Characteristics of Na^+-Ca^{2+} exchange in frog skeletal muscle

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1. Fluxes studies were carried out to investigate the Na^+-dependent outward movement of Ca^{2+} in intact frog sartorius muscle from *Leptodactylus ocellatus*, a preparation for which published data on the subject are sparse.

2. Under normal resting conditions the Na^+-Ca^{2+} exchange was not readily detectable.

3. When muscles were exposed to 4 mM caffeine, the rate of fractional loss of Ca^{2+} ($k_{Ca,o}$) increased by about 50%. Most of this increase exhibits characteristics typical of the Na^+-Ca^{2+} antiport working in the forward mode found in other cells.

4. The increase in $k_{Ca,o}$ promoted by caffeine was decreased by: (a) 72% in the absence of external Na^+ (Na_o^+); (b) 73% in Na^+-loaded muscles ($[Na^+]_i = 98$ mM); (c) 70% when fibres were depolarized to −27 mV ($[K^+]_o = 50$ mM); and (d) 80% in the presence of 5 mM amiloride.

5. Ni^{2+} (5 mM), an inhibitor of the Na^+-Ca^{2+} exchanger current, unexpectedly increased the caffeine-promoted rise in $k_{Ca,o}$. This effect of Ni^{2+} was associated with a concomitant caffeine-stimulated Ni^{2+} influx. In the absence of caffeine Ni^{2+}did not affect $k_{Ca,o}$.

6. It was concluded that: (a) under resting conditions the sarcolemmal Ca^{2+} pump suffices to handle the cytosolic calcium concentration ($[Ca^{2+}]_i$); (b) Na^+-Ca^{2+} exchange activity becomes apparent when $[Ca^{2+}]_i$ is substantially increased by caffeine-induced Ca^{2+} release from the sarcoplasmic reticulum; and (c) the blocking effect of Ni^{2+} on the current generated by a Na^+-Ca^{2+} exchange with a coupling ratio > 2 might actually represent a shift of the antiport mode toward an electroneutral $1Ni^{2+}-1Ca^{2+}$ exchange.

Na^+-Ca^{2+} exchange has been studied extensively in heart and vascular smooth muscle (see Sheu & Blaustein, 1992 for review), in barnacle muscle fibres (Rasgado-Flores & Blaustein, 1987; Rasgado-Flores, De Santiago & Espinosa-Tanguma, 1991) and in squid axons (see reviews by DiPolo & Beaugé, 1983, 1991). In skeletal muscle, on the other hand, the published data, whether obtained in intact cells (Caputo & Bolaños, 1978) or in membrane fractions (Gilbert & Meissner, 1982; Donoso & Hidalgo, 1989; Hidalgo, Cifuentes & Donoso, 1991) are limited. In most cells this exchange and the Ca^{2+} pump are the two mechanisms located in the cell membrane which contribute to maintain a cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of the order of 10^{-7} M. Depending on the Na^+ and Ca^{2+} electrochemical gradients, the system works as an extracellular Na^+– intracellular Ca^{2+} ($Na_o^+-Ca_i^{2+}$) exchanger (forward mode) or a $Na_i^+-Ca_o^{2+}$ exchanger (reverse mode). In all muscle cell types studied so far, the exchange was found to be electrogenic with a $3Na^+-1Ca^{2+}$ coupling ratio.

The purpose of this work was to characterize this transport system in intact frog skeletal muscle from *Leptodactylus ocellatus*. In our experiments, under resting physiological conditions the $Na_o^+-Ca_i^{2+}$ exchange was not readily demonstrable. However, when $[Ca^{2+}]_i$ was substantially increased, several properties of the exchanger became apparent in this preparation.

A preliminary account of some of the data reported here was presented at the 38th Annual Meeting of the Sociedad Argentina de Investigación Clínica (Hoya & Venosa, 1993).

METHODS

Experiments were performed on isolated small frog sartorius muscles from *Leptodactylus ocellatus*. Animals were chilled to full immobility in an ice–water mixture and subsequently double pithed. After dissection, muscles were fastened to *ad hoc* light stainless-steel holders by means of thin surgical thread attached to the tendons. In some control experiments the dorsal head of semi-

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Table 1. Composition of solutions (mM)

	Normal Ringer	A	B	C	D	E	F	G	H	I	J	K
NaCl	115·00	115·00	—	—	—	—	—	—	—	115·00	—	—
KCl	2·50	2·50	2·50	2·50	2·50	2·66	2·50	2·50	—	2·50	2·50	2·50
$CaCl_2$	1·80	0·10	1·80	1·80	1·80	—	1·80	1·80	—	—	—	0·10
$MgCl_2$	—	1·70	—	—	—	1·70	76·70	—	—	1·80	1·80	1·70
Na_2HPO_4	2·15	2·15	—	—	—	1·08	—	—	—	2·15	—	—
NaH_2PO_4	0·85	0·85	—	—	—	0·43	—	—	—	0·85	—	—
Choline chloride	—	—	115·00	—	—	—	—	—	—	—	115·00	115·00
LiCl	—	—	—	115·00	—	—	—	—	—	—	—	—
Sucrose	—	—	—	—	181·00*	12·00	—	—	—	—	—	—
TrisCl	—	—	5·00	5·00	5·00	—	5·00	120·00	5·00	—	5·00	5·00
Na_2SO_4	—	—	—	—	—	58·80	—	—	—	—	—	—
K_2SO_4	—	—	—	—	—	23·70	—	—	—	—	—	—
$CaSO_4$	—	—	—	—	—	1·56†	—	—	—	—	—	—
$LaCl_3$	—	—	—	—	—	—	—	—	60·00	—	—	—
EGTA	—	—	—	—	—	—	—	—	—	1·50	1·50	—

* Osmotically, 1 M NaCl = 1·6 M sucrose (Venosa, 1991).
† From the dissociation constants of SO_4^{2-} salts the ionic concentration of Ca^{2+} would be 0·1 mM.

tendinosus muscles was used. The compositions of the solutions (pH = 7·2) used are summarized in Table 1.

When needed, amiloride was added to the experimental media from a stock solution of the drug in dimethyl sulphoxide (DMSO) so that the concentration of DMSO in the experimental solutions was always less than 1%. Control experiments showed that this concentration of DMSO does not affect Ca^{2+} efflux. Similar precautions were taken with the stock solutions of monensin in ethanol (Hoya & Venosa, 1992). Caffeine was purchased from Fluka Chemie AG, Switzerland and all other drugs were purchased from Sigma Chemical Co. $^{45}Ca^{2+}$ and $^{63}Ni^{2+}$ were from New England Nuclear, USA.

Resting membrane potentials were measured by means of conventional electrophysiological techniques using glass microelectrodes filled with 3 M KCl (5–15 MΩ resistance).

Ca^{2+} efflux was measured using $^{45}Ca^{2+}$. Muscles were first loaded with $^{45}Ca^{2+}$ by bathing them in normal Ringer solution labelled with the isotope (1–10 μCi ml^{-1}) for 2·5 h. The preparations were prewashed for 80 min in unlabelled normal Ringer solution and the radioactivity released during this period was discarded. Subsequently, the preparations were washed in a series of tubes containing 3 ml of unlabelled media at intervals of 5–15 min. At the end of the experiment, muscles were dissolved overnight in 0·4 ml of HNO_3. After addition of 15 ml of scintillation cocktail (POPOP (1,4-di[2-(5-phenyloxazolyl)]benzene), 250 mg; PPO (2,5-diphenyloxazole), 5 g; ethanol, 500 ml; toluene, 2 l; Triton X-100, 1·25 l), the wash-out samples and dissolved muscles were assayed for radioactivity in a β-counter (Tracor Analytic 6892). Internal standards were used to correct for quenching. The rate of release of the isotope was calculated using a microcomputer and expressed in terms of rate of fractional loss ($k_{Ca,o}$; in units of min^{-1}).

