolytic inhibitors (0.1 mM PMSF, 10 µg/ml aprotinin, 0.8 µg/ml antipain, 2 µg/ml trypsin inhibitor) and 20 mM MES (pH 6.8) at a final protein concentration of 20-30 mg/ml. T-tubules and SR were separated by French press treatment followed by sucrose gradient centrifugation as described previously (3). The vesicular fractions were sedimented and homogenized in a solution containing 0.3 M sucrose, 0.15 M K gluconate, the proteolytic inhibitors and 20 mM MES (pH 6.8). The preparations were quickly frozen in liquid nitrogen and stored at -70°.

Induction and monitoring of T-tubule-mediated Ca^{2+} release: For polarization of the T-tubule moiety and active Ca^{2+} loading of the SR moiety, the triad vesicles (1.6 mg/ml) were incubated in a solution containing K 0.15 M (K, Na) gluconate, 200 µM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM Na_2 ATP, 5.0 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase, 9 µM arsenazo III, and 20 mM MES, pH 6.8 (Solution A) at 22° (cf. refs. 3, 5). It was found that 0.15 M gluconate contained ~ 3 mM endogenous Na^+, as determined by atomic absorption spectrophotometry. For depolarization, 5 - 7 min after the addition of ATP, Solution A was mixed with an equal volume of Solution B containing 0.15 M choline Cl, 9 µM arsenazo III, 20 mM MES (pH 6.8), and the time course of Ca^{2+} release was recorded using a stopped-flow spectrophotometer system as described previously (5).

Membrane potential assay: The assay was based upon the following principle. When a potential difference ($\Delta\psi$) exists across a membrane, a permeable ion moves across the membrane until electrochemical equilibrium is established. At equilibrium,

$$\Delta\psi = -(RT/zF)\ln(C_i/C_o) \quad \text{... Equation 1}$$

where C_i and C_o are the intravesicular and extravesicular concentrations of the permeable ion, respectively (review: ref. 6).

Then, general protocol of filtration assay of [^{14}C]SCN^- uptake was as follows. The basic reaction mixture contained 0.15 M (K, Na) gluconate, 13.7 µM [^{14}C]KSCN (56 mCi/mmol, Amersham), 5 mM $MgSO_4$, 1.0 mM EGTA, 20 mM MES (pH 6.8), and various vesicular fractions - triad (3.0 mg/ml), T-tubule (0.5 mg/ml), or SR (3.0 mg/ml). To create various levels of membrane potential, the vesicles were further treated as outlined in Table I. After allowing [^{14}C]SCN^- to redistribute to a stable level of C_i/C_o for 2 min ($t_{1/2}$ for establishing C_i/C_o leading to a stable potential was ~20 s), the reaction mixture was filtered through Whatman glass microfibre filter (Whatman type GF/F). The filter was immediately washed with a 5 ml volume of washing solution (0.15 M K gluconate, 20 mM MES, pH 6.8), air-dried, and the radioactivity retained on the filter was counted. The amount of [^{14}C]SCN^- uptake was determined by subtracting non-specific radioligand binding (determined in the absence of vesicles) from the total count.

The ([^{14}C]SCN^- uptake without ionophores)/([^{14}]SCN^- uptake with ionophore) ratio (**relative [^{14}C]SCN^- uptake**) was then calculated. If there is no potential-independent binding of [^{14}C]SCN^- to the membrane, the relative uptake value = C_i/C_o, because in the presence of added ionophores (viz. at zero potential) the intravesicular concentration of SCN^- = the extravesicular concentration of SCN^-. If there is non-specific binding, the relative uptake < C_i/C_o.

Table I. Procedures used to produce various levels of T-tubule membrane potential. Vesicles (triad, T-tubule, or SR) in the basic reaction mixture (0.15 M K gluconate, 3 mM endogeneous Na^+, 13.7 μM [^{14}C]KSCN (56 mCi/mmol), 1.0 mM EGTA, 5 mM $MgSO_4$, 20 mM MES, pH 6.8) were incubated at 22° for 3 min with various additions as indicated. One volume of the reaction mixture was mixed with nine volumes of dilution solution indicated. After allowing [^{14}C]SCN^- to redistribute for 2 min, the reaction mixtures were filtered, and the amount of the intravesicular [^{14}C]SCN^- was determined as described in Experimental Procedures.

