We studied the effect of adenosine on Na\(^+\)/Ca\(^{2+}\) exchange activity in ewe heart ventricular sarcolemmal vesicles. Adenosine was found to stimulate Na\(^+\)/Ca\(^{2+}\) exchange activity in a dose-dependent manner from 0.1 nM to 10 \(\mu\)M, with maximal stimulation (40\%) at 0.1 \(\mu\)M adenosine. The \(V_{\text{max}}\) of Na\(^+\)/Ca\(^{2+}\) exchange was increased, but the \(K_m\) for Ca\(^{2+}\) was not altered. The effect of adenosine was specific since 1 \(\mu\)M adrenaline, isoprenaline, and guanosine led to less than 15\% stimulation, and adenosine diphosphate had no effect. Caffeine antagonized the activation of Na\(^+\)/Ca\(^{2+}\) exchange by adenosine, and the order of potency of adenosine analogs was N\(^6\)-(L-2-phenylisopropyl)adenosine = N\(^6\)-cyclohexyladenosine = 5\-'(N-ethylcarboxamido)adenosine \(>\) N\(^6\)-(d-2-phenylisopropyl)adenosine, indicating the involvement of A\(_1\) subclass receptors. The effect of adenosine was mimicked by guanosine 5\-'O-(3-thiotriphosphate) (GTP\(\gamma\)S) and blocked by pertussis toxin treatment. Taken together, these results suggest that A\(_1\) subclass receptors coupled to a pertussis toxin-sensitive G protein mediate the activation of Na\(^+\)/Ca\(^{2+}\) exchange activity by adenosine. We conclude that the negative inotropic effect of adenosine in ventricular muscle, antagonistic toward cyclic AMP, may involve activation of Na\(^+\)/Ca\(^{2+}\) exchange.

The negative effects of adenosine on cardiac activity have been known since 1929 (1). However, the biochemical mechanisms underlying these effects remain unclear. P\(_1\) and P\(_2\) type adenosine receptors have been defined according to structural requirements of adenosine derivatives (2). P\(_1\) class sites require the integrity of the ribose moiety of the adenosine molecule, are most sensitive to adenosine, and can be blocked by methylxanthines such as caffeine. P\(_2\) class sites can be classified into A\(_1\) and A\(_2\) subclasses on the basis of the rank of potency of a series of agonists (3, 4). The P\(_2\) class sites only accept adenosine analogs with an intact purine moiety and are most sensitive to ATP (2).

A\(_1\) subclass adenosine receptors have been identified directly by radioligand binding in atrial (5, 6) and ventricular tissues (6, 7), and it is generally admitted that these receptors mediate the negative inotropic effects of adenosine (8–14). In atrial muscle, adenosine exerts a “direct” effect by activating K\(^+\) channels, which leads to a shortening of the action potential duration and, in turn, reduces the force of contraction (15–17). In ventricular muscle, adenosine produces a negative inotropic effect only when the force of contraction is stimulated with adenylylcyclase activators or phosphodiesterase inhibitors (12, 13, 18). This “indirect” effect is not linked to a change in K\(^+\) conductance (12), and adenylylcyclase inhibition has been proposed as the mechanism. However, conflicting results have been reported (6, 19–21). Moreover, inhibition of adenylylcyclase by adenosine analogs was shown to be mediated by a P\(_2\) site (22), which precludes this mechanism in the A\(_1\)-mediated negative inotropic effects of adenosine.

Na\(^+\)/Ca\(^{2+}\) exchange catalyzes electrogenic countertransport of Na\(^+\) ions for Ca\(^{2+}\) ions (23–26). It can operate in both directions, and its activity varies during the course of the cardiac cycle. It supports Ca\(^{2+}\) uptake during the early part of the action potential and plays an essential role in relaxation by extruding Ca\(^{2+}\) (27, 28). It has been suggested that Na\(^+\)/Ca\(^{2+}\) exchange is involved in a number of aspects of cardiac function including the force-frequency relationship, the beat-to-beat regulation of cardiac contraction, \(\beta\)-adrenergic relaxation, calcium-induced arrhythmias, the antiarrhythmic action of local anesthetics, cardiac glycoside-induced positive inotropic effects, the calcium paradox, and reperfusion injury (for a review, see Ref. 27). Defective Na\(^+\)/Ca\(^{2+}\) exchange activity has been associated with intracellular Ca\(^{2+}\) overload in myocardial ischemia (29), genetically linked or catecholamine-induced cardiomyopathies (30), and cardiac hypertrophy (31).