Ni^{2+} influx was measured using a procedure similar to that employed previously to measure Ca^{2+} influx in vascular smooth muscle (Amado Cattaneo, Gende, Cingolani & Venosa, 1991). Briefly, one member of a pair of muscles from the same animal was exposed to solution A containing 5 mM Ni^{2+} ($NiCl_2$) labelled with $^{63}Ni^{2+}$ (12–16 μCi ml^{-1}). The other member of the pair was exposed to an identical radioactive solution in which 4 mM caffeine had been added. In all the experiments, the Ni^{2+} uptake period in these solutions lasted 15 min. At the end of the exposure period in radioactive solutions the preparations were washed for 2 h in a series of tubes as in the Ca^{2+} efflux experiments. The wash-out tubes contained an isotonic unlabelled La^{3+} medium (solution H) to facilitate the release of Ni^{2+} from extracellular binding sites and to minimize Ni^{2+} release from the intracellular compartment. Thus a reasonably clear separation of those two components of the release could be obtained.

Wash-out tubes and muscles were assayed for $^{63}Ni^{2+}$ in a β-counter, as described above for $^{45}Ca^{2+}$. Curves of the radioactivity remaining in the preparation as a function of the wash-out time were obtained by back-addition of the counts per minute in the muscle at the end of the run and in the wash-out tubes. After an initial fast release from the extracellular space, the decay of radioactivity in the muscles became monoexponential. This last component represents the release of Ni^{2+} from the intracellular compartment and its extrapolation to time zero is a measure of the amount of intracellular Ni^{2+} at the end of the loading period (see Fig. 8). From the specific activity of the loading solution, the exposure time, and the area of the superficial sarcolemma (430 cm^2 g^{-1}, T-tubules excluded; Venosa, 1991), the influx was calculated and expressed in terms of picomoles per square centimetre per second. Ni^{2+} was measured under two experimental conditions (protocol 1 and 2) which are described in Results.

The internal Na^+ concentration was determined by atomic absoprtion spectrophotometry as described before (Hoya & Venosa, 1992).

If not stated otherwise, values from identical experiments are expressed as means ± 1 S.E.M. Student's t test was used to estimate statistical significance of differences.

RESULTS

In frog semitendinosus muscle from *Rana pipiens*, Caputo & Bolaños (1978) showed that Ca^{2+} efflux was reduced by about one-third when Na^+ in the external medium was replaced by a non-permeant cation (choline) and that Ca^{2+} efflux fell by about 66% when, in addition to Na^+ withdrawal, external Ca^{2+} was omitted. These results, which agree with early data of Cosmos & Harris (1961), clearly suggested that under resting physiological conditions about one-third of the outward movement of Ca^{2+} represented an exchange with external Na^+ and another third with external Ca^{2+}.

Figure 1*A* shows the effect of replacing the external Na^+ by Li^+ (solution C) on a mole-per-mole basis. The absence of Na^+ produced a marked and transient increase in the $^{45}Ca^{2+}$ efflux rate coefficient ($k_{Ca,o}$). Similar results were obtained when external Na^+ was replaced by choline (solution B), Tris (solution G), Mg^{2+} (solution F), or sucrose (solution D). We suspected that the difference between our results and those of Caputo & Bolaños (1978) could be due to the fact that we used sartorius rather than semitendinosus muscle. However, in our hands, the response of $k_{Ca,o}$ to 0 mM $[Na^+]_o$ in semitendinosus muscles was similar to that observed in sartorii (data not shown). At the moment we cannot account for this discrepancy. One possibility is that it might be the expression of structural differences between the exchanger proteins of the two species.

When the Na^+ replacement was done in the virtual absence of Ca^{2+} ($[Ca^{2+}]_o = 0$, $[Mg^{2+}]_o = 1{\cdot}8$ mM, 1·5 mM EGTA, solutions I and J), the increase in $k_{Ca,o}$ did not occur (Fig. 1*B*). Moreover, the surge of Ca^{2+} efflux produced by 0 mM $[Na^+]_o$ was also absent in media where $[Ca^{2+}]_o$ was 0·1 mM (1·7 mM replaced by Mg^{2+} and no EGTA; solutions A and K). Figure 2 illustrates this point. Most experiments were carried out in the presence of this calcium

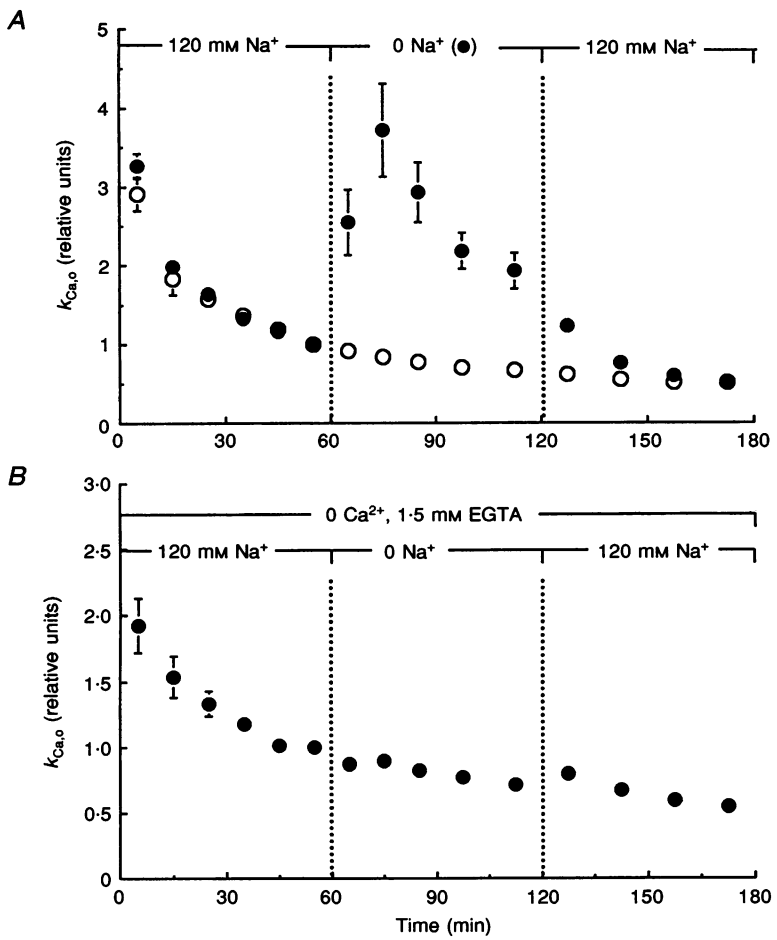

Figure 1. Effect of Na^+-free medium (Li^+ replacement, solution C) on $k_{Ca,o}$

A, data are normalized with respect to the last value of the first hour of the run and represent the mean from six paired (control, ○; experimental, ●) sartorii. *B*, the Na^+-free effect on $k_{Ca,o}$ shown in *A* is absent when $[Ca^{2+}]_o$ is virtually zero (solution J). The data represent the means of four identical experiments normalized with respect to the last value in solution I before changing to Na^+-free medium (solution J). In this and subsequent figures bars represent ± 1 S.E.M. (absent if smaller than symbol).

