A novel phospholipase C- and cAMP-independent positive inotropic mechanism via a P2 purinoceptor

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Podrasky, Ernest, David Xu, and Bruce T. Liang. A novel phospholipase C- and cAMP-independent positive inotropic mechanism via a P2 purinoceptor. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2380–H2387, 1997.—Although ATP, acting through a P2 purinoceptor, can stimulate a pronounced positive inotropic effect in cardiac ventricular myocytes, the receptor-effector mechanism that underlies this stimulatory cardiac action is not well understood. The objectives of the present study were to develop the cultured chick embryo ventricular myocytes as a novel model for the cardiac P2 purinoceptor and to determine the mechanism underlying its positive inotropic effect. ATP caused an 89 ± 8.9% (n = 14 cells) increase in the myocyte contractility, with an efficacy and potency order of ATP < ADP < AMP < adenosine. 2-Methylthio-ATP (2-MeS-ATP) but not α,βmethylene-ATP was able to stimulate myocyte contractility, with a maximal increase of 54 ± 2.6% (n = 11 cells). Although UTP potently stimulates phosphoinositide hydrolysis, it had an only modest positive inotropic effect (27 ± 7% maximal increase; n = 8 cells). In contrast to previous suggestions, the 2-MeS-ATP-stimulated positive inotropic response does not require the action of phospholipase C (PLC), such as that of the inositol phosphates; the UTP effect on contractility appears to be mediated via the 2-MeS-ATP-sensitive P2 receptor. The PLC inhibitor U-73122 had no effect on the 2-MeS-ATP-stimulated increase in contractility, providing further evidence against a role for PLC in the inotropic effect of 2-MeS-ATP. An adenosine 3',5'-cyclic monophosphate-independent Ca2+-entry-stimulating mechanism appears to underlie a direct coupling of the receptor to stimulation of the myocyte contractility. This new PLC- and adenosine 3',5'-cyclic monophosphate-independent positive inotropic mechanism represents a target for developing novel positive inotropic therapeutics.

ATP exerts a number of pronounced effects in the cardiovascular system (for reviews, see Refs. 21, 24). In the cardiac myocyte, ATP causes a pronounced positive inotropic effect (12, 18), which appears to be mediated by a cell surface ATP receptor or P2 purinoceptor. Activation of the P2 purinoceptor by ATP, which can be released from platelets, endothelial cells, or hypoxic cardiac tissues (5, 10, 14, 15, 22, 33) as a paracrine and autocrine regulatory agent, may provide important inotropic support in both healthy and diseased hearts. ATP can also act in synergy with a β-adrenergic agonist to augment myocyte contractility (37) as a cotransmitter released with norepinephrine from the sympathetic nerve endings. How ATP causes the increase in myocyte contractility is not well understood. A number of P2-purinoceptor cDNAs have been cloned, including at least four subtypes for the P2Y receptor and seven for the P2X receptor as well as that encoding the P2 receptor found on macrophages and platelets (7).

Activation of the P2Y receptor has been shown to cause either an inhibition of or no effect on adenylyl cyclase activity and adenosine 3',5'-cyclic monophosphate (cAMP) accumulation (26, 36). Coupling of the P2Y receptor to the inhibition of adenylyl cyclase and cAMP accumulation is mediated by the inhibitory G (Gi) protein. The P2Y receptor is also linked to stimulation of phosphatidylinositol 4,5-bisphosphate (PIP2)-phospholipase C (PLC) leading to an increase in inositol 1,4,5-trisphosphate ([Ins(1,4,5)P3]) and diacylglycerol (for a review, see Ref. 17). Ins(1,4,5)P3 by mobilizing intracellular Ca2+, can stimulate myocyte contractility. Diacylglycerol, by activating protein kinase C (PKC), can increase myofilament sensitivity to Ca2+ and thus enhance cardiac contractility. The P2X receptor subfamily represents a ligand-gated ion channel that permits entry of Na+ and Ca2+ into the cell (6, 8, 32). Although a P2 purinoceptor appears to mediate stimulation of Ca2+ entry and an increase in the cytosolic Ca2+ level in isolated cardiac myocytes, the subtype of the P2 purinoceptor that mediates the increase in cardiac myocyte contractility and the mechanism underlying this stimulatory effect remain unknown. A myocyte model system for the cardiac P2 purinoceptor is lacking. The role of P1P2-PLC in mediating the P2 agonist-stimulated myocyte contractility is not clear.