We show here that adenosine, at nanomolar concentrations, specifically stimulates Na\(^+\)/Ca\(^{2+}\) exchange in ewe ventricular sarcolemmas. The action of adenosine is mediated by A\(_1\) class adenosine receptors coupled to a pertussis toxin-sensitive G protein.

**MATERIALS AND METHODS**

**Sarcolemmal Vesicle Preparation**—Sarcolemmal vesicles were prepared from frozen heart ventricles of 2-year-old ewes, according to Frank et al. (32) with minor modifications. Minced frozen ventricles (80 g) were homogenized using a Waring Blender for 2 \(\times\) 20 s (high speed) in 20 mM Tris acetic acid, pH 7.6, containing 250 mM sucrose, 100 mM KCl, 25 mM sodium pyrophosphate, and 1 mM dithiothreitol. Vesicles harvested from the sucrose gradient were suspended in 160 mM NaCl, 20 mM Hepes, pH 7.4, and centrifuged at 160,000 X \(g\) for 75 min. The pellets were then resuspended in the same buffer at a concentration of 4 mg of protein/ml and stored in liquid nitrogen until use.

**Treatment of Sarcolemmal Vesicles with Pertussis Toxin**—Prior to use, the toxin was activated by incubation with 20 mg dithiothreitol

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1 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrito)tetraacetic acid; NECA, N\(^6\)-(N-ethylcarboxamido)adenosine; CHA, N\(^6\)-cyclohexyladenosine; 1-PIA, N\(^6\)-(L-2-phenylisopropyl)adenosine; d-PIA, N\(^6\)-(d-2-phenylisopropyl)adenosine; GTP\(\gamma\)S, guanosine 5\-'O-(3-thiotriphosphate).
for 30 min at 37 °C. Sarcolemmal vesicles (1 mg of protein) were incubated with 10 μg/ml activated pertussis toxin or vehicle, in a final volume of 0.5 ml containing 20 mM Hepes, pH 7.4, 160 mM NaCl, 1 mM ATP, 3 mM dithiothreitol, 0.1 mM GTP, and 10 μM NAD for 30 min at 30 °C. Samples were then washed twice by a 2-fold dilution in ice-cold 160 mM NaCl, 20 mM Hepes, pH 7.4, and centrifugation at 15,000 × g for 5 min. The final pellet was resuspended in 160 mM NaCl, 20 mM Hepes, pH 7.4.

**Na⁺/Ca²⁺ Exchange Assay**—Na⁺-dependent Ca²⁺ uptake was measured according to Philipson et al. (33) with slight modifications. Briefly, vesicles at a final concentration of 2 mg of protein/ml were loaded with Na⁺ by preincubation at 37 °C for 30 min in 160 mM NaCl, 20 mM Hepes, pH 7.4. The assay medium consisted of 160 mM KCl, 20 mM Hepes, pH 7.4, 0.4 mM EGTA, 550 μM CaCl₂ (150 μM free calcium), 1.5 μM of ⁴⁵CaCl₂, and 0.5 μM valinomycin (added as a 20 mM solution in acetone). Na⁺-dependent Ca²⁺ uptake was initiated by rapid vortexing of one droplet (6–10 μl) of Na⁺-loaded vesicles, suspended on the side of a polystyrene tube, with the assay medium. The final assay volume (from 250 to 420 μl, as specified in the figure legends) was adjusted so that the ratio of K⁺ to Na⁺ (respectively contained in the assay medium and vesicles) was 42 in all the experiments. The reaction was stopped after a 10-s incubation at 37 °C by the addition of 4.5 ml of ice-cold washing solution consisting of 20 mM Hepes, pH 7.4, 5 mM LaCl₃, 10 mM COCl₂, 1 mM EGTA, and 140 mM KCl. The samples were then filtered under vacuum through Millipore filters (HAWP 0.45 μm) soaked previously in the ice-cold washing solution for 1 h. The filters were then washed three times with 4.5 ml of the washing solution, dried, and counted in 10 ml of Beckman Ready Protein. Blank values were obtained by measuring Ca²⁺ uptake in an assay medium containing 160 mM NaCl instead of KCl. The blanks were subtracted from all data points to correct for superficial Ca²⁺ binding and Na⁺ gradient-independent Ca²⁺ fluxes. Results were obtained from triplicate determinations.

