J. Membrane Biol. 39, 57–73 (1978)

Mode of Action of Theophylline on Sodium Efflux in Barnacle Muscle Fibers

E. Edward Bittar and Hanna Benjamin

Department of Physiology, University of Wisconsin, Madison, Wisconsin 53706

Received 25 February 1977; revised 6 June 1977; revised again 1 September 1977

Summary. The response of the Na efflux in unpoisoned barnacle fibers to 10 mM theophylline is biphasic; i.e., inhibition is followed by stimulation. The stimulatory response is unaffected by ouabain. Fibers pretreated with ouabain show no transitory inhibition when 10 mM theophylline is applied, but show prompt stimulation the magnitude of which is comparable to that observed with unpoisoned fibers. The same holds true for lower concentrations of theophylline. Prior injection of 500 mM EGTA completely abolishes the biphasic action of 10 mM theophylline. External application of 10 mM theophylline following removal of external Ca^{2+} fails to bring about a biphasic effect. Ca^{2+} restoration, however, results in a moderate rise in the Na efflux. External application of 10 mM theophylline stimulates the Na efflux into Ca^{2+}-free artificial seawater (ASW) when the test fibers are pretreated with ouabain. Injection of the protein inhibitor of Walsh leads to reduced stimulation by 10 mM theophylline of the Na efflux in unpoisoned fibers. Injection of the protein inhibitor of Corbin into unpoisoned fibers leads to reduced stimulation by 10 mM theophylline. Injection of cAMP into ouabain-poisoned fibers, following internal application of Corbin's inhibitor and external application of 10 mM theophylline, fails to cause a marked rise in the ouabain-insensitive Na efflux. Injection of Corbin's inhibitor into ouabain-poisoned fibers, following the onset of peak stimulation by 10 mM theophylline, fails to reduce the Na efflux. Fibers injected with 1 mM and 100 mM EGTA and exposed to 10 mM theophylline show a marked reduction in the response of the ouabain-insensitive Na efflux to injected cAMP when the concentration of theophylline is 10 mM. A poor response to injected cAMP is also seen in fibers bathed in Ca-free ASW containing 10 mM theophylline. Theophylline (10 mM) fails to cause an enhanced stimulation of the ouabain-insensitive Na efflux into Ca-free 3 mM-HEPES ASW or 10 mM-Ca^{2+} – 3 mM-HEPES ASW following the addition of protons to the bathing medium. An enhanced response is similarly not observed with injected cAMP following the addition of theophylline to the bathing medium. Injection of 8-fluorotheophylline, 3-isobutyl-1-methylxanthine and doxantrazole leads to a marked reduction in the response of the ouabain-insensitive Na efflux to injected cAMP. Contraction always takes place upon injecting these substances. These results are in keeping with the theory that theophylline acts chiefly by reducing myoplasmic pCa ($pCa \equiv -\log_{10}[Ca^{2+}]$), and that a reduced pCa leads to stimulation of the ouabain-insensitive Na efflux as the result of activation of the cGMP-dependent protein kinase system by newly formed cGMP.

Key Words: Theophylline, Na efflux and barnacle fibers.

0022-2631/78/0039-0057 $3.40

This work forms a part of a wider study of the mechanisms by which myoplasmic cAMP and Ca^{2+} control the ouabain-insensitive Na efflux in barnacle muscle fibers. Its starting point is the finding that caffeine exerts a biphasic effect on Na efflux which depends on external Ca^{2+}, and that the delayed stimulation caused by caffeine involves the ouabain-insensitive component of the Na efflux (Bittar *et al.*, 1974). The problem whether the delayed stimulation is the result of inactivation of the cAMP-phosphodiesterase system or the result of a raised myoplasmic free Ca^{2+} concentration has now been examined by using 1,3-dimethylxanthine (theophylline). Theophylline, like caffeine, is known to cause the release of Ca^{2+} from sarcoplasmic reticulum vesicles (Johnson & Inesi, 1969) and to slow Ca^{2+} uptake by the sarcoplasmic reticulum (Chapman & Rutherford, 1976). In addition, theophylline is known to inactivate the phosphodiesterase system in a wide variety of tissues (Appleman, Thompson & Russell, 1973). However, since Ca^{2+} is a powerful activator of the phosphodiesterase system (Kakiuchi & Yamazaki, 1970), it seemed a matter of special interest to determine whether the biphasic response of the Na efflux to a xanthine derivative is due to a fall in myoplasmic pCa or to a rise in myoplasmic cAMP concentration or both. To obtain information on the possible role of pCa and cAMP, experiments were undertaken using EGTA and the cAMP-dependent protein kinase inhibitor. This inhibitor, when injected into barnacle fibers, abolishes to a large extent the stimulatory response of the Na efflux to injected cAMP (Bittar, Chambers & Schultz, 1976). Thus, experiments with the protein inhibitor not only have the advantage of simplicity, but also of yielding information about rapid changes in myoplasmic cAMP concentration.

Materials and Methods

Materials

Specimens of the barnacle, *Balanus nubilus* and *Balanus aquila* were supplied by David King at Friday Harbor, Washington. They were kept at 12 °C in a filtered aerated Instant Ocean aquarium which contained artificial seawater (ASW) of the following composition (mM): Na, 465; K, 10; Ca, 10; and Mg, 50.

Dissection and Cannulation

Single fibers measuring, on an average, 3 cm in length and 1 1/2 mm in diameter were isolated by dissection from the three pairs of depressor muscle bundles. Cannulation of these fibers was carried out as described by Caldwell and Walster (1963) in their work on *Maia* muscle fibers. A weight of 50–100 mg was attached to the tendon.

Microinjector

Cannulated fibers were loaded with Na-22 and injected with test solutions by means of a microinjector similar to that designed by Hodgkin and Keynes (1956) and modified by Caldwell and Walster (1963). A more recent type of microinjector described by Bittar and Tallitsch (1975) was also employed in the latter half of this work. Since the volume of test solution was approximately 0.3 µl and the intrafiber volume 30 µl, the dilution factor maybe taken as 100.

