

Strophanthidin-Sensitive Sodium Fluxes in Metabolically Poisoned Frog Skeletal Muscle

BRIAN G. KENNEDY and PAUL DE WEER

From the Department of Physiology and Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT Strophanthidin-sensitive and insensitive unidirectional fluxes of Na were measured in frog sartorius muscles whose internal Na levels were elevated by overnight storage in the cold. ATP levels were lowered, and ADP levels raised, by metabolic poisoning with either 2,4-dinitrofluorobenzene or iodoacetamide. Strophanthidin-sensitive Na efflux and influx both increased after poisoning, while strophanthidin-insensitive fluxes did not. The increase in efflux did not require the presence of external K but was greatly attenuated when Li replaced Na as the major external cation. Membrane potential was not markedly altered by 2,4-dinitrofluorobenzene. These observations indicate that the sodium pump of frog skeletal muscle resembles that of squid giant axon and human erythrocyte in its ability to catalyze Na-Na exchange to an extent determined by intracellular ATP/ADP levels.

INTRODUCTION

Caldwell et al. (1960) showed that, in the presence of cyanide, Na efflux from squid giant axons becomes insensitive to the removal of external K and, instead, requires Na in the bathing medium. They suggested that Na-Na exchange occurs after metabolic inhibition. Later, Baker et al. (1969) demonstrated a ouabain-sensitive Na influx in partially poisoned squid axons. De Weer (1970, 1974) further characterized this mode of exchange and showed that pump-catalyzed Na-Na exchange is unaffected by changes in internal P_i , but increases with rising intracellular ADP levels. In untreated human red blood cells, Garrahan and Glynn (1967) found a sizeable pump-mediated Na-Na exchange, which was inhibited by high levels of external K. In addition, there was evidence that Na-Na exchange increases with decreasing levels of ATP. Glynn and Hoffman (1971) further showed that high levels of ADP also promote Na-Na exchange. In view of these findings in squid giant axons and human red blood cells, it seemed important to determine whether the sodium pump of muscle will engage in Na-Na exchange after elevation of intracellular levels of ADP.

An Na-Na exchange component of pump activity in muscle, though small, has been well documented. Keynes and Steinhardt (1968) demonstrated 20% inhibition by ouabain of both influx and efflux of Na in K-free media. Horowicz et al. (1970) observed a ouabain-sensitive, external Na-dependent component of Na efflux in frog sartorius muscle. More recently, Sjodin and Beaugé (1973),

looking at net movement of Na as well as unidirectional fluxes, concluded that their findings were consistent with the existence of a pump-mediated Na-Na exchange.

On the other hand, reports concerning the effects of metabolic inhibition on Na fluxes in muscle have been conflicting. Keynes and Maisel (1954) reported no marked changes in Na efflux after poisoning with cyanide plus iodoacetic acid. Using the same protocol for inhibition, but working in a high K medium, Frazier and Keynes (1959) reported a decrease in efflux and a stimulation of influx. Portela et al. (1974), however, reported decreases in efflux as well as influx after iodoacetic acid poisoning. Beaugé and Sjodin (1975, 1976) measured Na efflux after treatment with azide, a known metabolic poison. A ouabain-sensitive increase in efflux, which was attenuated in a medium free of Na but not abolished by removal of K, was observed. In the present study two metabolic inhibitors, 2,4-dinitrofluorobenzene (DNFB) and iodoacetamide (IAM), were used to lower the ATP/ADP ratio. Portela et al. (1974) have reported that iodoacetic acid, which blocks glycolysis by inhibition of glyceraldehyde phosphate dehydrogenase, does decrease the ATP/ADP ratio. Infante and Davies (1965) have shown that 2,4-dinitrofluorobenzene inhibits all ATP generation in frog skeletal muscle: it inactivates creatine phosphokinase, and also interferes with oxidative phosphorylation and glycolysis. Mommaerts and Wallner (1967) demonstrated a decrease in the ATP/ADP ratio after application of DNFB.

METHODS

Muscle Preparation

Northern *Rana pipiens* were kept at room temperature (approx 23°C) in an aquarium with running water, and fed thrice weekly. Size ranged from 3.5 to 7.5 cm while isolated sartorii ranged in weight from 20 to 80 mg. The muscles were examined for parasites and blood clots before use. For all experiments except those in which membrane potential was to be determined muscles were carefully dissected free and 6-0 surgical thread was tied to both tendons. The muscles were then mounted on wire frames and stored for approximately 24 h (range, 20-30 h) at 3°C in K-free Ringer's solution in order to raise their internal Na levels. Muscles used for membrane potential measurements were taken immediately after dissection and pinned into Petri dishes containing a base of polymerized Sylgard 184.

Solutions

The composition of normal Ringer's fluid, (Na)Ri, was as follows (in millimoles/liter): NaCl 115; KCl 2.5; CaCl₂ 1.8; Na₂HPO₄ 1.08; and NaH₂PO₄ 0.43. In K-free Ringer's, OK(Na)Ri, the KCl was omitted. In Na-free Ringer's, (Li)Ri, LiCl was substituted for NaCl on a mole for mole basis and potassium phosphate was substituted for the sodium phosphate. The Na, K-free, magnesium-substituted Ringer's, OK(Mg)Ri, contained: MgCl₂ 86.3; CaCl₂ 1.8; and Tris phosphate buffer 1.5. The pH of all solutions was 7.3.