The objectives of the present study were to develop a myocyte model using cultured chick embryo ventricular myocytes to characterize the cardiac P2 purinoceptor and to investigate the cellular mechanism underlying the P2 receptor-mediated positive inotropic effect.

METHODS

Preparation of cultured cardiac cells. Ventricular cells were cultured from chick embryos 14 days in ovo according to previously described procedures (2, 19). After trypsin was neutralized with a medium containing horse serum, the cells were centrifuged and resuspended in culture medium containing 6% fetal bovine serum, 40% medium 199 (GIBCO), 0.1% penicillin-streptomycin, and a salt solution. The cells were plated at a density of 400,000 cells/ml and cultivated in a humidified 5% CO2-95% air mixture at 37°C. The cells grew to confluence on day 3 in culture and exhibited rhythmic spontaneous contraction.

Measurement of Ca2+ uptake into myocardial cells and cAMP level. Determination of Ca2+ uptake was made as previously described (20). Cultures were incubated with [3,4,5-3H(N)]-leucine (152.2 Ci/mmol) for 24 h before 45Ca uptake. [3H]Leucine incorporated into the cellular protein allowed normalization of Ca2+ content to milligrams of cell protein. After exposure to 45Ca, the cells were washed free of 45Ca with four rinses of ice-cold buffer containing (in mM) 5 N-2-hydroxy-
ethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1.0 CaCl$_2$, 4 KCl, 0.5 MgCl$_2$, 142 NaCl, and 1 lanthanum, pH 7.35. Such a washing procedure removed >99% of the extracellular marker $^{38}$Cr-EDTA and ensured complete removal of the extracellular $^{45}$Ca. Uptake of $^{45}$Ca was quantitated for 90 s. The cells were solubilized for 2 h in a solution containing 1% sodium dodecyl sulfate and 10 mM sodium borate. Aliquots of solution containing dissolved cells were assayed for radioactivity and protein content. cAMP was then extracted with the addition of a 0.1 volume of 1 N HCl to the medium, followed by boiling for 10 min. The extracted cAMP was assayed according to a previously described radioimmunoassay method (Ref. 19; Amersham, Arlington Heights, IL). The effect of the agonist on cAMP accumulation was linear for 10 min, at which time cAMP was extracted for assay.

Determination of contractile amplitude. Measurement of contractile amplitude in cultured cardiac cells was carried out via an optovideo motion-detection system as previously described (2, 20). Cells were paced at 2 Hz to focus on the change in contractile amplitude. Myocytes were exposed to a perfusion medium that contained the various nucleotide analogs indicated as well as the following components (in mM): 4 HEPES (pH 7.4), 137 NaCl, 3.6 KCl, 0.5 MgCl$_2$, 1.1 CaCl$_2$, and 5.5 glucose. Measurement of contractile amplitude was carried out on only one cell per coverslip. Both the basal contraction amplitude and the amplitude measured during adenine nucleotide exposure were determined.

Measurement of the phosphoinositide response. Inositol phosphates were determined according to the basic method of Berridge et al. (3), and further modified as described by Barnett et al. (1). Cells were preincubated with 5 µCi/ml of myo-$^3$H]inositol for 24 h and washed with Dulbecco’s modified Eagle’s medium containing 15 mM LiCl and incubated in this LiCl buffer for 10 min at 37°C before being exposed to ATP or other nucleotide analogs. After extraction with 1 ml of chloroform-methanol-HCl (at 1:2:0.05, vol/vol), the various inositol phosphates were separated on a 1.0-ml anion-exchange column (AGx8 resin, formate form), and inositol phosphates were eluted sequentially with 100 mM formic acid-200 mM ammonium formate, 100 mM formic acid-1 M ammonium formate, and 100 mM formic acid-1 M ammonium formate, respectively. The columns were calibrated with inositol phosphate standard to confirm complete separation of InsP$_1$, Ins(1,4)P$_2$, and Ins(1,4,5)P$_3$. Recovery of each inositol phosphate was >95%.