Solutions

The ASW used as the bathing medium had the following composition (mM): NaCl, 465; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 10; $NaHCO_3$, 10; and pH 7.8. Solutions of varying Ca^{2+} concentration were prepared by altering NaCl in osmotically equivalent amounts. In some experiments bicarbonate as buffer was replaced by N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES).

Measurement of Na-22

The method adopted for collecting samples of the effluent and for counting their activity and the activity of the fiber at the end of each experiment was basically that described by Bittar (1966) and Bittar, Caldwell and Lowe (1967). Most of the experiments were carried out at a room temperature of 22–25 °C. In the experiments done at ca. 0 °C, microbeakers of special design surrounded by ice were used. The data obtained was treated in two ways: (i) net efflux (dNa^*/dt) in cpm was plotted against time on semilog paper, and (ii) the fractional rate constant $(1/Na^*) \cdot dNa^*/dt$ was plotted against time on linear paper. Fractional rate constant plots formed the basis of calculation of the magnitude of the responses observed in this work. Stimulation as a percentage was computed by taking into account the difference between the maximum rate constant for Na efflux and the value immediately preceding treatment. Percentage inhibition was computed by taking into account the difference between the rate constant immediately preceding treatment and the post-treatment value obtained by extrapolating the last points on the curve back to the time of the immediately preceding treatment. Significance levels were computed by means of Student's *t*-test.

Agents

Theophylline, EGTA, cAMP, and HEPES were purchased from Sigma Chemical Company, St. Louis. The protein inhibitor in solvent containing 0.3 M K_2HPO_4 and 0.001 M EDTA was obtained as a gift from Dr. Donal Walsh of the University of California, Davis. A similar gift from Dr. Jackie Corbin of Vanderbilt University, Nashville, was obtained in a solvent containing 1 mM EDTA and 5 mM K_2HPO_4 (pH 6.8). This protein inhibitor was isolated from rabbit skeletal muscle and partially purified by both workers in accordance with the method of Walsh, Ashby, Gonzalez, Calkins, Fischer and Krebs (1971). 3-Isobutyl-1-methylxanthine was purchased from Aldrich Chemical Company, Milwaukee. 8-Fluoro-theophylline was a gift from Dr. J.T. Wilkowsky of ICN Nucleic Acid Research Institute, Irvine, California. Doxantrozole [3-(-5-Tetrazolyl) thioxanthone 10, 10-dioxide monohydrate] was obtained as a gift from Mr. A.F. Green of the Wellcome Research Laboratories, Beckenham, Kent.

Results

The Biphasic Action of External Theophylline. Lack of Effect with Internal Application

External application of 10 mM theophylline was often found to cause a biphasic effect on the Na efflux; i.e., inhibition was quickly followed by stimulation. This result is illustrated in Fig. 1*a* and *b*. The magnitude of the inhibition averaged $20.5 \pm 3.7\,\%$ ($n=5$) and the stimulation $126.0 \pm 14.2\,\%$ ($n=7$). When, however, a solution of 100 mM theophylline was injected, the result was only a transitory stimulation in the order of $38.0 \pm 5.2\,\%$ ($n=4$). This result is practically the same as that obtained by injecting 100 mM caffeine (Bittar *et al.*, 1974).

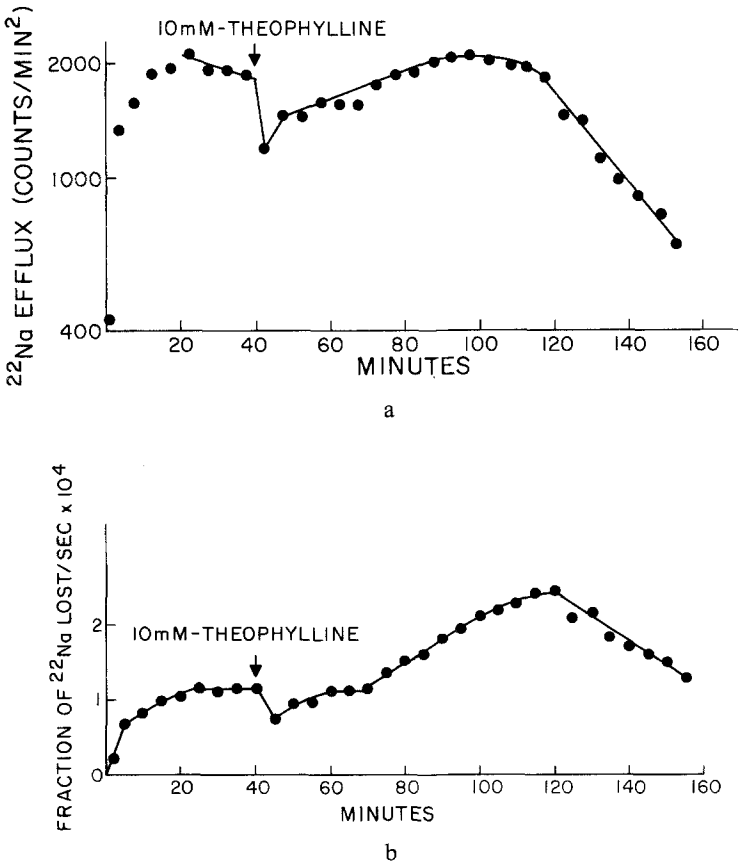

Fig. 1. The biphasic response of the Na efflux to external application of 10 mM theophylline. (*a*): Efflux plot; (*b*): Fractional rate constant plot

Stimulation by External Theophylline of the Ouabain-insensitive Na Efflux

The Na efflux in fibers treated with 10^{-4} M ouabain was markedly stimulated by external application of 10 mM theophylline. The average stimulation was in the order of 417.0 ± 48.4 % ($n=4$). To be certain that the ouabain-insensitive component of the Na efflux is the one modified by theophylline, experiments were designed in which 10^{-4} M ouabain was applied externally following 10 mM theophylline ($n=4$). Shown in Fig. 2 is the record of such an experiment. Clearly, ouabain was without effect, a result which confirms an earlier conclusion drawn from work with