DNFB was obtained from Sigma Chemical Co. (St. Louis, Mo.) and dissolved in the appropriate Ringer's solution by vigorous stirring for 1-2 h. DNFB (0.38 mM) and IAM (0.5 mM) solutions were always freshly made on the day of the experiment. Strophanthidin (40 μM) was added directly to the appropriate Ringer's and dissolved by stirring overnight. Carrier-free ²²Na was obtained in a neutral solution from New England Nuclear (Boston, Mass.) and added directly to the appropriate Ringer's (approximately 10 μCi/ml).

Efflux Experiments

Muscles, attached to their wire frames, were placed in an OK(²²Na)Ri labeling solution at 3°C for approximately 6 h (thus the last 6 h of the loading period were spent in labeled Ringer's). The paired muscles were then moved through a series of tubes containing 5 ml of unlabeled Ringer's at 23°C. The average efflux period in any one tube was 15 min. After an experiment, the muscle was placed in a platinum crucible and ashed at 500°C. The ash was dissolved in 1 N nitric acid, and the volume then brought to 5 ml. All tubes were counted in an Auto-gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) to approximately 2% counting error. By back calculation, the total counts remaining in the muscle and the instantaneous rate coefficient for efflux could be calculated for each efflux period.

Influx Experiments

Influx experiments employed paired muscles from the same frog. The control was preincubated in (Na)Ri while the experimental muscle was preincubated either for 30 min in (Na)Ri + DNFB or for 90 min in (Na)Ri + IAM. Muscles were next placed in [²²Na]Ri for 15 min. Washout was then followed as described in the previous section, except that muscles were blotted lightly and weighed before ashing. The washout solutions consisted of OK(Na)Ri plus 4×10^{-5} M strophanthidin plus 10^{-7} g/ml tetrodotoxin (TTX). Preliminary experiments had indicated that high-Na muscles occasionally twitched when placed in (Na)Ri + strophanthidin at room temperature. To circumvent this problem all incubation, labeling, and washout solutions used in the influx experiments contained 10^{-7} g/ml TTX. Data were analyzed by plotting total counts remaining in the muscle semilogarithmically against time. Total counts gained during the 15-min labeling period were determined by extrapolating the linear phase of washout to zero time. Influx is expressed as micromoles Na(grams wet weight)⁻¹h⁻¹.

Analysis for Total Na

This procedure followed Sjodin and Henderson (1964). Intracellular Na was elevated as described, and muscles were then allowed to recover for 3 h at room temperature in (Na)Ri, rinsed for 15 min in OK(Mg)Ri to remove extracellular Na, lightly blotted on ashless filter paper, weighed, and ashed at 500°C. The ash was dissolved in 1 N nitric acid and assayed for total Na by atomic absorption spectrometry.

ATP and ADP Determination

Muscles were Na loaded as usual. Muscle pairs were used: the control was incubated in (Na)Ri while the other member of the pair was held for 30 min in (Na)Ri + DNFB or for 90 min in (Na)Ri + IAM. After incubation the muscles were weighed, frozen in liquid nitrogen, and pulverized with mortar and pestle in liquid N₂. 2 vol of 0.1 N HCl in methanol were added to the powder at -20°C, followed by 7 vol of 0.3 N perchloric acid at 0°C. After centrifugation at 4,500 rpm for 20 min, an equal volume of neutralizing solution containing 0.25 M KOH, 0.15 M imidazole, and 0.15 M KCl was added to the supernate. Aliquots of the extract were assayed, in triplicate, for ATP and ADP on the same day they were extracted. The fluorometric assay procedures were taken from Lowry and Passonneau (1972).

Membrane Potential Determinations

As noted previously, muscles used for membrane potential determination were not Na loaded. Paired muscles were dissected and one was pinned directly into the Petri dish; the other remained at room temperature in (Na)Ri until measurements on the first were complete. One muscle served as control, potential measurements being taken in (Na)Ri,

while measurements in the experimental muscle were taken in (Na)Ri + DNFB. Potentials were recorded as a series of penetrations taken over time. Glass microelectrodes of 10–20 M Ω resistance were used.

Statistical Analysis

Values are reported as means \pm SEM. Except as noted, all *P* values were determined from two-tailed *t*-tests for paired groups.

RESULTS

Na Efflux

Washout of extracellular ^{22}Na required about 30 min. The rate constant then remained stable for over 90 min, as seen in Fig. 1. The mean rate constant for 56 determinations of Na efflux in (Na)Ri was $0.0160 \pm 0.0006 \text{ min}^{-1}$. This value can be compared to 0.016 min^{-1} obtained for Na-loaded muscles by Beaugé and Sjodin (1968) and 0.014 min^{-1} obtained by Keynes and Steinhardt (1968). Average drift over time was 0.001 min^{-1} per h.