Ins(1,4,5)P$_3$-radioreceptor assay. To complement results on the Ins(1,4,5)P$_3$ level determined by anion-exchange chromatography, the effect of ATP-receptor agonists on the Ins(1,4,5)P$_3$ level was quantitated via an Ins(1,4,5)P$_3$-radioreceptor assay. The growth medium in which the ventricular cells were grown was replaced with a HEPES-buffered solution (pH 7.35) containing (in mM) 1.0 CaCl$_2$, 4 KCl, and 0.5 MgCl$_2$, and then exposed to ATP. The reaction was terminated by a 0.2 volume of ice-cold trichloroacetic acid, which was removed by extraction with a solution of 1,1,2-trichloro-1,2,2-trifluoroethane-trioctylamine. The Ins(1,4,5)P$_3$ in the aqueous phase was then measured using the Ins(1,4,5)P$_3$ receptor supplied (DuPont, Boston, MA) (20).

Materials. Embryonic chick eggs were from Spafas (Storrs, CT). The cAMP radiodinoimmune assay kit was obtained from Amersham. [3H]leucine, myo-[3H]inositol, the Ins(1,4,5)P$_3$-radioreceptor assay kit, and $^{45}$Ca were obtained from DuPont (Boston, MA). Adenosine, ADP, AMP, α,β-methylene-ATP, β,γ-methylene-ATP, and UTP were obtained from Sigma Chemical (St. Louis, MO); 2-methylthio-ATP (2-MeS-ATP) was from RBI (Natick, MA). 1,3,5(10)-trien-17-yl)amino(heptyl)-1H-pyrrole-2,5-dione (U-73122) and 1,6(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)-amino(heptyl)-2,5-pyrrolidine-dione (U-73343) were from BIOMOL (Plymouth Meeting, PA).

RESULTS

Characterization of the positive inotropic response to ATP and adenine nucleotides. ATP stimulated a marked increase in the myocyte contractile amplitude, with a half-maximal effective concentration (EC$_{50}$) of 0.16 ± 0.01 (SE) µM and a maximal increase of 89 ± 8.9% occurring at 3 µM (n = 14 cells; Fig. 1). ADP also caused a significant increase in myocyte contractility, with a similar EC$_{50}$ (0.40 ± 0.3 µM; n = 8 cells) although it was less efficacious (maximal increase of 47 ± 10.5%; n = 8). AMP and adenosine were less effective in stimulating myocyte contractility (maximal percent increase in contractility...
tractile amplitude was 10 ± 4.3%, n = 7 cells and 16 ± 3.7%, n = 24 cells, respectively), indicating that the inotropic effect of ATP is mediated by the P2 rather than by the P1 purinoceptor (Fig. 1). To characterize the subtype of P2 purinoceptor that mediates the positive inotropic response to ATP, a number of P2 receptor subtype-selective agonists were tested. The P2-receptor agonist 2-MeS-ATP caused a large increase in the contractile amplitude (EC50 of 0.06 ± 0.05 µM; n = 11 cells), whereas α,β-methylene-ATP or β,γ-methylene-ATP, which are agonists at some of the P2X receptors, was ineffective at stimulating the myocyte contractility (Fig. 2). UTP, capable of activating the UTP-sensitive P2Y receptor, had a modest stimulatory effect on the myocyte contractility, with an EC50 of 0.3 ± 0.1 µM and a maximal percent increase in contractile amplitude of 27 ± 7% (n = 8 cells; Fig. 2), consistent with a role of an UTP-sensitive P2Y receptor in mediating the positive inotropic response to ATP. Because 2-MeS-ATP is a potent agonist at some of the P2X receptors such as the P2X2, P2X5, and P2X6 subtypes, it is possible that a P2X receptor can also mediate the ATP-induced positive inotropic effect in the cardiac myocyte.

Subtype of cardiac P2 purinoceptor coupled to stimulation of phosphatidylinositol hydrolysis. Because P2Y receptors can be coupled to activation of PIP2-PLC, with consequent stimulation of phosphatidylinositol (PI) hydrolysis (16), we determined whether a UTP-sensitive cardiac P2Y receptor can also couple to stimulation of PIP2-PLC and whether the increase in PIP2-PLC activity mediates the positive inotropic response. ATP caused a significant increase in the levels of InsP1, Ins(1,4)P2, and Ins(1,4,5)P3 (Fig. 3A), and after 30 min of stimulation by ATP, there was a nearly sixfold (570 ± 110%; n = 12 cells) increase in total inositol phosphates, with an EC50 of 15 ± 10 µM (n = 12 cells; Fig. 3B). The increase in the Ins(1,4,5)P3 isomer was confirmed by an Ins(1,4,5)P3-radioreceptor assay (basal level, 42 ± 6 pmol/mg; in the presence of ATP, 96 ± 4 pmol/mg; n = 3 experiments). The Ins(1,4,5)P3 increase was transient, peaking at 45 s. UTP is also coupled to a pronounced stimulation of inositol phosphate production, with an increase in total inositol phosphates of 500 ± 90% at its maximal concentration (300 µM) and an EC50 of 11 ± 10 µM.