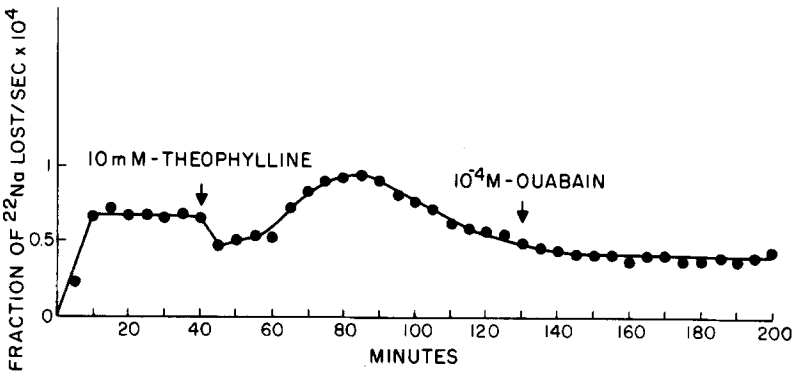

Fig. 2. Lack of effect of 10^{-4} M ouabain when applied externally following the full onset of the biphasic response to 10 mM theophylline

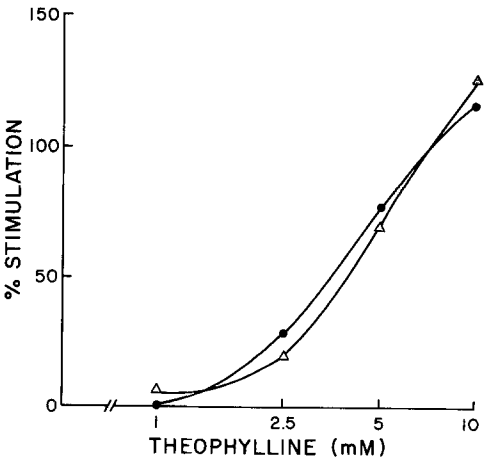

Fig. 3. Dose-response curve for the effect of varying external concentrations of theophylline on the Na efflux in unpoisoned ▲, and 10^{-4} M ouabain-poisoned ●, fibers. Each unfilled triangle represents the mean value of data obtained from the following number of fibers: 1 mM, $n=3$; 2.5 mM, $n=3$; 5 mM, $n=4$, and 10 mM, $n=7$. Each filled circle represents the following number of fibers: 1 mM, $n=3$; 2.5 mM, $n=3$; 5 mM, $n=3$; and 10 mM, $n=4$

cAMP, namely, that a see-saw mechanism underlies phosphorylation of the membrane as the result of activation of a cAMP-dependent protein kinase (Bittar, Chambers & Schultz, 1976). Summarized in Fig. 3 are the results obtained with varying concentrations of external theophylline employing both unpoisoned and ouabain-poisoned fibers. The baseline adopted for this comparison is the resting efflux found in both unpoisoned and ouabain-poisoned fibers. It can be seen that the two curves are practically superimposable.

Abolition of the Stimulatory Action of Theophylline by Preinjecting EGTA or by Removing External Ca^{2+}

Injection of fibers with 500 mM EGTA (at pH 7.0) before external application of 10 mM theophylline completely abolished the dual action of theophylline ($n=4$). It also prevented contraction from occurring provided the entire length of the fiber was injected with the chelator. In view of this, fibers were bathed in Ca^{2+}-free ASW before exposure to 10 mM theophylline. Though they contracted, a response of the Na efflux to theophylline failed to occur. Only when Ca^{2+} was restored to the bathing medium, was stimulation noticed. It averaged 72.5 ± 14.2 % ($n=16$) in size. These results differ from those observed with 10 mM caffeine only in that theophylline failed to produce inhibition of the Na efflux. Why this is so is unclear. To complete this comparison, the next group of experiments was conducted with ouabain-poisoned fibers. Shown in Fig. 4 is that the ouabain-insensitive Na efflux into Ca-free ASW was

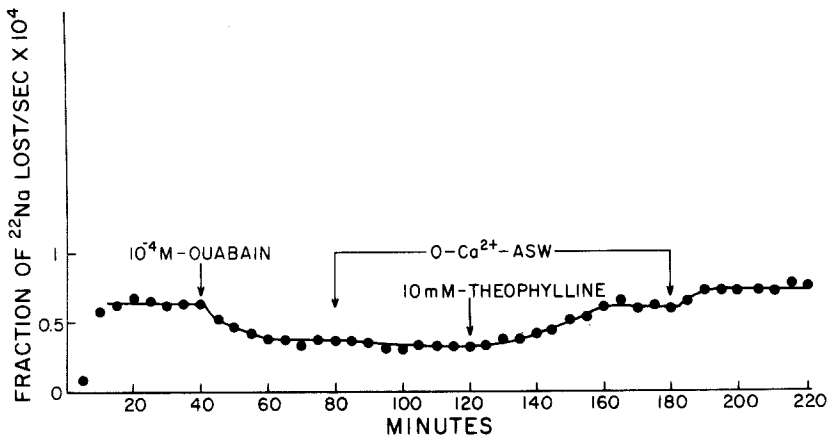

Fig. 4. Effect on the Na efflux of external application of 10^{-4} M ouabain, followed by removal of external Ca^{2+}, external application of 10 mM theophylline and then restoration of external Ca^{2+}

stimulated by 10 mM theophylline and that further stimulation took place as soon as external Ca^{2+} was restored (141.8 ± 34.2 % stimulation by theophylline and 48.4 ± 11.2 % stimulation following the addition of 10 mM-Ca^{2+}; $n=6$). This mode of behavior, that of dependency on external Ca^{2+}, is consistent with the concept that the ouabain-insensitive component of the Na efflux consists of Na:Ca exchange, as found in squid axons (Baker *et al.*, 1969) or that external Ca^{2+} acts as an allosteric regulator.