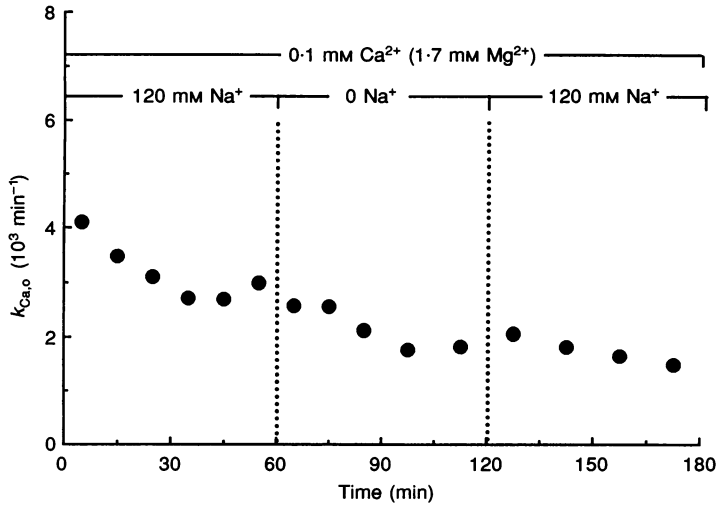

Figure 2. Effect of 0 mM $[Na^+]_o$ on $k_{Ca,o}$ in the presence of low $[Ca^{2+}]_o$

Representative experiment showing that in the presence of 0·1 mM $[Ca^{2+}]_o$ ($[Mg^{2+}]_o = 1·7$ mM), the change of $[Na^+]_o$ from 120 mM (solution A) to 0 mM (solution K) does not affect $k_{Ca,o}$.

concentration (a condition that favours the forward mode of the exchanger). It should be mentioned that single frog muscle fibres exposed to 0·1 mM $[Ca^{2+}]_o$ do not depolarize to any appreciable extent (Curtis, 1963). In the presence of this calcium concentration we found a mean membrane potential (V_m) of $-84·5 \pm 3·9$ mV (mean ± 1 S.D., $n = 89$) compared with a V_m of $-88·2 \pm 5·5$ mV (mean ± 1 S.D., $n = 31$) in the presence of 1·8 mM $[Ca^{2+}]_o$. In addition, the reduction of $[Ca^{2+}]_o$ to 0·1 mM (solution A) did not affect, *per se*, Ca^{2+} efflux (data not shown).

The lack of the Na^+-free effect on $k_{Ca,o}$ when $[Ca^{2+}]_o$ was absent or reduced to 0·1 mM suggests that in 0 mM $[Na^+]_o$ a $Ca_o^{2+}-Ca_i^{2+}$ exchange might be activated. Chen (1974) showed that, in barnacle muscle fibres, procaine blocks $Ca_o^{2+}-Ca_i^{2+}$ exchange and has little effect on Na^+-Ca^{2+} exchange. In control experiments, we tested the effect of another local anaesthetic, tetracaine, on the response of $k_{Ca,o}$ to Na^+-free medium and found that in Ringer solution ($[Ca^{2+}]_o = 1·8$ mM) containing 0·4 mM of the drug, the withdrawal of Na^+ (replaced by choline, solution B) produced no change in $k_{Ca,o}$ (data not shown). It is worth noting that this concentration of the anaesthetic did not affect Ca^{2+} efflux in normal Ringer solution.

It is apparent therefore that, under resting conditions, our preparations do not exhibit a detectable $Na_o^+-Ca_i^{2+}$ exchange.

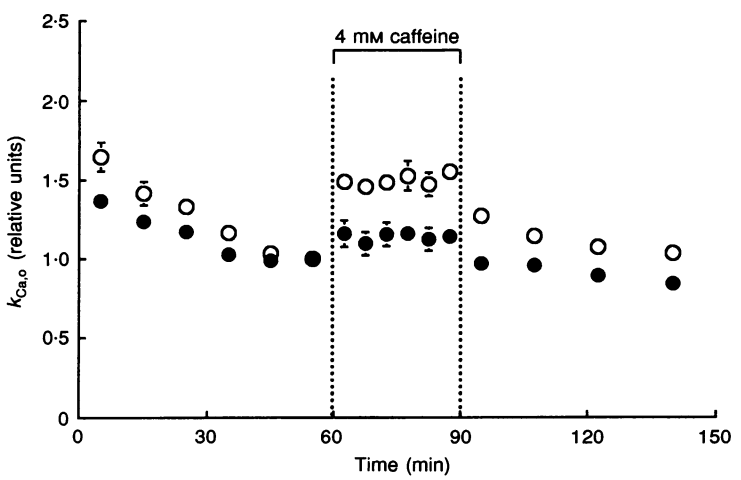

Figure 3. Effect of 4 mM caffeine on $k_{Ca,o}$

Effect of 4 mM caffeine in the presence (○) and absence (●) of normal (120 mM) Na^+ (solutions A and K). Data, normalized with respect to the last value before the 60 min mark, represent means from seven identical experiments using paired muscles. $P < 0·03$ for all paired circles during caffeine exposure.

Since in most cells Na^+–Ca^{2+} exchange behaves as a transport system of high transport capacity and low affinity for Ca_i^{2+}, it was thought that perhaps an increase in $[Ca^{2+}]_i$ could activate the exchange to a measurable extent. One simple way of increasing $[Ca^{2+}]_i$ in frog muscle fibres is to promote the release of this cation from the sarcoplasmic reticulum, typically by exposure to an adequate concentration of caffeine. If this manoeuvre were able to produce an increase in $k_{Ca,o}$, one might presume that it would be due to an activation of the exchanger, provided that this effect is: (1) diminished when the Na^+ gradient is reduced by either lowering $[Na^+]_o$ and/or increasing $[Na^+]_i$; (2) reduced by depolarization of the fibres; and (3) reduced by inhibitors, albeit not specific, like amiloride and Ni^{2+}. The following experiments were designed to test these hypotheses.

Effect of caffeine on $k_{Ca,o}$

Figure 3 shows the mean $k_{Ca,o}$ from seven sartorii exposed for 30 min to 4 mM caffeine in the presence of normal $[Na^+]_o$ and the response to the drug of their paired companions in Na^+-free medium. Clearly, the increase in $k_{Ca,o}$ produced by the drug is considerably larger in the presence of normal $[Na^+]_o$ than in its absence. In these experiments, in the presence of Na^+ (solution A), caffeine increased $k_{Ca,o}$ by 49·7 ± 1·5% while in Na^+-free medium (solution K) the increase was 11·4 ± 1·0% ($P < 0·03$ for all paired mean values during the application of caffeine in Fig. 3). As can be seen in Fig. 3 the absence of Na^+ in the external medium does not completely abolish the effect of caffeine on $k_{Ca,o}$. In 0 mM $[Na^+]_o$ and in the virtual absence of external Ca^{2+} (solution J) 4 mM caffeine still produced a small response (data not shown) which was not significantly