Protein concentrations were determined according to the procedure of Peterson (34) using bovine serum albumin as standard. Free calcium was calculated using the EQUIV program. Nucleosides, nucleotides, 5’-(N-ethylcarboxamido)adenosine (NECA), N⁴-cyclohexyladenosine (CHA), and caffeine were purchased from Sigma, pertussis toxin was from List Biological (Campbell, CA); 4SCa (lo-40 mCi/mmol), from Amersham Corp.; and NG-(L-2-phenylisopropyl)adenosine (D-PIA) and NG-(L-PIA), from Boehringer Mannheim. Frozen ewe hearts were obtained from Pelfreez Biologicals (Eurobio, Les Ulis France).

**RESULTS**

Na⁺/Ca²⁺ exchange activity was assayed in vesicles, prepared from frozen ewe heart ventricles according to Frank et al. (32), under optimal conditions defined previously by Philipson et al. (33). As illustrated by the Eadie-Hofstee plot in Fig. 1, Na⁺/Ca²⁺ exchange activity in vesicles preloaded with 160 mM NaCl was dependent on Ca²⁺. The apparent Kₐ for Ca²⁺ was 1.3 μM, and the maximal velocity of sodium-dependently Ca²⁺ uptake determined at 10 s approached 8 nmol/mg of protein. Ca²⁺ uptake increased as a function of the time (Fig. 2), but one may note that the measurement of Ca²⁺ uptake at 10 s resulted in an underestimation of the rate of Na⁺/Ca²⁺ exchange activity. However, this time of incubation was chosen since it gave better reproducibility between results obtained from different experiments. Ca²⁺ uptake in vesicles loaded with Na⁺ concentrations varying from 30 to 160 mM rose with increasing Na⁺, and reached a maximum at 80 mM NaCl. The apparent affinity for Na⁺ (Kₐ) was 44 mM, and a Hill coefficient of 2.4, characteristic of Na⁺/Ca²⁺ exchange systems, was obtained (not shown).

Adenosine at 1 μM evoked a 30–50% stimulation of the initial rate, measured at 1 s of Na⁺/Ca²⁺ exchange activity (Fig. 2). It is important to note that the same degree of stimulation was observed after a 10-s incubation (Fig. 2). The effect of adenosine was dose dependent, maximal activation being observed at 100 nM adenosine and half-maximal activation at 2.5 nM adenosine (Fig. 3). Adenosine acted via an increase in the maximal velocity of Na⁺/Ca²⁺ exchange activity, with no change in the apparent affinity of the system for Ca²⁺ (Fig. 1). The effect of adenosine was specific since 1 μM adenosine, inosine, and guanosine were associated with only 7, 15, and 12% activation of Na⁺/Ca²⁺ exchange activity, respectively, whereas adenosine diphosphate (0.01–100 μM) had no significant effect on Na⁺/Ca²⁺ exchange activity.
no effect (Table I). Na⁺/K⁺ ATPase activity was not altered by adenosine, and calcium pump activity in sarcolemma showed 10% inhibition in the presence of 10 μM adenosine (not shown).