Stimulation by Theophylline at 0 °C

Bittar, Chambers and Schultz (1976) produced evidence that injected cAMP stimulates the Na efflux in fibers cooled down to 0 °C, and that this mechanism depends on the presence of external Ca^{2+}. Experiments with fibers chilled to 0 °C show that 10 mM theophylline caused stimulation of the remaining efflux in the order of 191.8 ± 60.3 % ($n=3$). The stimulation by theophylline observed in fibers treated with 10^{-4} M-ouabain prior to cooling was in the order of 81.6 ± 12.6 % ($n=3$). An explanation as to why ouabain-poisoned fibers showed a reduced response is unavailable.

The Response to Theophylline Following Injection of the Protein Inhibitor of Walsh and Corbin

Fibers injected with the protein inhibitor of Walsh before external application of 10 mM theophylline showed a marked reduction in the response of the Na efflux to the xanthine derivative (40.3 ± 12.2 % stimulation ($n=3$) vs. 110.0 ± 10.2 % in controls, $n=3$, $p<0.02$). This led to experiments with 10^{-4} M ouabain-pretreated fibers. Estimates of the stimulatory response to external 10 mM theophylline in these fibers preinjected with the protein inhibitor gave an average value of 107.0 ± 45.6 % ($n=6$), as compared with 193.5 ± 74.5 % stimulation observed in controls injected only with the solvent. The difference is not statistically significant. In parallel experiments, however, carried out with the protein inhibitor supplied by Dr. J. Corbin (who also isolated the material from rabbit skeletal muscle) the results were as follows: (i) Fibers injected with Corbin's inhibitor, followed by external application of 10 mM theophylline, showed 81.3 ± 12.6 % stimulation ($n=8$). This is to be compared with 169.4 ± 24.2 % stimulation by theophylline observed in control

fibers ($n=8$) injected with solvent (containing 1 mM EDTA and 5 mM K_2HPO_4, pH 6.8) beforehand. ($P<0.01$). (ii) Fibers treated with 10^{-4} M ouabain, followed by internal application of Corbin's inhibitor, showed 101.1 ± 10.6 % stimulation by external 10 mM theophylline. Controls, by contrast, showed 470.2 ± 113.1 % stimulation ($n=4$). The difference is statistically significant ($P<0.05$). (iii) In further experiments, the same protocol was adopted except that 10^{-2} M cAMP was injected following the onset of (reduced) stimulation by external theophylline. Injected cAMP, in this case, caused only 36.6 ± 4.3 % stimulation ($n=3$), whereas in control fibers it caused 197.1 ± 4.6 % stimulation ($n=3$) ($P<0.001$). Such a result is not unexpected if (i) Corbin's inhibitor stops the protein kinase reaction, (ii) a fall in myoplasmic pCa causes activation of phosphodiesterase, and (iii) a fall in pCa impairs the action of cAMP on the protein kinase system. In view of the above results, it became necessary to check whether injection of Corbin's inhibitor following external application of 10 mM theophylline partially reverses stimulation of the ouabain-insensitive Na efflux caused by theophylline. The results obtained showed a lack of effect with injected Corbin's inhibitor ($n=6$). This is illustrated by Fig. 5*a* and *b* where it can be seen that injection of solvent into the companion control fiber causes the stimulated Na efflux to taper-off slightly in the same manner as does Corbin's inhibitor. The tapering effect is most likely due to the EDTA present in the solvent.

Response of the Ouabain-Insensitive Na Efflux to Injected cAMP Following Injection of EGTA and External Application of Theophylline

To find out whether theophylline reduces the stimulatory action of injected cAMP by raising the myoplasmic free Ca^{2+} concentration, experiments were designed in which graded amounts of EGTA were injected prior to the application of theophylline and cAMP. EGTA when injected usually caused a very transitory rise in the basal efflux. As shown in Fig. 6, the response of the ouabain-insensitive Na efflux in a fiber preinjected with 1 mM EGTA is small not only to 10 mM theophylline, but also to injected 10^{-2} M cAMP. Estimates gave an average value of 44.8 ± 8.3 % and 68.9 ± 28.4 %, respectively ($n=6$), or a total stimulation of about 144 % if it is assumed that theophylline acts only by increasing cAMP. The latter estimate, however, cannot be compared with the control value, since the stimulation caused by theophylline was barely affected by Corbin's inhibitor when it was applied following the onset of peak stimulation. The control experiments in which theophylline was not