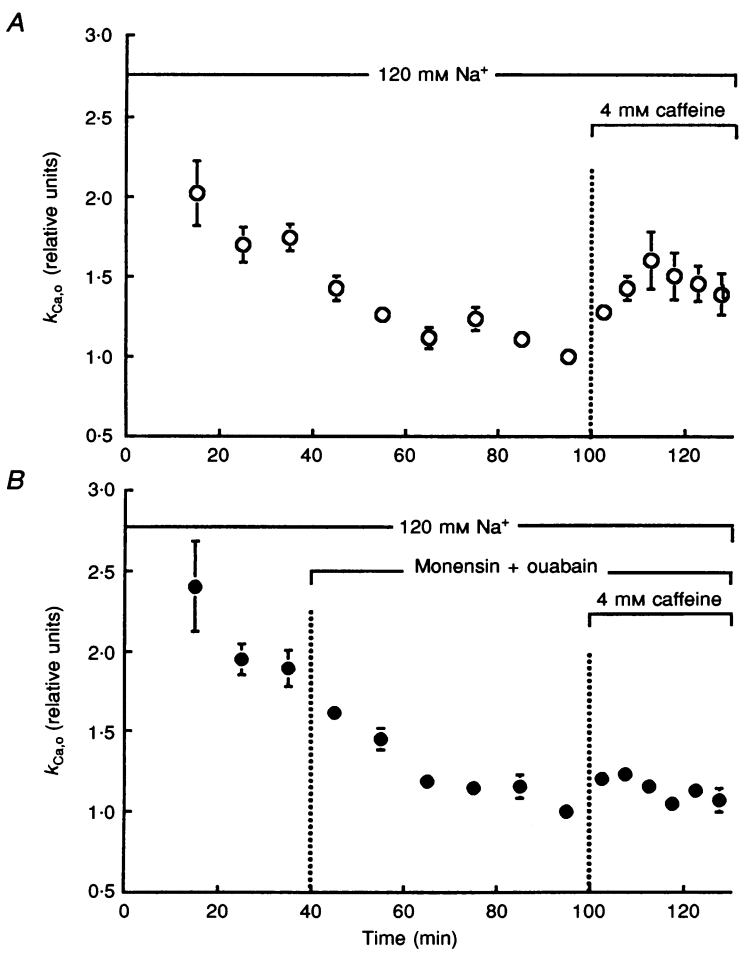

Figure 4. Effect of raising $[Na^+]_i$ on the response of $k_{Ca,o}$ to caffeine

A, $k_{Ca,o}$ as a function of the time in the presence of solution A. At the 100 min mark the preparations were exposed to 4 mM caffeine which produced the usual response. *B*, $k_{Ca,o}$ from paired muscles of those shown in *A*. At the 40 min mark monensin (40 μM) and ouabain (50 μm) were added to the medium (solution A), and, subsequently, as *A*, the preparations were exposed to caffeine (4 mM). The mean $[Na^+]_i$ of muscles in *B* at the end of the experiment was 98 ± 6·1 mM, as compared with a value of 13·5 mM in untreated muscles (Hoya & Venosa, 1992). Data from six identical experiments.

different from that observed in 0·1 mM $[Ca^{2+}]_o$, 0 mM $[Na^+]_o$, suggesting that the remaining effect seen in the absence of Na_o^+ might be due, at least in part, to an activation of the sarcolemmal Ca^{2+} pump.

If the observed increase in $k_{Ca,o}$ produced by caffeine in the presence of normal $[Na^+]_o$ (= 120 mM) was due to the activation of the exchanger working in the forward mode (Na_o^+–Ca_i^{2+}), it should be expected that an increase in $[Na^+]_i$, by a reduction in the driving force, would decrease the effect of caffeine on $k_{Ca,o}$. This indeed occurred and is shown in Fig. 4. The effect of caffeine was tested in paired sartorii. Thus one member of the pair was used as a control while its companion was exposed to 40 μM monensin, a Na^+ ionophore, and 50 μM ouabain to block the activation of the Na^+ pump by the increase in $[Na^+]_i$ due to monensin. As previously shown, this treatment produced a marked

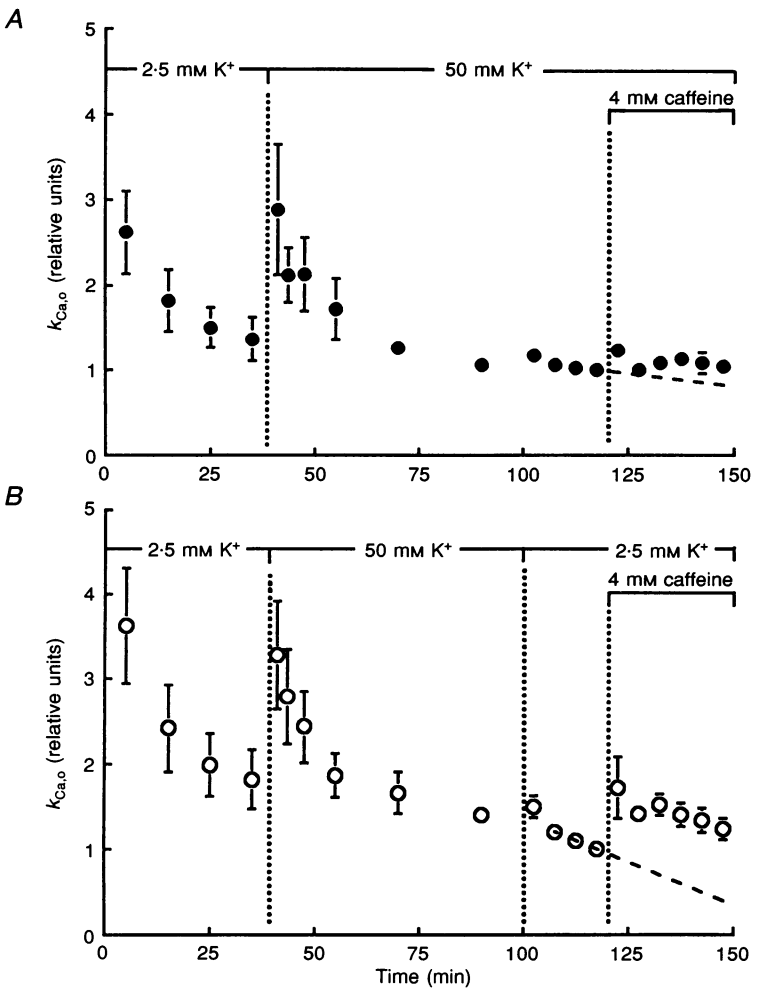

Figure 5. Depolarization reduces the response of $k_{Ca,o}$ to caffeine

A, muscles were depolarized to −27 mV ($[K^+]_o$ = 50 mM) at the 40 min mark (solution A changed to solution E at constant $[Cl^-]_o \times [K^+]_o$ = 300 mM^2). Once the transient increase of $k_{Ca,o}$ subsided, at the 120 min mark caffeine (4 mM) was added to the depolarizing medium. *B*, response to 4 mM caffeine in normally polarized companion muscles of those in *A*. These muscles were also exposed to 50 mM $[K^+]_o$ for 60 min. At the 100 min mark the muscles were repolarized before the exposure to caffeine at the 120 min mark. Note the substantial reduction in the response of $k_{Ca,o}$ to caffeine produced by depolarization (*A*). Data, normalized with respect to the last value before caffeine exposure, represent the means from four identical experiments using paired preparations. All solutions contained 0·1 mM ouabain to avoid any reduction in $[Na^+]_i$ due to the activation of the Na^+ pump in the presence of high $[K^+]_o$. The solutions also contained *d*-tubocurarine Cl (10^{-5} g ml^{-1}) to preclude the probable firing of action potentials due to the increased release of acetylcholine from the nerve terminals under these conditions. To account for the declining trend of $k_{Ca,o}$, the last three points before the exposure to caffeine were fitted to a straight line which was used as a baseline to estimate the changes in $k_{Ca,o}$. The mean increases in $k_{Ca,o}$ calculated from the last four points of the caffeine exposure were 67·3 ± 3·8 and 20·0 ± 3·1 % in control and depolarized muscles, respectively.

increase in $[Na^+]_i$ in relatively short periods (Hoya & Venosa, 1992). In six identical experiments the mean $[Na^+]_i$ in muscles exposed to the monensin–ouabain combination measured at the end of the experiment was 98 ± 6·1 mM as compared with a mean control value in this preparation of 13·5 ± 1·3 mM (Hoya & Venosa, 1992). It is apparent that the increase in $k_{Ca,o}$ produced by caffeine is significantly smaller in high $[Na^+]_i$ muscles than in the controls.

It has been shown (Kotsias, Venosa & Horowicz, 1984) that 3·5 mM caffeine produces a depolarization of about 6 mV which remains steady for at least 40 min. We confirmed this observation. In five muscles equilibrated in solution A, the addition of 4 mM caffeine produced a depolarization of 4·6 ± 0·8 mV in 37 ± 4 min, at which time ten to fourteen fibres per muscle were impaled. It seems possible that this small effect on V_m might be due, in part, to an inward current produced by the activation of the Na^+–Ca^{2+} exchange.