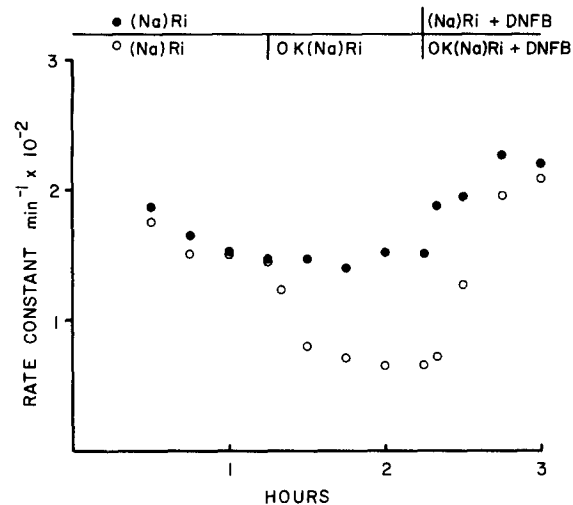


FIGURE 1. Effects of 0.38 mM DNFB on the rate constant for efflux of ^{22}Na . The control (●) efflux was followed in (Na)Ri for 145 min, at which point 0.38 mM DNFB was applied. The paired muscle (○) displayed an unchanging rate constant in (Na)Ri for 30 min, after which it was exposed to OK(Na)Ri. When the rate constant had again stabilized, OK(Na)Ri + 0.38 mM DNFB was applied. The increase in Na efflux in DNFB did not require external K.

DNFB Effects on Na Efflux

The effects of DNFB were determined in (Na)Ri, OK(Na)Ri, and (Li)Ri. Fig. 1 shows that when a muscle bathed in (Na)Ri (solid symbols) is exposed to DNFB, a marked increase in the rate constant for Na efflux occurs. The stimulation was transient, reaching a peak value of 1.5 times control level. In the paired muscle, the effect of DNFB in OK(Na)Ri can be seen. Removal of K produced a marked

(56%) decrease in the rate constant for efflux. In six normal muscles the magnitude of the rate constant in OK(Na)Ri relative to that in (Na)Ri was 0.47 ± 0.05 , comparable to the value of 0.48 reported by Keynes and Steinhardt (1968). The significant point for this study is that, even in a K-free medium, a marked DNFB-induced increase in Na efflux, with rate of rise similar to that in (Na)Ri, is evident: peak response is more than double the base-line level. Clearly, the stimulating effect of DNFB on Na⁺ efflux is larger in the absence of external K than in its presence.

A prompt, transient increase in Na efflux occurred upon replacement of external Na with Li, as expected for a sartorius muscle with elevated internal Na levels (see Horowicz et al. [1970] and Beaugé and Ortiz [1972] for a discussion of this phenomenon). In 16 muscles the average Na-free effect (ratio of rate constant in (Li)Ri to that in (Na)Ri) was 1.63 ± 0.045 , similar to the ratios of 1.57,

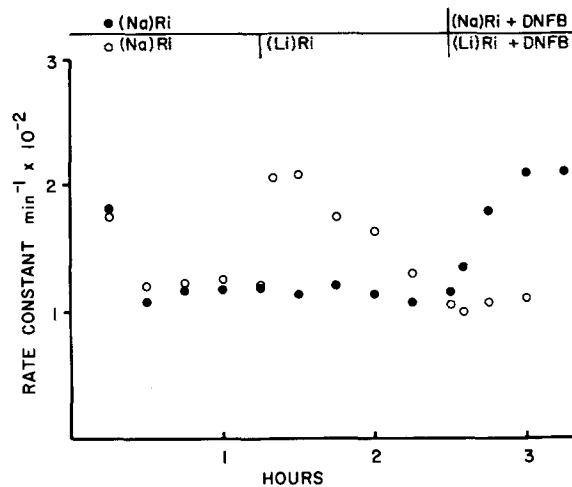


FIGURE 2. Effect of 0.38 mM DNFB and Li substitution on the rate constant for efflux of ^{22}Na . DNFB was applied after 150 min of efflux. The control muscle (●) was held in (Na)Ri while the paired muscle (○) was exposed to (Li)Ri after 75 min in (Na)Ri. The typical DNFB effect is markedly attenuated in the absence of external Na.

reported by Keynes and Steinhardt (1968), and 1.52, obtained by Beaugé and Sjödin (1968). Fig. 2 illustrates one experiment in which DNFB was applied to a muscle that had been exposed to (Li)Ri for 75 min. The DNFB effect is greatly attenuated compared to that observed in the paired control kept in (Na)Ri (solid symbols). Since a muscle in Na-free medium will be losing internal Na, the attenuation of the DNFB effect in (Li)Ri could conceivably be due to decreased levels of internal Na rather than to the absence of external Na. To test this point five experiments were performed, one of which is shown in Fig. 3. Paired muscles were used and both were exposed to (Li)Ri for approximately 90 min. One muscle, indicated by the filled symbols, was then returned to (Na)Ri just long enough to establish a stable efflux, whereupon both muscles were exposed

to DNFB. Although both muscles were depleted of internal Na, only the muscle bathed in Na containing Ringer's exhibited the characteristic stimulation upon application of DNFB.

Finally, the strophanthidin sensitivity of the DNFB-stimulated Na efflux was studied. Fig. 4 shows virtually no effect on the rate constant for Na efflux when DNFB is applied in the presence of strophanthidin. (The paired muscle, indicated with filled symbols, shows a normal DNFB effect in (Na)Ri.)