Intended states	Additions to the basic reaction mixture	Composition of Dilution solution
Zero potential	10 μM valinomycin, 10 μM monensin	0.15 M K gluconate
Background potential	none	0.15 M K gluconate
Polarized	5 mM Na_2ATP	0.15 M K gluconate
Depolarized	5 mM Na_2ATP	0.067 M K gluconate & 0.083 M choline Cl

RESULTS

To create various levels of membrane potential, different procedures outlined in Table I were applied to each of three vesicular fractions (T-tubules, triads, and SR vesicles). Table II lists the relative [^{14}C]SCN^- uptake values (see Experimental Procedures) corresponding to various levels of membrane potential in these vesicles. As seen in Table II, upon incubation of T-tubules and triads with ATP in a (K, Na) gluconate-containing solution, the value of relative [^{14}C]SCN^- uptake became significantly higher than that without ATP, indicating that upon the incubation with ATP the membrane became polarized making the lumenal side of the vesicle (≡ the extracellular side in an intact muscle cell) more positive. Upon mixing with choline Cl-containing solution of the T-tubules or triads, which had been incubated with ATP (polarized vesicles), the relative uptake value decreased, indicating that the membrane became depolarized. Neither incubation with ATP nor mixing with choline Cl produced any appreciable effect on the [^{14}C]SCN^- uptake by the SR. These results suggest that both ATP-dependent membrane polarization and choline Cl-induced membrane depolarization occur in the T-tubule moiety, but not in the SR moiety. Although the

Table II. Relative [^{14}C]SCN^- uptake* by T-tubules, triads and SR vesicles at four potential levels (cf. Table I). *Relative value taking the uptake value at zero potential = 1.0.

Intended states	T-tubules	Triads	SR vesicles
Zero potential	1.0	1.0	1.0
Background potential	3.0 ± 0.6	1.5 ± 1.0	1.1 ± 0.9
Polarized	8.9 ± 1.5	6.1 ± 1.3	1.3 ± 0.5
Depolarized	1.9 ± 0.8	2.0 ± 1.1	1.4 ± 0.8

The numbers: average ± standard deviation (n=9, triads; n=7, T-tubules; n=6, SR vesicles).

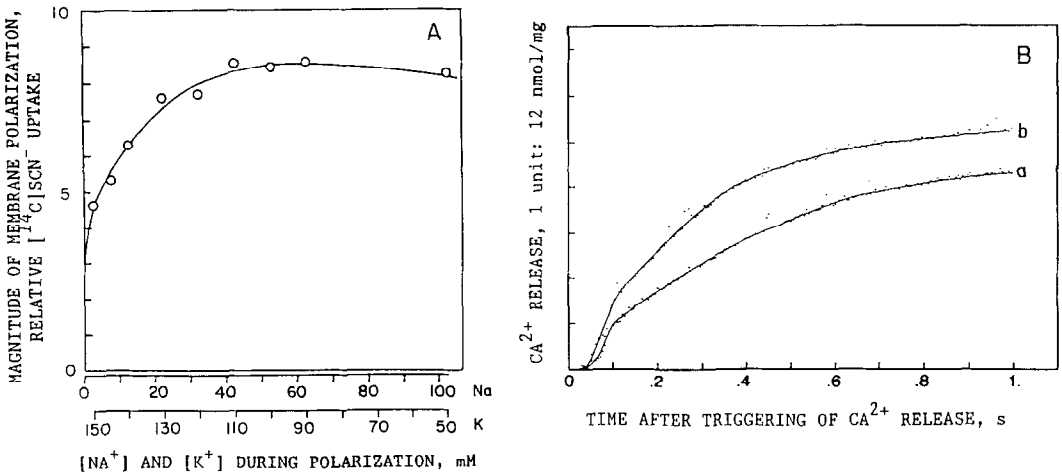

Fig. 1. The magnitude of T-tubule membrane polarization, as determined by the ATP-dependent [^{14}C]SCN$^-$ uptake by triads, increased with an increase of the Na^+ concentration during polarization (A), leading to activation of choline Cl-induced Ca^{2+} release (B). For the assay of ATP-dependent polarization (A), triads in the basic reaction mixture (see Experimental Procedures) were incubated with 5 mM K_2ATP in the presence of various concentrations of Na^+ (added Na^+ plus 3 mM endogeneous Na^+) and K^+ for 3 min. The reaction mixture was diluted ten times with 0.15 M K gluconate, 20 mM MES (pH 6.8). Immediately after dilution, one ml portion of the mixture was filtered through Whatman glass microfibre filter (Whatman type GF/F), and the [^{14}C]SCN$^-$ uptake was determined as described in Experimental Procedures. For choline Cl-induced Ca^{2+} release assay (B), triads were incubated in Solution A containing various concentrations of Na^+ and K^+ (see below), and Ca^{2+} release was induced by dilution with choline Cl and monitored. **curve a:** 4 mM Na^+, 149 mM K^+; **curve b:** 54 mM Na^+, 99 mM K^+. The potential data were obtained by averaging two experiments, and each stopped-flow trace was obtained by signal-averaging a total of ~90 traces originating from four triad preparations.

relative uptake value is virtually indistinguishable between the T-tubule and the triad (Table II), the specific amount of [^{14}C]SCN$^-$ uptake, in pmol/mg protein, is much larger in purified T-tubules (e.g. 28.0, at C_o=1.37 μM in the polarized state) than in triads (viz. 3.0) under equivalent conditions. This further supports the above notion that the potential-dependent movement of [^{14}C]SCN$^-$ is localized in the T-tubule membrane.