Fig. 2. Effects of P2 purinoceptor-selective agonists on cardiac myocyte contractility. Ventricular myocyte cultures were prepared and effect of varying concentrations of 2-methylthio-ATP (2-MeS-ATP; □), UTP (○), and α,β-methylene-ATP (■) on myocyte contractile amplitude was determined as in Fig. 1. Data are means ± SE; n = 11 cells for 2-MeS-ATP, 8 cells for UTP, and 10 cells for α,β-methylene-ATP from 3–4 cultures.

Fig. 3. Effect of ATP, UTP, and 2-MeS-ATP on inositol phosphate levels. Cardiac ventricular myocytes were cultured as described in METHODS. A: myocytes were exposed to 100 µM ATP (●) for 30 s, and levels of inositol 1-phosphate (InsP1), inositol 1,4-bisphosphate [Ins(1,4)P2], and inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] were measured as described in METHODS. □, Control. cpm, Counts/min. Data are typical of 3 other experiments. B: effects of varying concentrations of ATP (●), UTP (○), and 2-MeS-ATP (■) on total level of inositol phosphates [InsP1 + Ins(1,4)P2 + Ins(1,4,5)P3]. Data are means ± SE of 3 experiments.
(n = 12 cells; Fig. 3B). Neither the ATP- nor the UTP-stimulated PI response was attenuated by prior treatment of the myocytes with 5 ng pertussis toxin/ml over 24 h (data not shown), a treatment protocol that caused complete ADP ribosylation of G, by the endogenous NAD+ in these cultures (19, 20).

Because ATP is a potent agonist at both the P2Y and P2X receptors, it is possible that the positive inotropic effect of ATP is mediated by both a PIP2-PLC-coupled, UTP-sensitive P2Y receptor and a PLC-independent P2X or P2Y receptor. To test this notion, the effect of UTP and 2-MeS-ATP on PI hydrolysis was examined. UTP was as effective as ATP in stimulating PI hydrolysis, whereas 2-MeS-ATP caused only a small increase in PI hydrolysis (52 ± 9%; n = 4 cells; Fig. 3B). The P2X receptor-selective agonists α,β-methylene-ATP and β,γ- methylene-ATP were ineffective in stimulating PI hydrolysis (data not shown). The data are consistent with the notions that a UTP-sensitive cardiac P2Y receptor is closely coupled to the activation of PIP2-PLC, whereas a separate 2-MeS-ATP-sensitive P2 receptor is potently coupled to stimulation of myocyte contractility but is inefficiently coupled to PIP2-PLC. Alternatively, a P2X or P2Y receptor, activated by 2-MeS-ATP, may be selectively coupled to the stimulation of myocyte contractility, whereas the UTP-sensitive P2Y receptor is coupled only to PIP2-PLC, the activation of which has no effect on the myocyte contractility. If this notion is correct, the stimulatory effect of 2-MeS-ATP on PLC activity is due to its agonist activity at the PLC-coupled P2Y receptor, whereas the positive inotropic effect of UTP is due to its agonist activity at a 2-MeS-ATP-sensitive P2 purinoceptor. To provide further evidence for this notion, a number of cross-desensitization experiments were carried out.