Effect of depolarization

Thermodynamics dictates that for a tightly coupled Na^+–Ca^{2+} counter-transport at equilibrium the following equation must hold:

$$n(E_{Na} - V_r) = z_{Ca}(E_{Ca} - V_r), \quad (1)$$

where E_{Na} and E_{Ca} are the equilibrium potentials for Na^+ and Ca^{2+} respectively, n is the Na^+–Ca^{2+} coupling ratio, V_r is the reversal potential and z_{Ca} is the Ca^{2+} valency (i.e. 2). In most preparations $n = 3$. In T-tubule membrane vesicles the effect of changing the membrane potential suggested a value of $n > 2$ (Hidalgo *et al.* 1991). Therefore the activity of the exchanger should be affected by the magnitude of the membrane potential. For $[Na^+]_o = 120$ mM, $[Na^+]_i = 13·5$ mM (Hoya & Venosa, 1992), $[Ca^{2+}]_o = 1·8$ mM and assuming $[Ca^{2+}]_i = 2 \times 10^{-7}$ M (Tsien & Rink, 1980; Harkins, Kurebayashi & Baylor, 1993), eqn (1) becomes:

$$V_r = 3E_{Na} - 2E_{Ca} = (3 \times 55) - (2 \times 115) = -65 \text{ mV}. \quad (2)$$

For $[Ca^{2+}]_o = 0·1$ mM, the calcium concentration that we used in these experiments, the calculated V_r is 9 mV. In the presence of this calcium concentration (and also in 1·8 mM $[Ca^{2+}]_o$) and V_m close to −85 mV, the inward driving force on Na^+ is greater than on Ca^{2+} ($3(E_{Na} - V_m) > 2(E_{Ca} - V_m)$), a condition which favours the Na_o^+–Ca_i^{2+} exchange (forward mode). Depending on the value of V_r, depolarization of the fibres would slow down or even reverse the Na_o^+–Ca_i^{2+} exchange. Figure 5 represents an attempt to test the effect of depolarization on $k_{Ca,o}$. Muscles were depolarized by exposing them to 50 mM $[K^+]_o$ (solution E). $[K^+]_o$ was changed, keeping the product $[K^+]_o \times [Cl^-]_o$ constant (300 mM^2) to minimize the time taken for the stabilization of V_m after a sudden change in $[K^+]_o$ (Hodgkin & Horowicz, 1959). In the presence of normal $[K^+]_o$ (2·5 mM; solution A), the mean V_m was −84·5 mV (see above). Under these conditions the system works in the forward mode, and the net driving force is:

$$3(E_{Na} - V_m) - 2(E_{Ca} - V_m) = 3(55 + 84·5) - 2(78 + 84·5)$$
$$= 93·5 \text{ mV}.$$

In the presence of 50 mM $[K^+]_o$, which in control experiments depolarized the fibres to −26·7 ± 1·3 mV (mean ± 1 S.D.; $n = 38$), the net driving force will be reduced to $3(55 + 26·7) - 2(78 + 26·7) = 35·7$ mV. Therefore, upon depolarization one would expect a measurable reduction in the activation of ${}^{Ca}k_o$ by caffeine. The data shown in Fig. 5 support this expectation. Figure 5*A* shows that, at the 40 min mark, the increase of $[K^+]_o$ from 2·5 to 50 mM produced a transient increase in $k_{Ca,o}$ with a decay half-time of about 5 min. After 80 min in this condition, muscles were exposed to 4 mM caffeine, and a rather small increase of $k_{Ca,o}$ (20·0 ± 3·1%) was observed in these depolarized muscles. The response of paired preparations in the presence of 2·5 mM $[K^+]_o$ is shown in Fig. 5*B*. Here the muscles were also exposed to 50 mM $[K^+]_o$ at the 40 min mark for a period of 60 min followed by a 20 min period in 2·5 mM $[K^+]_o$ to repolarize the muscles. The muscles were then exposed to 4 mM caffeine which produced a 67·3 ± 3·8% increase in $k_{Ca,o}$. The aim of this protocol was to subject the control muscles to conditions as close as possible to those of their paired companions in Fig. 5*A* mainly with regard to the transient increase in $k_{Ca,o}$ promoted by high $[K^+]_o$. This transient probably represents the exit, through different sarcolemmal pathways (i.e. Na_o^+–Ca_i^{2+} exchange, voltage-gated Ca^{2+} channels, active transport), of ${}^{45}Ca^{2+}$ released from the sarcoplasmic reticulum by T-tubule membrane depolarization. The effect of caffeine in Fig. 5*B* is clearly similar to the effects under control conditions shown in other figures, suggesting that the exposure to high $[K^+]_o$ for a relatively long period does not affect *per se* the stimulation of $k_{Ca,o}$ by caffeine in repolarized fibres. In this regard it is worth noting that in depolarized fibres ($[K^+]_o = 50$ mM), $[Ca^{2+}]_i$ might be somewhat higher than in normally polarized fibres. However, the fact that upon repolarization (change from 50 mM $[K^+]_o$ to 2·5 mM $[K^+]_o$ in Fig. 5*B*), $k_{Ca,o}$ did not rise, despite the increase in driving force on the Na_o^+–Ca_i^{2+} counter-transport suggests that $[Ca^{2+}]_i$ was below the level at which the activity of the exchanger becomes apparent.

It should be noticed that the difference between the driving force in the presence of 2·5 mM $[K^+]_o$ and that in the presence of 50 mM $[K^+]_o$, essentially, would not be affected by the small depolarizing effect of caffeine commented upon in the previous section.

Effect of amiloride

It has been shown that amiloride and its derivatives inhibit the exchanger (Siegl, Cragoe, Trumble & Kaczorowski, 1984; Slaughter, García, Cragoe, Reeves & Kaczorowski, 1988;

Donoso & Hidalgo, 1989). We tested the effect of 5 mM amiloride on the increase in $k_{Ca,o}$ promoted by 4 mM caffeine. This concentration of amiloride, as shown in Fig. 6*A*, has no effect on $k_{Ca,o}$ in the absence of caffeine.

Figure 6*B* illustrates the effect of 4 mM caffeine on $k_{Ca,o}$ in the absence and presence of 5 mM amiloride in paired preparations. Clearly, the increase in $k_{Ca,o}$ promoted by caffeine was significantly reduced by the drug. It can be seen, in good agreement with the data in Fig. 6*A*, that before and after the exposure of the muscles to caffeine there was no significant difference between the $k_{Ca,o}$ of muscles exposed to amiloride and of their paired companions kept in amiloride-free media throughout the experiment.

Effect of Ni^{2+}

Electrical measurements have shown that external Ni^{2+} quite effectively abolishes the current due to the activity of the exchanger in cardiac cells (Kimura, Miyamae & Noma, 1987; Ehara, Matsuoka & Noma, 1989). Thus this cation is considered, in practice, a rather specific blocker of the exchanger. In order to characterize further the Na^+-Ca^{2+} antiport in skeletal muscle we studied the effect of 5 mM Ni^{2+} on the caffeine stimulation of the Ca^{2+} efflux. Surprisingly, it was found that in the presence of caffeine, Ni^{2+} actually increases $k_{Ca,o}$. This is shown in Fig. 7*A* where the addition of 5 mM Ni^{2+} to solution A containing 4 mM caffeine produced a very significant increase in $k_{Ca,o}$ rather than the expected reduction. The increase in $k_{Ca,o}$ produced by Ni^{2+} during activation of the exchanger by caffeine does not exclude the likelihood of an increase of calcium efflux unrelated to the activity of the exchanger itself. This possibility, however, was readily discarded because, as shown in Fig. 7*B*, 5 mM Ni^{2+} did not alter $k_{Ca,o}$ in muscles where the exchanger was quiescent (i.e. in the absence of caffeine).