Pooled data for all experiments concerning the effects of DNFB on the rate constant for ^{22}Na efflux are presented in Fig. 5. In these comparisons, each muscle served as its own control, base-line efflux being determined immediately before drug application. The rate constant in DNFB was taken as the peak value reached after application of the inhibitor. 23 determinations were made in

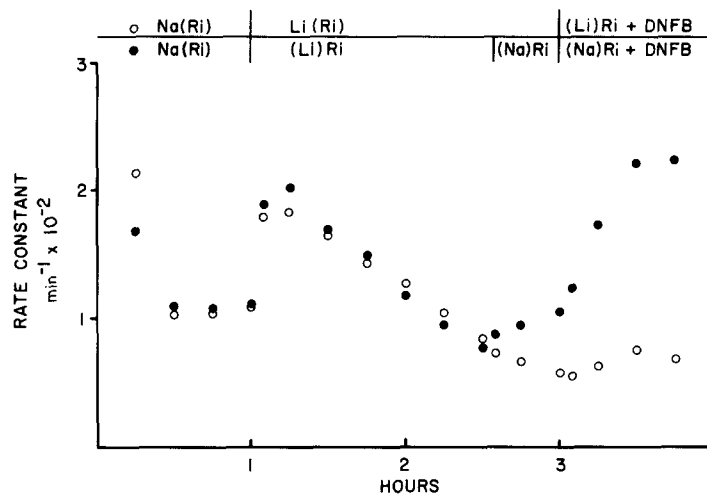


FIGURE 3. Effect of 0.38 mM DNFB and Li substitution on the rate constant for efflux of ^{22}Na for low Na muscles. Both muscles were depleted of internal Na by exposure to (Li)Ri—the control muscle (○) for 120 min, the paired experimental muscle (●) for 90 min. The experimental muscle was then re-exposed to (Na)Ri for 30 min before addition of 0.38 mM DNFB. Attenuation of the DNFB effect in the absence of external Na is not due to depletion of internal Na levels.

(Na)Ri. The average effect of DNFB application was a significant 50% increase in the rate constant for Na efflux ($P < 0.001$). In OK(Na)Ri, DNFB also produced a significant 150% stimulation ($P < 0.001$). Also shown in Fig. 5 are pooled data for the DNFB effect in (Li)Ri. The effect, though small, is significant at the 0.02 level. However, it should be noted that the rate constant in (Li)Ri was still falling when the inhibitor was applied. The control rate constant was thus estimated by extrapolation of a falling base line; this procedure may introduce some error. The results from five control experiments performed in Na-depleted muscles are also shown. The DNFB stimulation of Na efflux into (Na)Ri for these *low Na* fibers was similar to that observed in *loaded* fibers; the mean rate constants in the presence of DNFB were not significantly different for the two groups. (The P value, as determined by a t -test for independent groups,

was greater than 0.2). Finally, Fig. 5 also presents the pooled data for six experiments on the effect of DNFB on Na efflux in the presence of strophanthidin. The mean rate constants are virtually identical ($P > 0.2$). It should be noted that the base-line efflux was still decreasing slightly when the inhibitor was applied (as can be seen in Fig. 4); again, this made evaluation of small inhibitor induced effects difficult. Hence, it is possible that DNFB did produce a small decrease in strophanthidin-insensitive Na efflux. The conclusion is that the marked DNFB-induced stimulation of Na efflux is greatly reduced in the absence of external Na and abolished in the presence of strophanthidin.

Iodoacetamide Effects on Na Efflux

Marked transient stimulation of the rate constant for Na efflux was observed after application of 0.5 mM IAM. In the experiment shown in Fig. 6, stimulation

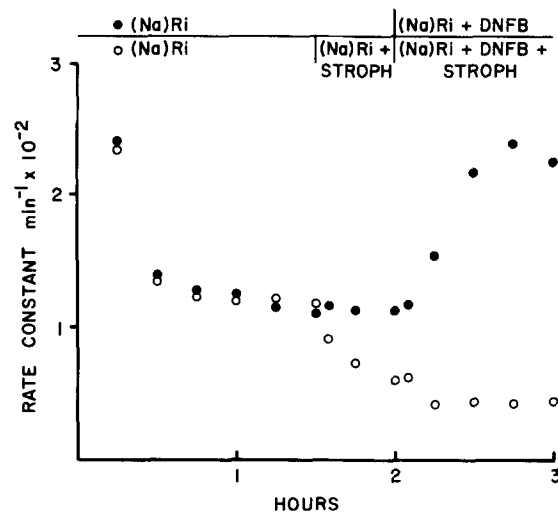


FIGURE 4. Effect of 0.38 mM DNFB and 4×10^{-5} M strophanthidin on the rate constant for efflux of ^{22}Na . The control muscle (●) was held in (Na)Ri for 120 min and then exposed to (Na)Ri + DNFB. The experimental muscle (○) was held in (Na)Ri for 90 min and then exposed to 4×10^{-5} M strophanthidin for 30 min. DNFB was added in the presence of strophanthidin. The DNFB effect is strophanthidin sensitive.

was about 60%. Compared to DNFB, IAM acted with a much longer delay, average time to peak being about 90 min. The effect of IAM was less pronounced and more variable than that observed with DNFB. Fig. 7 summarizes the results for efflux determinations in IAM. 15 determinations in (Na)Ri indicated an average 40% increase ($P < 0.001$) in rate constant due to IAM. Similar measurements made in K-free Ringer's show a twofold increase ($P < 0.001$); thus, as for DNFB treatment, the stimulation produced by IAM is not blocked by removal of K. The effect of IAM is greatly attenuated, however, in an Na-free medium. Finally, when applied in the presence of strophanthidin, IAM had no significant effect on the rate constant for Na efflux, which suggests that the extra efflux is pump mediated.