The effect of caffeine (a specific antagonist of P₁ type receptors) was also examined. As reported in Table I, caffeine totally antagonized the activation of Na⁺/Ca²⁺ exchange by adenosine whereas it had no effect on basal activity. In addition, the action of adenosine was mimicked by the P₁ type receptor agonists L-PIA, CHA, and NECA (Fig. 4), which all led to a maximal 35–45% activation of Na⁺/Ca²⁺ exchange at a concentration of 0.1 μM. Half-maximal effects were obtained with 1, 3.7, and 5.2 nM for L-PIA, CHA, and NECA, respectively. D-PIA had a lower potency and elicited half-maximal activation at 60 nM.

According to the literature, the rank order of potency of the adenosine agonists at the A₂ subclass of P₁ receptors is NECA > L-PIA > CHA > D-PIA. L-PIA being active at nanomolar concentrations and only 5–15 times more potent than D-PIA (2–7). This differs from the ranking order at A₁ subclass receptors, i.e. CHA = L-PIA = NECA >> D-PIA, with L-PIA being active at nanomolar concentrations and 30–100 times more potent than D-PIA (8, 10). A particularity of A₁ receptors in heart and in brain is their similar affinity, in the range of nanomolar concentrations, for CHA, L-PIA, and NECA (8, 10, 11). Our observations that L-PIA is 60 times more potent than D-PIA and that L-PIA, CHA, and NECA have similar potency (in the nanomolar range) strongly argue in favor of the involvement of A₂ subclass receptors in mediating the activation of Na⁺/Ca²⁺ exchange.

The coupling of adenosine receptors to pertussis toxin-sensitive nucleotide-binding proteins (G protein) has been reported repeatedly in the literature (4, 15, 21). We therefore examined the possible involvement of G proteins in the activation of Na⁺/Ca²⁺ exchange by adenosine. In a first approach we examined the effect of the nonhydrolyzable analog of GTP,

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**TABLE I**

Specific activation of Na⁺/Ca²⁺ exchange by adenosine compounds and antagonistic effect of caffeine

<table>
<thead>
<tr>
<th>Compound</th>
<th>Na⁺-dependent Ca²⁺ uptake (nmol calcium/mg protein/10 s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>8.1 ± 0.5</td>
</tr>
<tr>
<td>0.1 μM</td>
<td></td>
</tr>
<tr>
<td>1 μM</td>
<td>8.6 ± 0.5</td>
</tr>
<tr>
<td>Inosine</td>
<td>8.2 ± 0.2</td>
</tr>
<tr>
<td>0.1 μM</td>
<td></td>
</tr>
<tr>
<td>1 μM</td>
<td>9.2 ± 0.5</td>
</tr>
<tr>
<td>Guanosine</td>
<td>8.4 ± 0.2</td>
</tr>
<tr>
<td>0.1 μM</td>
<td></td>
</tr>
<tr>
<td>1 μM</td>
<td>8.9 ± 0.5</td>
</tr>
<tr>
<td>ADP</td>
<td>8.1 ± 0.3</td>
</tr>
<tr>
<td>0.01 μM</td>
<td></td>
</tr>
<tr>
<td>1 μM</td>
<td>8.2 ± 0.1</td>
</tr>
<tr>
<td>100 μM</td>
<td>8.1 ± 0.2</td>
</tr>
<tr>
<td>Caffeine</td>
<td>8.0 ± 0.5</td>
</tr>
<tr>
<td>0.01 μM</td>
<td></td>
</tr>
<tr>
<td>0.1 μM</td>
<td>8.2 ± 0.5</td>
</tr>
<tr>
<td>1 μM</td>
<td>7.3 ± 0.4</td>
</tr>
<tr>
<td>Adenosine</td>
<td>11.2 ± 0.6</td>
</tr>
<tr>
<td>(1 μM)</td>
<td></td>
</tr>
<tr>
<td>+Caffeine</td>
<td>9.2 ± 0.6</td>
</tr>
<tr>
<td>0.01 μM</td>
<td></td>
</tr>
<tr>
<td>0.1 μM</td>
<td>8.2 ± 0.5</td>
</tr>
<tr>
<td>1 μM</td>
<td>7.6 ± 0.5</td>
</tr>
</tbody>
</table>

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FIG. 4. Effects of adenosine analogs on Na⁺/Ca²⁺ exchange activity. Na⁺-loaded vesicles (42 μl) were preincubated at 37 °C for 5 min with varying concentrations of either CHA or NECA (top) or L-PIA or D-PIA (bottom) in a final volume of 56 μl. Droplets of 8 μl of the vesicle suspension were then mixed with 332 μl of the assay medium and incubated for 10 s at 37 °C as described under "Materials and Methods." Results are the mean ± S.E. of three experiments and are expressed as a percentage of control activity determined in the absence of agonist (8.0 ± 1 nmol of calcium/mg of protein/10 s).