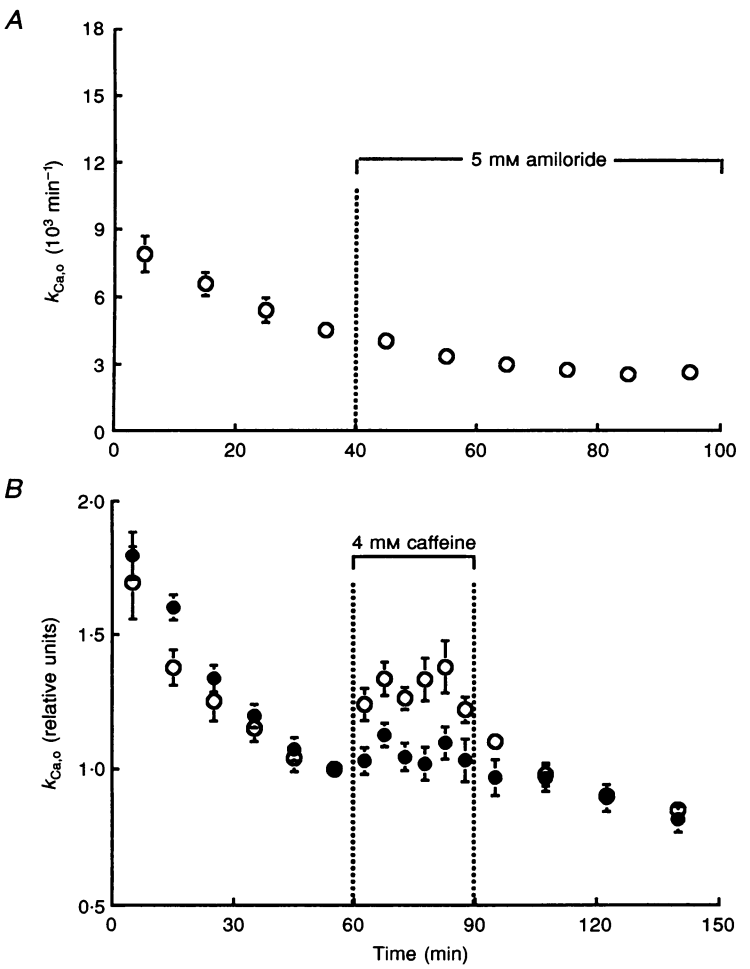

Figure 6. Effect of amiloride on $k_{Ca,o}$

A, amiloride (5 mM) has no effect on $k_{Ca,o}$ in the presence of solution A. Data are from four identical experiments. *B*, effect of 5 mM amiloride on the response of $k_{Ca,o}$ to 4 mM caffeine. Data represent the means from eight identical experiments, and were normalized with respect to the last value before the exposure to caffeine. The response to caffeine was significantly reduced in the presence of the drug ($P < 0{\cdot}05$ for all pairs of values except the last one where $0{\cdot}1 > P > 0{\cdot}05$). ○, control; ●, 5 mM amiloride.

In view of this finding, we hypothesized that one possibility was that in the presence of external Ni^{2+} the exchanger might switch from an electrogenic $3Na^+$–$1Ca^{2+}$ exchange mode to an electroneutral $1Ni^{2+}$–$1Ca^{2+}$ exchange with a higher turnover rate. This would reconcile the two observed effects of Ni^{2+}: (a) the abolishment of the exchanger current found in electrical measurements (Kimura *et al.* 1987); and (b) the increase in Ca^{2+} efflux. If this scheme were correct, Ni^{2+} influx should be increased upon activation of the exchanger by caffeine.

Experiments using $^{63}Ni^{2+}$ as a tracer showed that Ni^{2+} influx in the presence of 4 mM caffeine is indeed considerably higher than in its absence.

We used two different protocols. In the first one (protocol 1), two paired muscles were pre-exposed to solution A, containing 5 mM Ni^{2+} for 30 min; subsequently, the control muscle was exposed to the same solution labelled with $^{63}Ni^{2+}$ for a 15 min period, while the experimental one was exposed for the same period to a similarly labelled solution which in addition contained 4 mM caffeine. A wash-out period (see Methods) followed immediately. Figure 8*A* illustrates typical wash-out curves. It shows the semi-logarithmic plots of the Ni^{2+} content (nmol g^{-1}) from both muscles in a La^{3+}-containing medium (solution H) as a function of the wash-out time. Clearly, the intracellular Ni^{2+} content at the end of the loading period, as determined from the back extrapolation of the slow monoexponential component of the curve (see Methods), was appreciably higher when the uptake medium contained 4 mM caffeine. The mean values for Ni^{2+} influx from four identical experiments were $0{\cdot}600 \pm 0{\cdot}036$ pmol cm^{-2} s^{-1} for control (no caffeine) and $1{\cdot}66 \pm 0{\cdot}40$ pmol cm^{-2} s^{-1} in 4 mM caffeine ($P < 0{\cdot}04$; Fig. 8*B*). In the second protocol

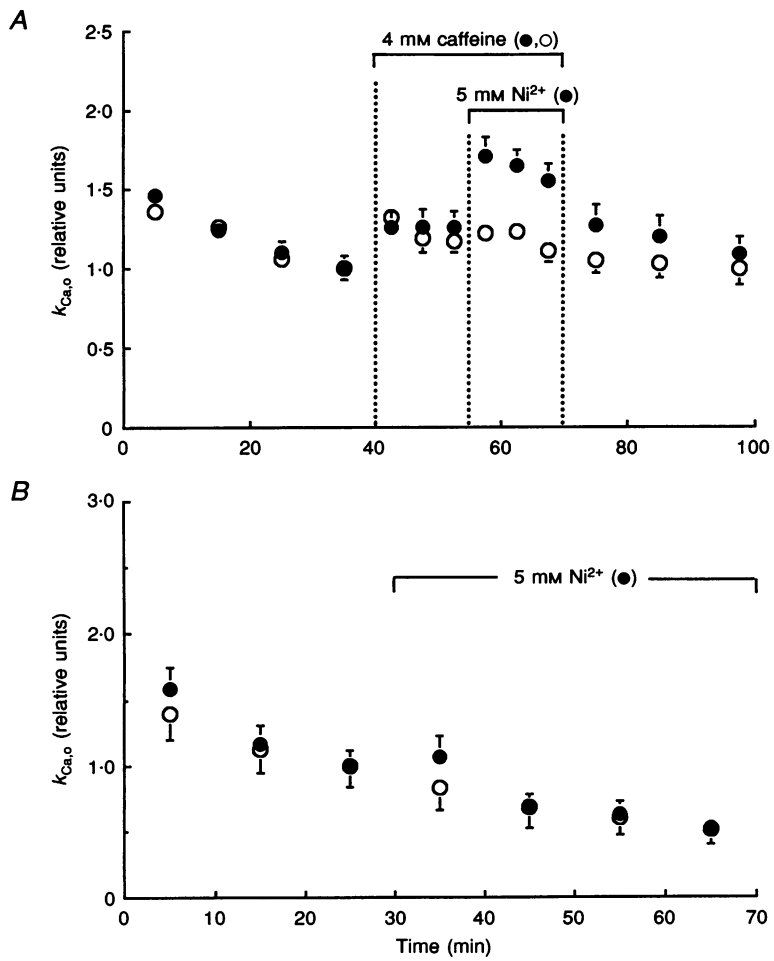

Figure 7. $k_{Ca,o}$ in the presence of Ni^{2+}

A, effect of 5 mM Ni^{2+} on the response of $k_{Ca,o}$ to 4 mM caffeine (both added to solution A). Means from four identical experiments using paired muscles (○) and normalized with respect to the last value before the addition of caffeine. Instead of inhibiting the increase in calcium efflux promoted by caffeine, the exposure to Ni^{2+} further increased it very significantly ($P = 0{\cdot}002$). *B*, lack of effect of 5 mM Ni^{2+}on Ca^{2+} efflux under resting conditions in the presence of solution A. Means from four identical experiments (paired muscles; ○, ●) normalized with respect to the last point before the exposure of the muscles to Ni^{2+}.