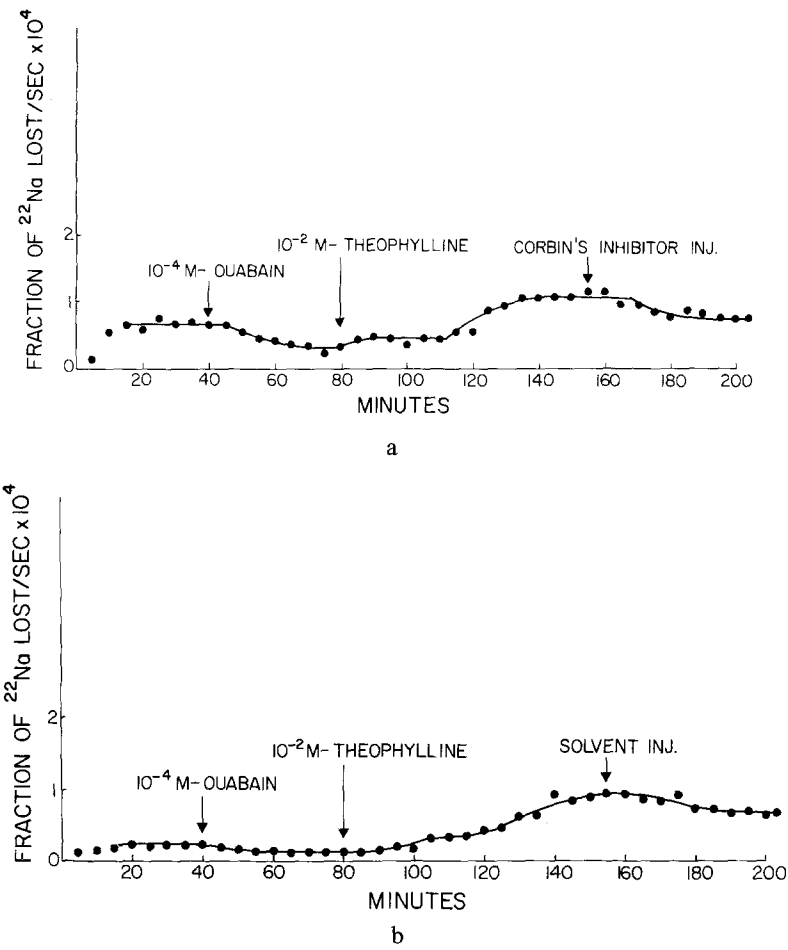

Fig. 5. (*a*): Effect of internal application of Corbin's inhibitor on the ouabain-insensitive Na efflux following external application of 10^{-4} M ouabain and 10^{-2} M theophylline. (*b*): Control fiber injected with the solvent free of Corbin's inhibitor

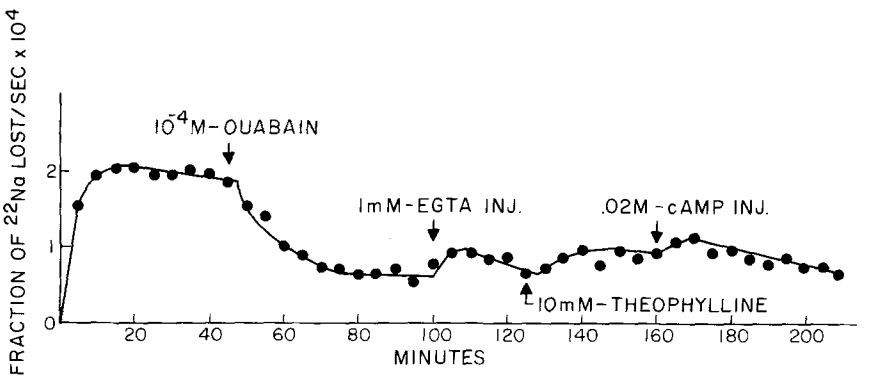

Fig. 6. Effect on the ouabain-insensitive Na efflux of external application of 10 mM theophylline, followed by internal application of 0.02 M cAMP in a fiber pretreated with 10^{-4} M ouabain and injected with 1 mM EGTA (pH 7.0)

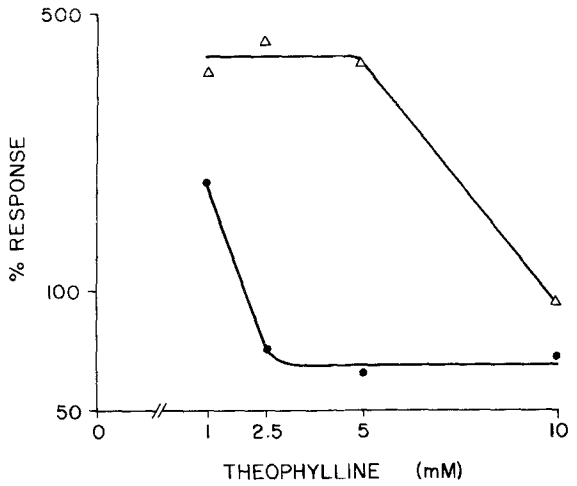

Fig. 7. Response curves for the stimulatory effect on Na efflux of injected 0.02 M cAMP using fibers exposed to a varying concentration of external theophylline following pretreatment with 10^{-4} M ouabain and injection with 1 or 100 mM EGTA. ●, fibers injected with 1 mM EGTA; each plotted point is the mean of data obtained from the following number of experiments: 1 mM, $n=7$; 2.5 mM, $n=4$; 5 mM, $n=4$; and 10 mM, $n=6$. △, fibers injected with 100 mM EGTA; each plotted point is the mean of data obtained from four fibers (log-log plot)

applied showed 234.3 ± 66.5 % stimulation caused by injected cAMP ($n=10$). The difference between 68.9 % (and not 144 %) and this value is significant, $P<0.05$. Parallel experiments were run using 100 mM EGTA. These showed 93.7 ± 33.6 % ($n=4$) stimulation caused by injected cAMP. Comparing this result with that obtained with 1 mM EGTA, the difference turns out not to be statistically significant. To substantiate the idea that myoplasmic pCa (as affected by theophylline) governs the response of the ouabain-insensitive Na efflux to injected cAMP, it became necessary to demonstrate that suppression of the cAMP effect depends on the concentration of theophylline used. Hence, two groups of experiments were undertaken: in one group 10^{-4} M-ouabain-poisoned fibers were injected with 1 mM EGTA and exposed to varying concentrations of theophylline before being injected with 0.02 M cAMP. In the other group a similar protocol was followed, except that the fibers were injected with 100 mM EGTA. Summarized in Fig. 7 are the results of these experiments. The main point which emerges is that only with 10 mM theophylline was it possible to drastically block the response of the ouabain-insensitive Na efflux to injected 0.02 M cAMP in both groups of fibers. It is perhaps also of significance that marked stimulation by injected

cAMP was observed in both groups of fibers when pre-exposed to 1 mM theophylline. To ascertain whether the blocking action of 10 mM theophylline on the response of the ouabain-insensitive Na efflux to injected cAMP depends on external Ca^{2+}, measurements of the efflux were made using Ca-free ASW. Following external application of 10^{-4} M ouabain, external Ca^{2+} was omitted, and 10 mM theophylline added; this was followed by injection of 0.02 M cAMP. The stimulation caused by cAMP was in the order of 100.9 ± 20.5 % ($n=10$), as compared with 414.0 ± 11.7 % stimulation in control fibers unexposed to theophylline ($n=2$) ($P<0.001$). Restoration of external Ca^{2+} following the onset of stimulation caused by cAMP resulted in 27.2 ± 4.1 % stimulation ($n=6$), an effect which is less than half that observed in unpoisoned fibers unexposed to theophylline but injected with cAMP (Bittar, Chambers & Schultz, 1976), or more than half that observed in fibers exposed to theophylline (p. 62). In this connection, it should be mentioned that the percentage change method of analysis adopted here is valid since the omission of external Ca^{2+} failed to alter the basal efflux.