Inhibitor Effects on Na Influx

The appearance of a Na-dependent Na *efflux* in response to metabolic poisoning, as described above, leads one to expect the concomitant induction of a strophanthidin-sensitive Na *influx*. Increased Na influx into metabolically poisoned muscles was indeed found. Fig. 8 A illustrates a typical washout experiment after a 15-min period of exposure to ^{22}Na . A slow phase of washout is evident in both muscles after 30 min, and extrapolation of this phase to zero time

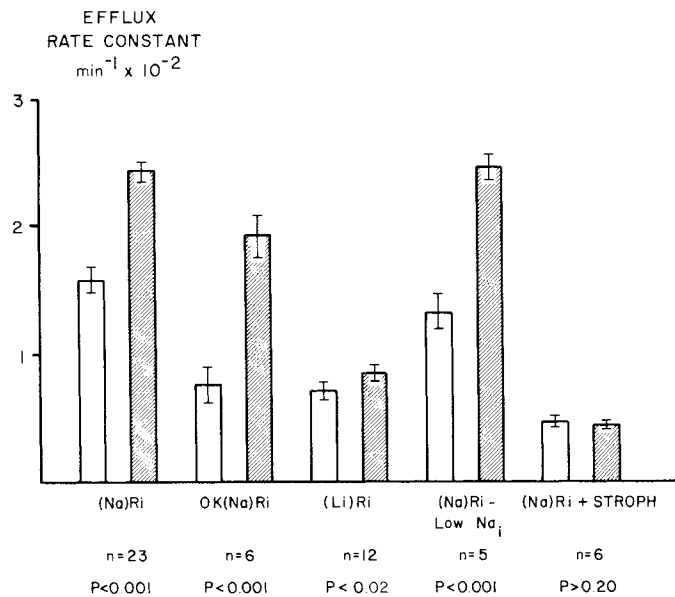


FIGURE 5. Summary of the effects of 0.38 mM DNFB on the rate constant for ^{22}Na efflux. Each muscle served as its own control. Open bars refer to the control efflux while the hatched bars refer to the maximum value attained by the rate constant after DNFB application. In (Li)Ri and in (Na)Ri + strophanthidin the rate constant was falling gradually when DNFB was applied; it was thus necessary to extrapolate this decline to the point in time at which the DNFB-stimulated efflux was maximal, to estimate a base-line efflux. In all other conditions a stable base line was obtained, and the control rate constant could conveniently be determined immediately before DNFB application.

indicates the magnitude of prior influx. The DNFB-treated muscle (open symbols) shows higher intracellular levels of radioactivity. The bar graphs in Fig. 8 B summarize the effects of DNFB on Na influx. Paired muscles were used, one muscle serving as control. In (Na)Ri DNFB produced, on the average, a 60% increase in Na influx, ($P < 0.001$), which was blocked by strophanthidin. There was actually a small but significant ($P = 0.02$) decrease in influx produced by DNFB in the presence of strophanthidin. Our influx determinations were not corrected for efflux during the labeling period. This correction, approximately 10%, was not made because all comparisons were between paired muscles, and because absolute influx measurements in whole sartorius muscles are subject to

errors of comparable magnitude due to extracellular space equilibration. Since DNFB stimulates Na efflux, the correction would be greater in poisoned muscles; the DNFB effect on Na influx has thus probably been slightly underestimated.

IAM treatment produced a 50% increase in influx (first pair of bar graphs in Fig. 9), which was abolished in the presence of strophanthidin, as shown in the second pair of bars. Fig. 9 also gives the results of two control experiments. First, it seemed important to determine whether strophanthidin itself decreases Na influx, thereby masking any inhibitor-induced effects. This is not the case since the influxes from (Na)Ri into Na-loaded frog sartorius fibers in the presence or absence of strophanthidin are indistinguishable. Second, since all influx experiments were performed in the presence of TTX, the effect of TTX on Na influx was also determined. The drug had no effect on Na influx, as illustrated by the last pair of bar graphs in Fig. 9.

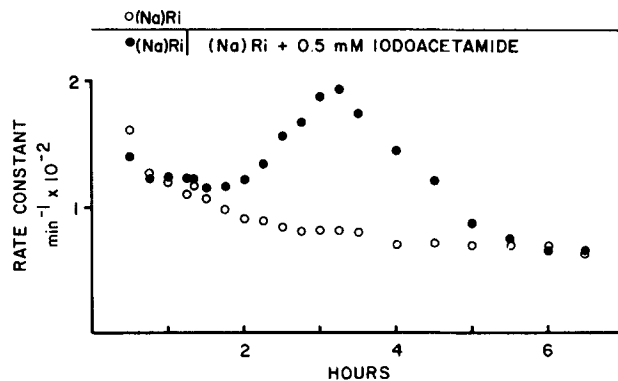


FIGURE 6. Effect of 0.5 mM IAM on the rate constant for efflux of ^{22}Na . The control muscle (O) was held in (Na)Ri for 390 min. The paired experimental muscle (●) had established a relatively stable efflux by 75 min at which point (Na)Ri + 0.5 mM IAM was applied.