Little information is available about the dependence of the T-tubule polarization on [Na^+] and [K^+] during incubation with ATP. The [Na^+] used for T-tubule polarization in skinned fiber experiments ranges from several mM (7, 8) to ~35 mM (9). In our previous Ca^{2+} release experiments with triad vesicles, the [Na^+] was not more than 4 mM even if the amount of endogeneous Na^+ (~ 3 mM, see Experimental Procedures) is considered. In the experiments shown in Fig. 1A, the [Na^+]/[K^+] ratio in the polarizing solution was varied while keeping the sum of [Na^+] and [K^+] constant (153 mM). An appreciable level of membrane polarization was achieved with several mM Na^+, the magnitude of polarization further increased with [Na^+], and leveled off at approximately 30 mM. Fig. 1B shows that the increase in the magnitude of polarization with [Na^+] led to an

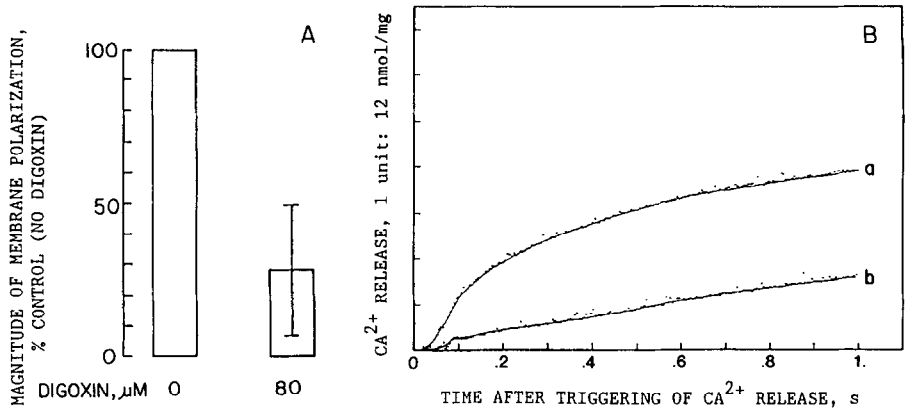

Fig. 2. Digoxin prevented membrane polarization (A), and consequently choline Cl-induced Ca^{2+} release was inhibited (B). In both A and B, the treatment for polarization (in the presence of 10 mM Na^+ and 143 mM K^+) was performed with or without addition of digoxin (80 μM added). Then, the magnitude of T-tubule polarization and choline Cl-induced Ca^{2+} release were investigated as described in the legend to Fig. 1. The data (both potential and stopped-flow) were obtained by averaging four experiments; the bar in the $[^{14}C]SCN^-$ uptake data represents the standard deviation. **Curve a:** control (no digoxin added); **curve b:** with 80 μM digoxin added.

increase of the choline Cl-induced Ca^{2+} release. Conversely, incubation with ATP in the presence of 80 μM digoxin, a membrane-permeable analog of ouabain, prevented membrane polarization (Fig. 2A), and consequenly choline Cl-induced Ca^{2+} release was inhibited (Fig. 2B).

DISCUSSION

Two-step treatment of triads - (i) incubation of the vesicles with ATP in the presence of (K, Na) gluconate and Ca^{2+}, and (ii) dilution with choline Cl - leads to rapid Ca^{2+} release from SR by mediation of the attached T-tubule (3, 4). The most important finding in this paper is that uptake of $[^{14}C]SCN^-$ used as an indicator of membrane polarization increased upon incubation of T-tubules or triads with ATP, while it decreased upon dilution with choline Cl. This indicates that the membrane was polarized by treatment (i), and depolarized by treatment (ii).

Importantly, the potential-dependent changes of the $[^{14}C]SCN^-$ uptake occur in T-tubules and triads, but not in the SR vesicles. Furthermore, the magnitude of polarization increased with $[Na^+]$ and decreased with digoxin, which are specific activator and inhibitor, respectively, of the (Na^+, K^+)-pump localized in the T-tubule. Thus, the potential changes detected with the $[^{14}C]SCN^-$ probe seem to represent an event localized within the T-tubule moiety of the triad.

Since the relative uptake (the parameter used to express $[^{14}C]SCN^-$ uptake in this study) is equal to, or smaller than, the C_i/C_o ratio (see Experimental Procedures), $\Delta\psi$ can be tentatively calculated from the present data using

Equation 1. For example, in the polarized T-tubule with relative uptake = 8.9 (Table II), $C_i/C_o \geq 8.9$; then $\Delta\psi \leq -55$ mV. In order to verify this estimate, however, we will have to perform appropriate calibration experiments. Although the use of the radio-ligand as a potential probe permits very sensitive assays of membrane potential at equilibrium as demonstrated here, the method will not be suitable for the studies of the time course of rapid changes in the potential, because potential-dependent movement of the probe across the membrane is rather slow.

In conclusion, the assay of potential-dependent movement of $[^{14}C]SCN^-$ has provided evidence that choline Cl-induced Ca^{2+} release is in fact produced by depolarization of the T-tubule moiety. Thus, the isolated triad vesicles retain both of the features essential for physiological e-c coupling; (i) depolarization of the T-tubule membrane, and (ii) resultant Ca^{2+} release from the SR.

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