(protocol 2), one of the muscles (experimental) was pre-exposed to solution A containing 4 mM caffeine for 15 min and was subsequently exposed to a similar medium containing, in addition, 5 mM Ni^{2+} labelled with $^{63}Ni^{2+}$ for another 15 min period. The companion muscle (control) was directly exposed for 15 min to solution A containing 5 mM Ni^{2+} labelled with $^{63}Ni^{2+}$ but in the absence of caffeine. Using this protocol the mean Ni^{2+} influx from four identical experiments was $0{\cdot}704 \pm 0{\cdot}182$ pmol cm^{-2} s^{-1} in the absence of caffeine and $2{\cdot}45 \pm 0{\cdot}17$ pmol cm^{-2} s^{-1} in the presence of the drug ($P < 0{\cdot}004$; Fig. 8*B*).

The mean Ni^{2+} influx in the presence of caffeine with respect to that in the absence of the drug, using protocol 2, appears to be larger and statistically more significant than that measured using protocol 1. This might reflect the fact that in muscles pre-exposed to caffeine for 15 min (protocol 2), the Na_o^+–Ca_i^{2+} exchange was already fully activated in the presence of 4 mM caffeine before the exposure to Ni^{2+} ($^{63}Ni^{2+}$), while in protocol 1 the activation of the exchanger began simultaneously with the Ni^{2+} uptake period. In all probability the reason for the difference in uptake rates is that, although the sarcolemma is highly permeable to caffeine (Kotsias *et al.* 1984), there is an unavoidable diffusion time lag for the drug to reach the sarcoplasmic reticulum to produce the Ca^{2+} release.

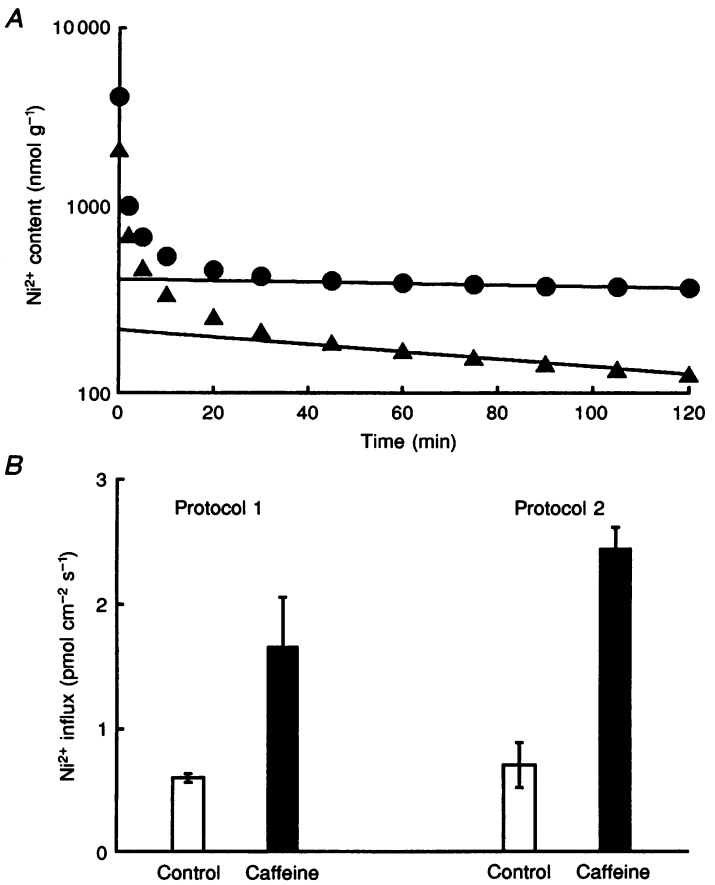

Figure 8. Effect of caffeine on Ni^{2+} influx

A, typical semilogarithmic plot of Ni^{2+} content as a function of time (carried out in the presence of solution H; see Methods) from a pair of muscles. One muscle was exposed for 15 min to solution A containing 5 mM Ni^{2+} labelled with $^{63}Ni^{2+}$ (▲). The other muscle (●) was exposed to a similar radioactive solution which contained, in addition, 4 mM caffeine (protocol 1; see Methods). The straight lines correspond to single exponentials fitted to the last four points of each curve. The values extrapolated to time = 0 represent the intracellular amount of Ni^{2+} (nmol g^{-1}) at the end of the loading period in each muscle. *B*, open columns represent the mean Ni^{2+} influx (in pmol cm^{-2} s^{-1}) under control conditions (solution A plus 5 mM Ni^{2+} labelled with $^{63}Ni^{2+}$). Filled columns correspond to the mean Ni^{2+} influx in the presence of a similar loading solution which contained, in addition, 4 mM caffeine (experimental muscles). In protocol 1, caffeine was present only in the loading solution. In protocol 2, prior to the loading period, the experimental muscles were exposed for 15 min to unlabelled solution A containing 4 mM caffeine. In each protocol four identical experiments were carried out using paired preparations. Values of Ni^{2+} influx (pmol cm^{-2} s^{-1}) are as follows. Protocol 1: control, $0{\cdot}600 \pm 0{\cdot}036$; caffeine, $1{\cdot}66 \pm 0{\cdot}40$ ($P < 0{\cdot}04$). Protocol 2: control, $0{\cdot}704 \pm 0{\cdot}182$; caffeine, $2{\cdot}45 \pm 0{\cdot}17$ ($P < 0{\cdot}004$).

DISCUSSION

The purpose of this work was to characterize the Na^+–Ca^{2+} antiport in intact frog skeletal muscle fibres. The simplest experiment to detect the presence of the exchanger working in the forward mode would be to investigate if the reduction or replacement of external Na^+ by another cation known to have little or no affinity for the exchanger, decreases Ca^{2+} efflux. In fact, this was observed by Caputo & Bolaños (1978) in semitendinosus muscle from *Rana pipiens*. In addition, they showed that the absence of external Ca^{2+} reduced Ca^{2+} efflux as expected from the exchanger working in the Ca^{2+}–Ca^{2+} exchange mode. Using muscles from *L. ocellatus* we were unable to reproduce their results. It seems possible that this functional discrepancy might reflect the different behaviour of two isoforms of the exchanger protein with different affinities for cytosolic Ca^{2+} (Rahamimoff, Dahan, Furman, Spanier & Tessari, 1991).

The large increase in $k_{Ca,o}$ produced in our preparation by Na^+-free media was absent when $[Ca^{2+}]_o$ was reduced to 0·1 mM or less. A possible mechanism for this effect could be the following. In Na^+-containing media the external binding sites of the exchanger protein at rest are mostly occupied by Na^+ ions. It is reasonable to assume that, under these conditions, because of its low affinity for Ca^{2+}, the activity of the Na_o^+–Ca_i^{2+} exchange would be negligible, and the compensator for the passive inward Ca^{2+} leak would mainly be the sarcolemmal Ca^{2+}-ATPase. We may further speculate that the absence of Na_o^+ causes an increase in the apparent affinity of the exchanger for Ca^{2+}, which promotes its activity in the Ca_o^{2+}–Ca_i^{2+} mode, thus producing the observed rise in $k_{Ca,o}$ upon withdrawal of Na^+ from the external medium. In connection with this, it is noteworthy that in squid axons (Blaustein & Russell, 1975) and barnacle muscle fibres (Lederer & Nelson, 1983), the Ca_i^{2+}–Ca_o^{2+} exchange can be much higher than Na^+–Ca^{2+} exchange. As would be expected, following this line of reasoning, the drastic reduction of $[Ca^{2+}]_o$ abolished the Na^+-free effect. Tetracaine (0·4 mM), on the other hand, suppressed the effect of Na^+-free media on $k_{Ca,o}$ in the presence of normal $[Ca^{2+}]_o$ (data not shown). This is in good agreement with the finding that procaine, a local anaesthetic closely related to tetracaine, inhibits Ca^{2+}–Ca^{2+} exchange in barnacle muscle fibres (Chen, 1974).