ATP and ADP Levels at Rest and after Inhibition

18 Na-loaded muscles were assayed for ATP and ADP. Resting levels were $3.43 \pm 0.19 \mu\text{mol/g}$ for ATP and $0.48 \pm 0.03 \mu\text{mol/g}$ for ADP. The resting ratio of ATP/ADP was thus 7.48 ± 0.62 . For *Rana pipiens sartorii* that had been stored in the cold, Dydynska and Harris (1966) report resting ATP levels of $2.6 \mu\text{mol/g}$ while Mommaerts and Wallner (1967) report $2.55 \mu\text{mol/g}$ ATP and $0.42 \mu\text{mol/g}$ ADP. When muscles were exposed for 30 min at room temperature to 0.38 mM DNFB, their levels of ATP were decreased, and those of ADP increased significantly (Fig. 10). These changes are in the direction reported by Mommaerts and Wallner (1967), although the present effects are more pronounced; the discrepancy is probably due to the fact that Mommaerts and Wallner (1967) performed their experiments at 0°C . Fig. 10 also summarizes eight experiments in which one muscle of a pair was exposed for 90 min to 0.5 mM IAM. The decrease in ATP and the increase in ADP as well as the decrease in the ATP/ADP ratio were

all significant ($P < 0.01$). In comparison to the DNFB experiments, the longer delay (necessitating 90 min preincubation) and the less pronounced effects seen with iodoacetamide should be noted.

DNFB Effects on Membrane Potential

Any large membrane potential changes produced by DNFB in the experimental muscle would complicate paired comparisons. Determination of membrane potential also provides a control for nonspecific membrane damage. Fresh

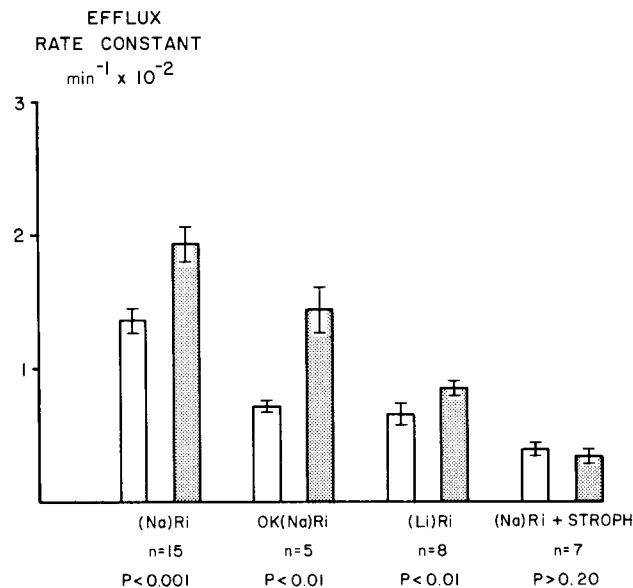


FIGURE 7. Rate constants for ²²Na efflux as affected by application of 0.5 mM IAM. See legend of Fig. 5 for protocol for rate constant determination. Stippled bars refer to the maximum rate constant attained after addition of 0.5 mM IAM.

muscles were used to circumvent problems posed by the large, transient net fluxes and electrogenic pumping which accompany recovery of loaded fibers in (Na)Ri at room temperature. Membrane potential in the presence of DNFB is virtually indistinguishable from that in (Na)Ri during 75 min of exposure to the inhibitor (Fig. 11). Thus for 75 min in (Na)Ri the mean resting potential of two freshly dissected muscles was -81.5 ± 0.66 mV (111 penetrations). For the same time period, two muscles in (Na)Ri + DNFB gave a mean resting potential of -81.3 ± 0.53 mV (110 penetrations). It will be recalled that flux measurements were not taken for more than 60 min in DNFB.

DISCUSSION

Na-Na Exchange

These experiments were devised to determine whether an Na-Na exchange, catalyzed by the sodium pump, could be elicited in frog sartorius muscle by metabolic inhibition. If the increase in Na efflux observed after poisoning with

DNFB or IAM represents Na-Na exchange, then this increase should not require the presence of external K, but be dependent on the presence of external Na. We found that, in both the presence and the absence of external K, metabolic poisoning caused an increase in strophanthidin-sensitive Na efflux.