The Response to Theophylline following Acidification of a Non-HCO_3^- Medium

Bittar *et al.*, (1976) reported that the sensitivity of the ouabain-insensitive Na efflux to injected cAMP is increased when protons are added to Ca^{2+}-free ASW containing HEPES as buffer instead of HCO_3^-. Similar experiments were therefore run with theophylline in which fibers bathed in 3 mM HEPES ASW were exposed to 10^{-4} M ouabain. This was followed by a reduction in external pH (pH*e*) from 7.8 to 6.8, 6.3, 5.8, 5.5, 5.3 and 5, omission or no omission of external Ca^{2+}, and then addition of 10 mM theophylline. Fig. 8, which gives the results of these experiments, shows that with only one exception (at pH*e* 5.3) the response of the ouabain-insensitive Na efflux into 10 mM-Ca ASW or Ca-free ASW remained relatively the same at low external pH and pH*e* 7.8. This was taken to mean that the pH*e*-sensitive component is inactive in the presence of theophylline. But to be certain that this is so, two groups of experiments were run in which 0.02 M cAMP was injected following the onset of stimulation caused by external 10 mM theophylline. In both groups external pH was lowered to 6.0, but in the second group external Ca^{2+} was omitted immediately before injection of cAMP. The results showed that cAMP caused 24.1 ± 9.6 % stimulation of the ouabain-insensitive Na efflux into 10 mM Ca-ASW ($n=4$) (controls in which ASW

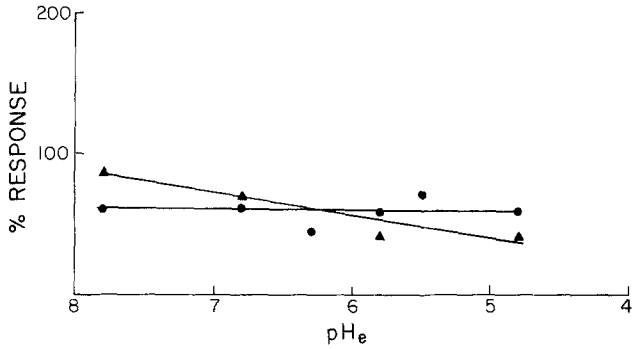

Fig. 8. Relation between the response of the ouabain-insensitive Na efflux into 10 mM-Ca^{2+} – ASW and Ca^{2+}-free ASW to external 10 mM-theophylline, and external pH. Buffer used was HEPES (3 mM) ▲, experiments with 10 mM-Ca^{2+} – ASW; each plotted point is the mean of data obtained from four fibers. ●, experiments with Ca-free ASW; each point, except that at pHe 7.8 (which is based on data from eight fibers), is the mean of data obtained from four fibers

without theophylline was used: 526.0 ± 86.2 %; $n=2$) and 27.8 ± 11.1 % stimulation in the absence of external Ca^{2+} ($n=4$) (controls: 182.1 ± 14.3 %; $n=2$). Failure of any appreciable stimulation of the ouabain-insensitive Na efflux to occur in the presence of a low external pH implies impairment of Na:H exchange as the result of the blocking actions of theophylline.

Response to Injected cAMP Following Injection of Xanthine Derivatives and Doxantrazole

The object of these experiments was to see if xanthine derivatives, e.g., 8-fluoro-theophylline and 3-isobutyl-1-methyxanthine, when injected, will reduce the response of the ouabain-insensitive Na efflux to injected cAMP. Experiments with doxantrazole [3-(5-tetrazolyl)-thioxanthrone 10, 10-dioxide], an antiallergic agent, which acts as a phosphodiesterase inhibitor (Tateson & Trist, 1976), were also undertaken. Summarized in Table 1 are the results obtained when the fibers used where pretreated with 10^{-4} M-ouabain and injected with 0.02M cAMP following injection of the xanthine derivative or doxantrazole. It can be seen that in all four groups of experiments a marked reduction in the response to injected cAMP took place. In every instance the fibers tested underwent contraction when injected with the derivative or doxantrazole. It seems, therefore, very likely that these substances behave just like theophylline.

Table 1. Response of the ouabain-insensitive Na efflux to 0.02 M cAMP injected in fibers preinjected with various phosphodiesterase inhibitors

	Substance injected	% Response		P value	Solvent	pH of solution	Contraction
		Test fibers	Control fibers				
1	Theophylline (10^{-1} M)	$n=6$ $\bar{x}=248.0\pm78.6$	$n=2$ $\bar{x}=910.4\pm118.1$	$P<0.01$	H_2O	9.0	+
2	8-Fluoro-Theophylline (10^{-2} M)	$n=8$ $\bar{x}=249.9\pm63.4$	$n=3$ $\bar{x}=1063.9\pm284.3$	$P<0.05$	H_2O	9.0	+
3	3-Isobutyl-1-Methyl-xanthine (10^{-2} M)	$n=7$ $\bar{x}=88.4\pm35.4$	$n=3$ $\bar{x}=661.2\pm122.0$	$P<0.01$	50% ETOH	8.0	+
4	Doxantrazole (10^{-2} M)	$n=4$ $\bar{x}=61.8\pm22.8$	$n=3$ $\bar{x}=661.2\pm122.0$	$P<0.01$	25% ETOH	10.0	+