FIG. 5. Effect of GTPγS on Na⁺/Ca²⁺ exchange activity. Na⁺-loaded vesicles (42 μl) were preincubated at 37 °C for 5 min with varying concentrations of GTPγS in a final volume of 56 μl. Droplets of 8 μl of the vesicle suspension were then mixed with 332 μl of the assay medium and incubated for 10 s at 37 °C as described under "Materials and Methods." Results are the mean ± S.E. of triplicate determinations and are expressed as a percentage of control activity determined in the absence of GTPγS (8.0 ± 1 nmol of calcium/mg of protein/10 s). The experiment was repeated twice with similar results.

GTPγS, which leads to irreversible activation of G proteins. As shown in Fig. 5, GTPγS mimicked the effect of adenosine and caused a dose-dependent activation of Na⁺/Ca²⁺ exchange. A maximal activation of 50–60% was obtained at 0.1 μM GTPγS. In another series of experiments, sarcolemmal vesicles were subjected to treatment with pertussis toxin in the presence of 10 μM NAD, under conditions that induce ADP-ribosylation of a 41,000-Da protein, identified as the α-subunit(s) of G(s) (not shown). Activation of Na⁺/Ca²⁺ exchange by adenosine was totally abolished in pertussis toxin-treated vesicles, but it was rather enhanced in control vesicles, in which 90% maximal stimulation was observed (Fig. 6), this being perhaps due to the extensive washings inherent to the control treatment. These results suggest that a pertussis toxin-sensitive G protein couples A₁ adenosine receptors to
FIG. 6. Consequences of in vitro treatment of sarcolemma vesicles with pertussis toxin on the response to adenosine of Na+/Ca2+ exchange activity. Sarcolemmal vesicles were subjected to a treatment with pertussis toxin (PT) or vehicle as control as described under "Materials and Methods." Na+-loaded vesicles (42 μl) were then preincubated at 37 °C for 5 min with varying concentrations of adenosine in a final volume of 56 μl. Droplets of 8 μl of the vesicle suspension were mixed with 332 μl of the assay medium and incubated for 10 s at 37 °C as described under "Materials and Methods." Results are the mean ± S.E. of triplicate determinations.

The experiment was repeated twice with similar results.

the Na+/Ca2+ exchange in heart ventricle and mediates activation by adenosine.

**DISCUSSION**

We report here that adenosine stimulates Na+/Ca2+ exchange activity through A1 subclass adenosine receptors coupled to a pertussis toxin-sensitive G protein. Vesicles obtained from ewe heart exhibit characteristic Na+/Ca2+ exchange activity. The apparent K_m for Ca2+ (1.3 μM) is in the physiological range (23) and within the range of the K_m values of the Na+/Ca2+ exchange cellular inner Ca2+ site determined previously in electrophysiological experiments, which varies from 0.7 to 2 μM (35). It is, however, rather low as compared with others biochemical studies using vesicles that report K_m varying between 1.5 and 140 μM. As discussed by Philipson (23) and Kevess and Sutko (25), such a large variability may be due either to the degree of membrane phosphorylation, alteration of Na+/Ca2+ exchange by limited proteolysis resulting from the preparation procedures, or to the physiological conditions of the hearts, as well as to the use and the concentration of EGTA in the assay medium. One also may note that the rate of Na+/Ca2+ exchange was determined at 10 °C. This results in an underestimated of the velocity of the reaction (a 3-4-fold decrease as compared with V_max determined at 1 s, as shown in Fig. 2) and gives a low V_max value (0.8 nmol/mg/s) as compared with other values reported in the literature, which vary from 1.4 to 30 nmol/mg/s (25, 26, 30, 33, 44). The 10-s period for incubation was nevertheless chosen because it gave a high reproducibility among the data obtained from different experiments and greatly facilitated the study of the regulation of Na+/Ca2+ exchange by adenine compounds and guanine nucleotides.