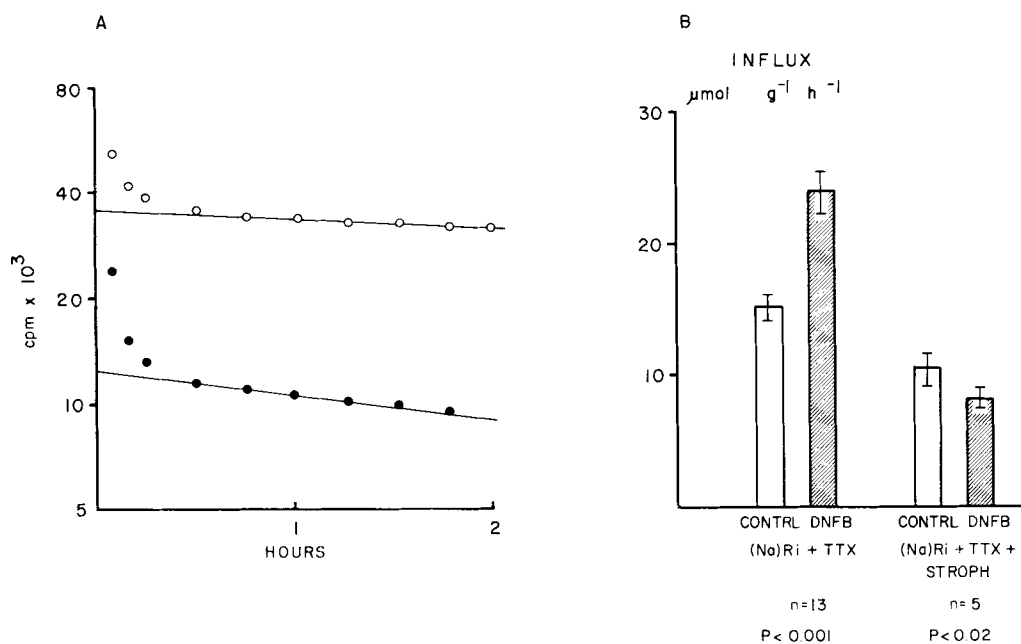


FIGURE 8 A. Technique for determination of ^{22}Na influx. Paired, Na-loaded frog sartorius muscles were labeled with ^{22}Na for 15 min at room temperature. Efflux into unlabeled OK(Na)Ri + 4×10^{-5} M strophanthidin + 10^{-7} g/ml TTX was followed as described in Methods. Total cpm remaining in each muscle is plotted semilogarithmically against time in minutes. The test muscle (○) was pretreated in (Na)Ri + 0.38 mM DNFB while the paired control (●) was held in (Na)Ri. Straight lines were fitted to the last six efflux points, and total intrafiber radioactivity was determined from extrapolation to zero time.

FIGURE 8 B. ^{22}Na influx from (Na)Ri. After 30 min preincubation in the given solution, muscles were transferred for 15 min to an identical solution containing ^{22}Na . Total intrafiber radioactivity was then determined as described in Fig. 8 A. From total intrafiber radioactivity, muscle wet weight and the specific activity of the loading solution, influx was calculated as $\mu\text{mol Na g}^{-1}\text{h}^{-1}$. All solutions contained 10^{-7} g/ml TTX.

On the other hand, this increment was greatly attenuated in the absence of external Na. The small residual stimulation of Na efflux seen in nominally Na-free media could represent Na-Na exchange dependent on remaining traces of Na, or result from a nonspecific leak produced by the inhibitor, though the latter possibility seems unlikely since strophanthidin-insensitive Na *influx* decreased after exposure to DNFB. Furthermore, the metabolic inhibitors stimulated Na influx, again as expected for an exchange mechanism. Finally, our

findings suggest that inhibitor-induced Na efflux and influx are catalyzed by the sodium pump, since both were strophanthidin sensitive. The extra Na efflux induced by metabolic poisoning is larger in the absence of external K than in its presence. Possible reasons are: (a) that the poisons are more effective in the absence of external K; (b) that metabolic poisoning induces a fixed amount of Na-Na exchange while inhibiting existing Na-K exchange; and/or (c) that external K inhibits Na-Na exchange. Further experiments, including measurements of K influx, are required to test these and other possibilities.

The DNFB effect on Na efflux is marked, but transient. Its brief duration makes it impossible to measure net Na movement accurately. A rough estimate of net flux can be made by comparing unidirectional fluxes, however. Average

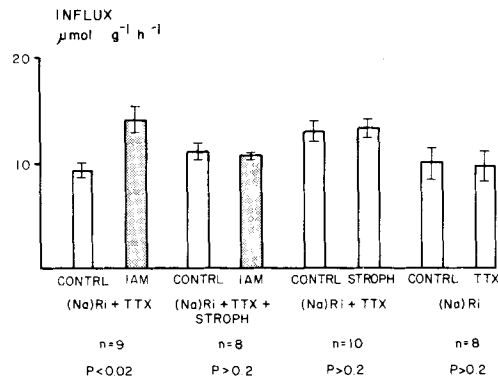


FIGURE 9. ²²Na influx from (Na)Ri. Influx was determined as described for Fig. 8. Preincubation periods of 90 min were used in the IAM experiments. Here all solutions contained 10⁻⁷ g/ml TTX, except for the preincubation and loading solutions used with the control muscles in the TTX experiment (last pair of bar graphs). Scatter in control values for influx is evident in comparison with the results of Fig. 8 B and is due to seasonal variability in the experimental animals.

influx of ²²Na in DNFB was 24 μmol/g·h. Given a rate constant for efflux of 0.024 min⁻¹, and an internal Na content of 20 mmol/kg (estimated by analysis for total Na of two muscles which had been loaded for 20 h and then allowed to recover for 3 h at room temperature in (Na)Ri), the efflux could be calculated as 29 μmol/g·h. This indicates that efflux and influx were, in fact, of comparable magnitude.

Metabolic Effects

This work was primarily designed to test the effects of altered metabolism on Na-Na exchange. As noted above, treatment with both DNFB and IAM did stimulate Na-Na exchange. Nucleotide assays indicated that significant alterations in [ATP], [ADP], and the ATP/ADP ratio did follow DNFB and IAM treatment. Thus, any observed flux changes could be due to increased [ADP], decreased [ATP], some combination of the two variables, or even a different metabolic alteration such as, for example, an increase in [AMP] which must accompany increases in [ADP], via the myokinase reaction. Since these factors

cannot be isolated, the ATP/ADP ratio was used as a convenient index. The time allowed for metabolic inhibition (30 min for DNFB and 90 min for IAM) was compatible with the time course of the respective efflux transients and influx determinations. It should also be noted that DNFB, which produces a greater change in the ATP/ADP ratio, also caused a more marked stimulation of Na efflux.