Lack of Response to External Theophylline in Fibers pretreated with Procaine

Bittar, Hift, Huddart and Tong (1974) showed that external procaine blocks caffeine not only from causing contracture in barnacle fibers, but also from altering the Na efflux. This is also the case when 10 mM procaine is applied before 10 mM theophylline. In the first group of experiments, external application of 10 mM procaine completely prevented 10 mM theophylline from modifying the Na efflux. Subsequent omission of procaine resulted in a slow rise of the Na efflux, the peak of which was in the order of 17.5 ± 10.3 % ($n=4$). Contracture was noticed to occur some 15 min after omission of the procaine. (Controls showed 154.5 ± 54.5 % stimulation and a strong contracture; $n=2$.) In the second group, fibers were exposed to 10^{-4} M ouabain before 10 mM procaine and 10 mM theophylline were applied. Such an experiment is shown in Fig. 9. It will be appreciated that theophylline is indeed without effect, and only following the removal of external procaine does the ouabain-insensitive Na efflux respond with some delay and rather slowly. Contracture was seen to develop some 10 min prior to the stimulation. The delayed stimulation was in the order of 31.8 ± 23.6 % ($n=4$) (controls showed 248.5 ± 143.1 % stimulation and a strong contracture; $n=2$). These results strengthen the argument that the biphasic action of theophylline on unpoisoned fibers and its monophasic action on ouabain-poisoned fibers are related to a fall in myoplasmic pCa.

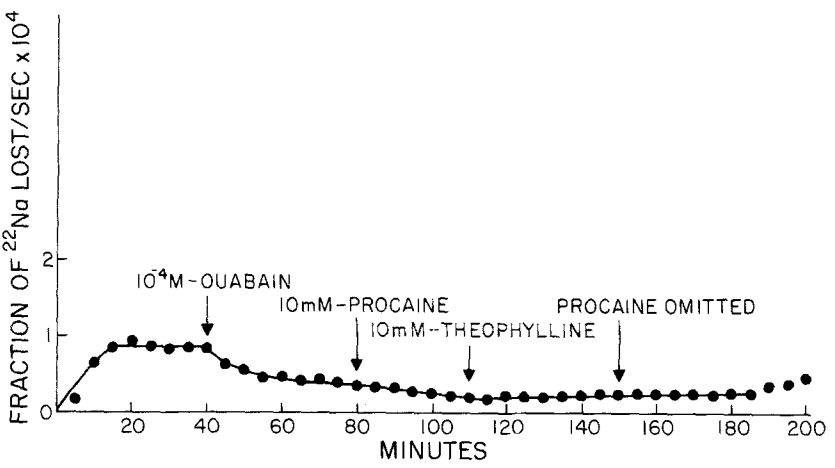

Fig. 9. Lack of effect of 10 mM theophylline when applied externally to a fiber pretreated externally with 10^{-4} M ouabain and 10 mM procaine

Discussion

The observation that the biphasic response of the Na efflux to external theophylline fails to occur in the absence of external Ca^{2+} and is not seen in fibers pretreated with a sufficient amount of injected EGTA has led to the inference that theophylline acts by reducing myoplasmic pCa. Transitory inhibition, which is often observed to occur before stimulation, can be accounted for in at least two ways. One is that it is due to activation by Ca^{2+} of cAMP-phosphodiesterase. This explanation is supported by evidence that barnacle fibers possess phosphodiesterase (F. Siegel, *private conversation*). The other is that inhibition is due to inactivation by Ca^{2+} of membrane adenyl cyclase (Drummond & Duncan, 1970; Severson, Drummond & Sulakhe, 1972).

The application of theophylline to poisoned fibers has been found to cause rapid stimulation of the ouabain-insensitive Na efflux, a situation strikingly similar to that seen when $CaCl_2$ (Danielson *et al.*, 1971; Bittar and Schultz, 1977), cAMP (Bittar *et al.*, 1976) or cGMP, (Sharp & Bittar, 1977) is injected into ouabain-poisoned fibers. The question may thus be asked whether the mechanism responsible for the stimulation can be satisfactorily interpreted in terms not only of a fall in myoplasmic pCa, but also of membrane phosphorylation. Three available clues to the answer are: Firstly, that Ca^{2+} is a powerful activator of the guanylate cyclase system found in a wide variety of tissues (Chrisman *et al.*, 1975; Garbers, Dyer & Hardman, 1975). Secondly, that depolarization of

barnacle fibers with high K leads to a threefold increase in internal cGMP content (Beam, Nestler & Greengard, 1977). And thirdly, that barnacle fibers contain a protein kinase which is activated by cGMP, the K_m for cGMP being 0.1 μM (J.P. Kuo, *private communication*). The idea that activation by newly formed cGMP of cGMP-dependent protein kinase results in phosphorylation of the fiber membrane helps to explain the failure of ouabain to reduce the stimulation observed with theophylline. This is consistent with evidence that the stimulatory response caused by injecting cAMP (Bittar *et al.*, 1976), cGMP (Sharp & Bittar, 1977) or $CaCl_2$ (Bittar & Schultz, 1977) is wholly unaffected by applying ouabain following the onset of peak stimulation.

One salient but puzzling feature of the experiments carried out with the protein inhibitor of Corbin is that its injection prior to the application of theophylline reduces the size of the stimulation in both unpoisoned and poisoned fibers. Such a result implies that theophylline acts in part by raising the myoplasmic cAMP concentration. This could be explained by postulating that the initial rise in myoplasmic free Ca^{2+} concentration is insufficient to inactivate adenyl cyclase but enough to activate it. The requirement for low Ca^{2+} is to some extent borne out by the finding that the protein inhibitor of Walsh is able to promptly reverse the stimulatory response of the Na efflux to injection of $CaCl_2$ in low concentration, but not in high concentration (Bittar & Schultz, 1977). Additional support for this suggestion is obtained from studies involving adenyl cyclase from glial tumor cells, which show that the enzyme is activated by low Ca^{2+} (Brostrom *et al.*, 1976).

The experiments with procaine yielded data which show that procaine stops theophylline not only from causing contracture, but also from causing a biphasic alteration in the Na efflux. The most plausible explanation of how procaine generally acts is to suppose that it blocks the release of Ca^{2+} from the sarcoplasmic reticulum (SR) (Feinstein, 1963; Carvalho, 1968; Suko *et al.*, 1976). Whether procaine interrupts the inward movement of Ca^{2+} is unknown, but it does reduce the efflux of ^{45}Ca before or after the removal of external Ca^{2+} (Chen, 1974).