The characteristics of the caffeine-induced increase in $k_{Ca,o}$ clearly suggest an activation of a Na_o^+–Ca_i^{2+} exchange (forward mode). This was supported by several tests that have previously been used to define operationally the Na^+–Ca^{2+} antiport. Our results indicate that in intact frog (*L. ocellatus*) skeletal muscle the exchanger behaves as a low affinity, high capacity system, which agrees with data obtained in frog (*Caudiverbera caudiverbera*) T-tubule vesicles by Donoso & Hidalgo (1989) and Hidalgo *et al.* (1991). In this membrane fraction, they found that the extent of the exchange is a saturable function of $[Ca^{2+}]_i$ with a $K_{0.5}$ (ion concentration required for half-maximal activation) of about 3 μM, that is, an order of magnitude greater than the $K_{0.5}$ (0·3 μM) of the active Ca^{2+} transport in a similar preparation from rabbit skeletal muscle (Hidalgo, Parra Riquelme & Jaimovich, 1986). This is consistent with our failure to detect any Na^+–Ca^{2+} exchange activity under resting conditions. In quiescent muscles $[Ca^{2+}]_i$ is about 0·1–0·2 μM (Tsien & Rink, 1980;

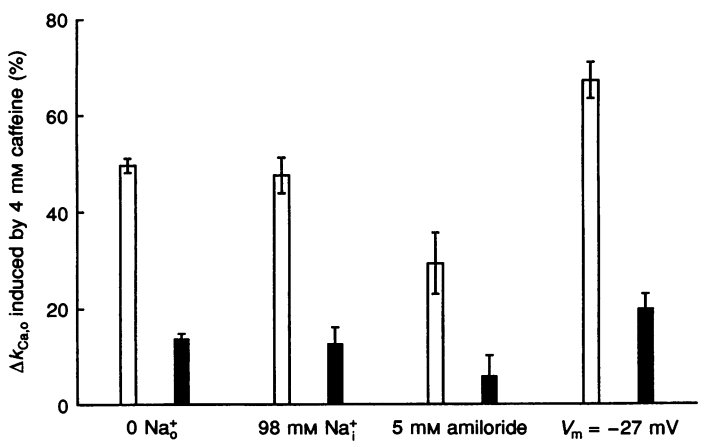

Figure 9. Summary of the effects of different interventions on the increase in $k_{Ca,o}$ promoted by caffeine

The first pair of columns shows the 72% reduction produced by reversing the Na^+ gradient across the sarcolemma in a nominally Na^+-free medium (from data in Fig. 3). The second pair of columns illustrates the reduction of 73% induced by raising $[Na^+]_i$ to about 98 mM in the presence of normal $[Na^+]_o$ (from data in Fig. 4). The third pair of columns shows the 80% decrease produced by 5 mM amiloride (from data in Fig. 6). The last pair of columns represents the 70% reduction promoted by depolarization to about −27 mV (from data in Fig. 5). All differences between paired control (□) and experimental muscles (■) are statistically significant.

Harkins *et al.* 1993) and, presumably, is kept at that level by the SR and sarcolemmal Ca^{2+} pumps. In addition, the lack of effect of 5 mM amiloride on $k_{Ca,o}$ in muscles under resting conditions supports further the notion that the exchanger does not play a relevant role in the control of submicromolar $[Ca^{2+}]_i$.

We did not measure $[Ca^{2+}]_i$ in the presence of 4 mM caffeine, but because this concentration of the drug produces a submaximal contracture of about 75% of the maximum (Kotsias *et al.* 1984) we may safely assume an increase in $[Ca^{2+}]_i$ close to one order of magnitude with respect to the value at rest. The rise of $[Ca^{2+}]_i$ promoted by 4 mM caffeine most probably overwhelms the relatively small transport capacity of the sarcolemmal Ca^{2+} pump and activates Na^+-Ca^{2+} exchanger. The results, summarized in Fig. 9, show that the increase in $k_{Ca,o}$ produced by caffeine exhibits the properties expected from a Na^+-Ca^{2+} antiport. As can be seen the increase in Ca^{2+} efflux promoted by the drug was reduced by 72% in the absence of external Na^+ and by 73% in Na^+-loaded muscles ($[Na^+]_i = 98$ mM). These two experimental conditions have in common changes in Na^+ gradient which should reduce the forward mode activity of the exchanger.

Amiloride, which did not affect Ca^{2+} efflux in quiescent muscles (Fig. 6), produced an 80% reduction in the $k_{Ca,o}$ increase generated by caffeine. This constitutes further circumstantial evidence of the stimulation of the Na^+-Ca^{2+} exchanger under conditions that increase $[Ca^{2+}]_i$ to levels which activate the contractile mechanism.

In different muscle types the Na^+-Ca^{2+} coupling ratio was found to be > 2 (i.e. $3Na^+-1Ca^{2+}$) and therefore should be affected by changes in V_m. V_m dependence is another criterion used to characterize the exchanger. The caffeine-induced increase in $k_{Ca,o}$ was reduced by 70% in muscles depolarized to -27 mV ($[K^+]_o = 50$ mM). This is the expected behaviour from a Na^+-Ca^{2+} exchange ratio greater than 2.

Overall, our results on the caffeine-promoted activity of Na^+-Ca^{2+} exchange are consistent with data previously obtained in studies of the exchanger current and of changes in $[Ca^{2+}]_i$ produced by the drug in ventricular myocytes (Callewaert, Cleeman & Morad, 1989; O'Neill, Valdeolmillos, Lamont, Donoso & Eisner, 1991).

With regard to the effect produced by Ni^{2+}, the results presented here clearly suggest that its inhibitory role, at least in frog muscle, should be redefined. It has been clearly shown that Ni^{2+} abolishes the exchanger current (Kimura *et al.* 1987). Against all expectations based on electrophysiological data, in frog muscle Ni^{2+} produced a very significant increase of Ca^{2+} efflux in muscles after the $Na^+_o-Ca^{2+}_i$ exchange had been activated by 4 mM caffeine. This stimulation of $k_{Ca,o}$ promoted by Ni^{2+} was accompanied by a Ni^{2+} influx considerably higher than in the absence of caffeine. The simplest conclusion from these results is that in frog skeletal muscle fibres in the presence of 5 mM Ni^{2+}, the exchanger, rather than being inhibited, switches its *modus operandi* from an electrogenic, i.e. $3Na^+_o-1Ca^{2+}_i$, exchange to an electrically silent $1Ni^{2+}_o-1Ca^{2+}_i$ antiport. In support of this notion, it should be mentioned that in bullfrog ventricle, Brommundt & Kavaler (1987) found an ATP-dependent Ca^{2+} efflux produced by external Ni^{2+} which was observed inconsistently and whose occurrence was favoured by Na^+-free media. This is at variance with the conclusion of Ehara *et al.* (1989) based on patch clamp experiments on ventricular myocytes, according to which Ni^{2+} blocks Na^+-Ca^{2+} exchange without being transported by the exchanger. Incidentally, if the Na^+-Ca^{2+} exchange current is defined by its Ni^{2+} sensitivity, such a definition would be valid whether Ni^{2+} acts by inhibiting the transport capacity of the exchanger protein or by promoting a shift of the exchange mode to an electroneutral $Ni^{2+}-Ca^{2+}$ antiport.

Since Ca^{2+} efflux, through the exchanger, is increased by the presence of Ni^{2+} in the external solution, it is reasonable to assume that the turnover rate of the $Ni^{2+}_o-Ca^{2+}_i$ exchange is higher than that of the Na^+-Ca^{2+} exchange. The lack of effect of Ni^{2+} on $k_{Ca,o}$ of quiescent muscles (caffeine-free medium) further shows the virtual absence of exchanger activity under resting conditions (Fig. 7).

In summary, the Na^+-Ca^{2+} exchanger in skeletal muscle from *L. ocellatus* behaves as a high capacity, low affinity system which is sensitive to amiloride and dependent on Na^+ gradient and V_m. In addition, in the presence of Ni^{2+} (5 mM), a blocker of the exchanger current, the antiport apparently switches from an electrogenic Na^+-Ca^{2+} mode to an electroneutral $Ni^{2+}-Ca^{2+}$ mode.

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