Two intracellular pools of ADP, bound and free, exist in the muscle cell. The assay procedure employed here measures total ADP. Only free ADP should influence pump operation, so the relation between bound and free ADP after metabolic inhibition must be considered. First, it would seem unlikely that both IAM and DNFB could act to increase the level of bound ADP, keeping free ADP

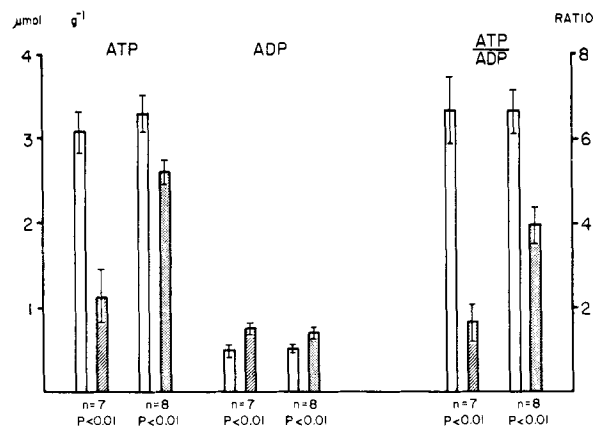


FIGURE 10. Effect of 0.38 mM DNFB on ATP and ADP content. Hatched bars refer to muscles treated for 30 min with (Na)Ri + 0.38 mM DNFB and then extracted and assayed as described in Methods. Stippled bars refer to muscles treated for 90 min with (Na)Ri + 0.5 mM iodoacetamide. Open bars refer to the paired control muscles.

constant as total ADP increased. Second, in the case of DNFB, Mommaerts and Wallner (1967) have presented direct evidence that poisoning with DNFB does not alter the amount of bound ADP. Thus, during metabolic poisoning, total ADP can be used as an index for changing levels of free ADP.

Controls for possible nonspecific effects of DNFB and IAM should be noted. Membrane potential was determined in normal and inhibitor-treated muscles for periods of time long enough to encompass any flux experiment. DNFB-treated muscles were virtually identical to untreated controls for the first 75 min of exposure. Similarly, Ling and Gerard (1949) report a virtually unchanged resting potential after 8 h in 0.3 mM iodoacetic acid. In addition it should be recalled that strophanthidin-insensitive Na efflux was unaffected by either IAM or DNFB. This would indicate that neither poison caused a large, nonspecific change in Na permeability. In fact a small *decrease* in Na influx, produced by DNFB in the presence of strophanthidin, was noted (Fig. 10). Given the fact that DNFB would be expected to react with all free amino groups, further comment

regarding its use as a specific metabolic inhibitor is in order. Mommaerts and Wallner (1967), Infante and Davies (1965), and Dydynska and Wilkie (1966) all came to the conclusion that DNFB can be used in studies of the mechanics and energetics of muscular contraction. Thus Infante and Davies (1965) reported that DNFB-treated muscle could develop normal isometric tension (though duration was curtailed due to ATP exhaustion), and could produce isotonic contractions normal in speed of shortening, speed of relaxation, and work performed. Also, fiber bundles prepared from DNFB-treated muscles by glycerol extraction shortened normally in response to ATP application. Finally,

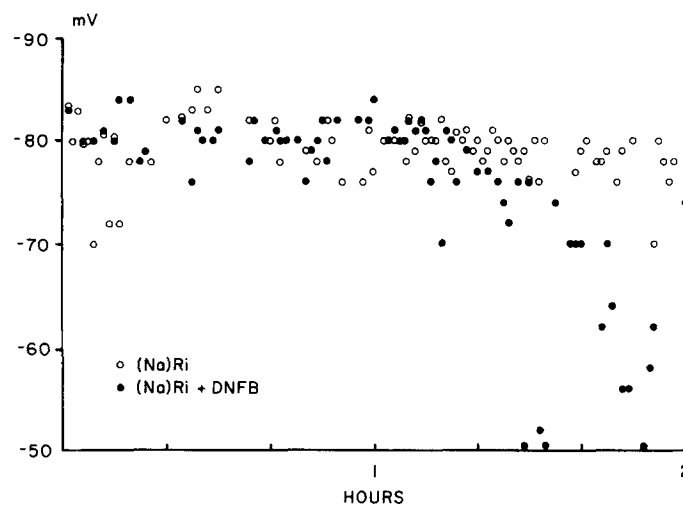


FIGURE 11. Effect of 0.38 mM DNFB on membrane potential. Solid symbols refer to penetrations in the DNFB-treated muscle; open symbols refer to the untreated, paired control muscle. The control (○) remained in (Na)Ri and a series of penetrations, over time, were taken. Timing for the experimental muscle (●) began when it was exposed to (Na)Ri + 0.38 mM DNFB, and all penetrations for this muscle were made in the presence of DNFB. Resting membrane potential is virtually unaffected by at least 1 h of exposure of DNFB.

myokinase and actomyosin ATPase were extracted after DNFB treatment; a slight (20–50%) inhibition of myokinase was noted while a slight (20–50%) stimulation of the ATPase was reported. In sum, DNFB does not appear to produce marked damage in frog sartorius muscle, indicating that it can be employed as a specific metabolic inhibitor.

Our conclusion, that increased Na-Na exchange is linked to a decreased ratio of ATP/ADP is based on the temporal correlation between the two kinds of effects produced by metabolic inhibitors. The case for a causal relationship seems strengthened by the fact that two chemically unrelated inhibitors of different potency, operating on different time scales, both caused similar alterations in pumped fluxes. The common link would seem to be the increase in [ADP] and/or the decrease in [ATP] concomitant with inhibition.

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