This work was supported in part by grants from the Graduate School Research Committee, The Wisconsin Heart Association and The Office of Naval Research.

References

Appleman, M.M., Thompson, W.J., Russell, T.R. 1973. Cyclic nucleotide phosphodiesterases. *Adv. Cyclic Nucleotide Res.* **3**:65

Baker, P.F., Blaustein, M.P., Hodgkin, A.L., Steinhardt, R.A. 1969. The influence of calcium on sodium efflux in squid axons. *J. Physiol. (London)* **200**:431

Beam, K.G., Nestler, E.J., Greengard, P. 1977. Increased cyclic GMP levels associated with contraction in muscle fibers of the giant barnacle. *Nature (London)* **267**:534

Bittar, E.E. 1966. Effect of inhibitors and uncouplers on the Na pump of the *Maia* muscle fibre. *J. Physiol. (London)* **187**:81

Bittar, E.E., Caldwell, P.C., Lowe, A.G. 1967. The efflux of sodium from single crab muscle fibres. *J. Mar. Biol. Assoc. U.K.* **47**:709

Bittar, E.E., Chambers, G., Schultz, R. 1976. Mode of stimulation by adenosine 3′:5′-cyclic monophosphate of the sodium efflux in barnacle muscle fibres. *J. Physiol. (London)* **257**:561

Bittar, E.E., Hift, H., Huddart, E., Tong, E. 1974. The effects of caffeine on sodium transport, membrane potential, mechanical tension and ultrastructure in barnacle muscle fibres. *J. Physiol. (London)* **242**:1

Bittar, E.E., Schultz, R. 1977. Studies of the mode of stimulation by external acidification and raising internal free calcium concentration of the sodium efflux in barnacle muscle fibers. *Pfluegers Arch. (in press)*

Bittar, E.E., Tallitsch, R.B. 1975. Stimulation by aldosterone of the sodium efflux in barnacle muscle fibres: Effects of RNA inhibitors and spironolactone. *J. Physiol. (London)* **250**:331

Brostrom, M.A., Brostrom, C.O., Breckenridge, B.M., Wolff, D.J. 1976. Regulation of adenyl cyclase from glial tumor cells by calcium and calcium binding protein. *J. Biol. Chem.* **251**:4744

Caldwell, P.C., Walster, G.E. 1963. Studies of the microinjection of various substances into crab muscle fibres. *J. Physiol. (London)* **169**:353

Carvalho, A.P. 1968. Calcium-binding properties of sarcoplasmic reticulum as influenced by ATP, caffeine, quinine, and local anesthetics. *J. Gen. Physiol.* **52**:622

Chapman, R.A., Rutherford, N.G. *Cited by:* Chapman, R.A. and Leoty, C. 1976. Which of caffeine's chemical relatives are able to evoke contractures in mammalian heart? *Recent Adv. Stud. Card. Struct. Metab.* **7**:425

Chen, S.S. 1974. Studies of radiocalcium efflux in single barnacle muscle fibres: Effects of procaine and external divalent cations. *J. Physiol. (London)* **238**:313

Chrisman, T.D., Garbers, D.L., Parks, M.A., Hardman, J.G. 1975. Characterization of particulate and soluble guanylate cyclase from rat lung. *J. Biol. Chem.* **250**:374

Danielson, B.G., Bittar, E.E., Chen, S., Tong, E. 1971. The influence of low pH, high K and microinjected $CaCl_2$ on the ouabain-insensitive component of sodium efflux in barnacle muscle fibers. *Life Sci.* **10**:833

Drummond, G.I., Duncan, L. 1970. Adenyl cyclase in cardiac tissue. *J. Biol. Chem.* **245**:976

Feinstein, M.B. 1963. Inhibition of caffeine rigor and radiocalcium movements by local anesthetics in frog sartorius muscle. *J. Gen. Physiol.* **47**:151

Garbers, D.L., Dyer, E.L., Hardman, J.G. 1975. Effects of cations on guanylate cyclase of sea urchin sperm. *J. Biol. Chem.* **250**:382

Hodgkin, A.L., Keynes, R.D. 1956. Experiments on the injection of substances into squid axons by means of a microsyringe. *J. Physiol. (London)* **131**:592

Johnson, P.N., Inesi, G. 1969. The effect of methylxanthines and local anesthetics on fragmented sarcoplasmic reticulum. *J. Pharmacol. Exp. Ther.* **169**:308

Kakiuchi, S., Yamazaki, R. 1970. Stimulation of the activity of cyclic 3′,5′-nucleotide phosphodiesterase by calcium ion. *Proc. Jpn. Acad.* **46**:387

Kuo, J.F., Krueger, B.K., Sanes, J.R., Greengard, P. 1970. Cyclic nucleotide-dependent protein kinases. V. Preparation and properties of adenosine 3′,5′-monophosphate-dependent protein kinase from various bovine tissues. *Biochim. Biophys. Acta* **212**:79

Severson, D.L., Drummond, G.I., Sulakhe, P.V. 1972. Adenylate cyclase in skeletal muscle. *J. Biol. Chem.* **247**:2949

Sharp, D.M., Bittar, E.E. 1977. On the mode of stimulation by cyclic GMP of sodium efflux in barnacle muscle fibers. *Physiologist* **20**:86

Suko, J., Winkler, F., Scharinger, B., Hellmann, G. 1976. Aspects of the mechanism of action of local anesthetics on the sarcoplasmic reticulum of skeletal muscle. *Biochim. Biophys. Acta* **443**:571

Tateson, J.E., Trist, D.G. 1976. Inhibition of adenosine-3′,5′-cyclic monophosphate phosphodiesterase by potential antiallergic compounds. *Life Sci.* **18**:153

Walsh, D.A., Ashby, C.D., Gonzalez, C., Calkins, D., Fischer, E.H., Krebs, E.G. 1971. Purification and characterization of a protein inhibitor of adenosine 3′,5′-monophosphate-dependent protein kinases. *J. Biol. Chem.* **246**:1977