Activation of Na+/Ca2+ exchange by adenosine at nanomolar concentrations described here is compatible with plasma levels of adenosine reported in the literature, which vary from 50 nM to 1 μM (36, 37). In isolated organs, however, the effects of adenosine are only found at relatively high micromolar concentrations (9, 10, 14) whereas the adenosine analogs NECA and L-PIA are effective at concentrations ranging from 10 to 100 nM (11-14). It has been suggested that the lesser potency of adenosine is due to its rapid uptake by the cardiac tissue, which may strongly influence the concentration of adenosine at receptor sites (9, 10).

Up to now the involvement of G proteins in the control of Na+/Ca2+ exchange activity has been overlooked. Our studies with pertussis toxin and GTPγS, the nonhydrolyzable analog of GTP, show that activation of Na+/Ca2+ exchange in heart ventricle is mediated by a pertussis toxin-sensitive G protein. It is now recognized that there are a number of closely related pertussis toxin substrates with similar molecular weight. These include transducin which is found only in retina, Goα and three G proteins all present in heart. It is conceivable that a G protein rather than Goα may be involved in the activation of Na+/Ca2+ exchange by adenosine since it has been shown recently that Goα adenosine agonist PIA evoked down-regulation of the three forms of Gi in adipocyte (38), which implies that the three forms may interact with the A1 subtype adenosine receptors.

Although activation of Na+/Ca2+ exchange by GTPγS occurs for nanomolar concentrations of nucleotide, inhibition of the adenylylcyclase system (39, 40), mediated by pertussis toxinsensitive G proteins (41), and activation of phospholipase C (42) are seen at micromolar concentrations of GTPγS, one may, however, consider the possibility that Na+/Ca2+ exchange activation by adenosine mediated by a pertussis toxin-sensitive G protein is indirect and consequent on adenylylcyclase inhibition or phospholipase C activation. It is unlikely that activation of Na+/Ca2+ exchange is secondary to inhibition of adenylylcyclase since this system is not susceptible to phosphorylation by cyclic AMP although it is substrate for a Ca2+-calmodulin dependent protein kinase-phosphatase system (49). Conversely, activation of Na+/Ca2+ exchange by exogenous addition of purified phospholipase C to cardiac sarcolemma has been reported (44), suggesting that Na+/Ca2+ exchange activity might be controlled by phosphatidylinositol hydrolysis. However, activation of phospholipase C by adenine compounds is mediated by P2 class adenosine receptors (45) and thus cannot account for the activation of Na+/Ca2+ exchange by adenosine mediated through A1 subclass adenosine receptors.

In conclusion, we show that adenosine stimulates Na+/Ca2+ exchange activity through A1 subclass adenosine receptors coupled to a pertussis toxin-sensitive G protein. Activation of Na+/Ca2+ exchange is relevant to the indirect negative inotropic effect of adenosine in ventricular muscle, manifested only after an elevation of intracellular cyclic AMP, following stimulation of adenylylcyclase or inhibition of phosphodiesterase (12, 13, 18). The primary effect of cyclic AMP in heart cells is the activation of Ca2+ channels through phosphorylation, leading to an increase in Ca2+ influx. Activation of Na+/Ca2+ exchange during repolarization should lead to an acceleration of Ca removal from the cell, thus antagonizing cytosolic Ca2+ elevation evoked by cyclic AMP. Taken together these results provide suggestive evidence that Na+/Ca2+ exchange may participate in the negative effects of adenosine in heart.

**Acknowledgments**—We wish to thank Dr. Jacques Hanoune for his continued support, Dr. Guy Vassort for his most helpful comments, and E. Grandvilliére and L. Rosario for their skilful secretarial assistance.

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