Role of PIP2-PLC in mediating the P2 agonist-induced positive inotropic response. The UTP- and 2-MeS-ATP-mediated PI hydrolysis was partially desensitized by a 90-min prior incubation of the myocytes with 100 µM UTP (Fig. 4). However, prior treatment of the myocytes with 100 µM 2-MeS-ATP for 80 min had no effect on the basal level of inositol phosphates (control cells: 9,883 ± 320 counts/min (cpm), n = 3 experiments; 2-MeS-ATP-treated cells: 9,214 ± 410 cpm, n = 3 experiments) or on the increase in PI hydrolysis that was subsequently stimulated by either UTP (control cells: 45,313 ± 1,820 cpm, n = 3 experiments; 2-MeS-ATP-treated cells: 46,576 ± 1,694 cpm, n = 3 experiments) or 2-MeS-ATP (control cells: 15,842 ± 2,010 cpm, n = 3 experiments; 2-MeS-ATP-treated cells: 14,265 ± 1,902 cpm, n = 3 experiments). The role of the PLC-coupled P2Y receptor in mediating the positive inotropic response was examined next. An 80-min exposure to 100 µM 2-MeS-ATP caused a significant desensitization in the ATP-induced positive inotropic response (73 ± 4% decrease in the extent of stimulation in myocyte contractility; n = 8 cells). The 2-MeS-ATP-treated myocytes also showed a diminished positive inotropic response to 2-MeS-ATP and virtually no increase in myocyte contractile amplitude in response to UTP (Fig. 5A). However, a 90-min exposure to 100 µM UTP had no effect on the 2-MeS-ATP- or UTP-stimulated increase in myocyte contractility (Fig. 5B). These data are consistent with the notion that a P2Y receptor, activated with a potency order of UTP >> 2-MeS-ATP, is coupled to stimulation of PIP2-PLC, whereas a P2X or P2Y receptor, activated with a potency order of 2-MeS-ATP >> UTP, is coupled only to stimulation of the myocyte contractility. The
aminosteroid U-73122, a known inhibitor of the receptor-mediated activation of PLC, was used to further determine whether PLC plays a role in the 2-MeS-ATP-stimulated increase in myocyte contractility. U-73122 at 1 (data not shown) or 10 µM (Fig. 6) had no effect on the 2-MeS-ATP-stimulated increase in myocyte contractility. Ten micromolar U-73122, a concentration known to completely inhibit the PLC-mediated PI hydrolysis (data not shown; Refs. 29, 31), caused a slight depression in myocyte contractility but did not affect 2-MeS-ATP-induced stimulation of contractility (increase in contractile amplitude in response to 2-MeS-ATP plus U-73122, 48.4 ± 8%; n = 12 cells from 3 cultures). The inactive structural analog of U-73122, U-73343, also had no effect on the 2-MeS-ATP-stimulated increase in myocyte contractility (data not shown).

A cAMP-independent 45Ca entry underlies the P2 receptor-mediated positive inotropic response. Previous studies indicated that ATP can induce an increase in myocyte contractile amplitude (3) as well as an increase in Ca2+ entry and cytosolic Ca2+ (9, 13, 26). Both 2-MeS-ATP and ATP caused a pronounced increase in the transsarcolemmal uptake of 45Ca, whereas α,β-methylene-ATP (Fig. 7) or β,γ-methylene-ATP had virtually no stimulatory effect on the 45Ca uptake (data not shown). Neither 2-MeS-ATP nor ATP was able to stimulate cAMP accumulation (basal cAMP level: 12.2 ± 2 pmol/mg, n = 9 cells; cAMP levels in the presence of 2-MeS-ATP and ATP: 13.1 ± 1.6 pmol/mg, n = 5 cells and 13.2 ± 1.1 pmol/mg, n = 5 cells, respectively). None of the other P2-receptor agonists such as UTP, α,β-methylene-ATP, or β,γ-methylene-ATP was able to induce cAMP accumulation (data not shown). As a positive control, isoproterenol caused a 6.7 ± 1.4-fold increase in the level of cAMP (n = 5 cells). Such data indicate that a cAMP-independent Ca2+ entry-stimulating mechanism mediates the P2-receptor agonist-induced stimulation of myocyte contractility.

**DISCUSSION**

ATP exerts a number of stimulatory effects in the heart, including vasodilatation of the coronary vasculature (21, 24), stimulation of transsarcolemmal Ca2+ entry (25, 26, 28), acidification (23), depolarization (27), cytosolic Ca2+ transients (12–14), and contractility of the cardiac myocyte (12, 18). These studies have largely investigated the effects of ATP on cardiac Ca2+ current and cytosolic Ca2+ transients that appear to be...
mediated by a purinoceptor of the P2 subtype, whereas others studied ATP-induced Na\(^+\) current, Cl\(^-\)/HCO\(_3\)\(^-\) exchange, acidification, and depolarization, which appear to require high concentrations of ATP and the presence of Mg\(^{2+}\) diffring to ATP release, adenosine, consistent with the notion that this positive inotropic response is mediated by a P2 purinoceptor. That the order of potency and efficiency is ATP > 2-MeS-ATP > UTP > \(\alpha,\beta\)-methylene-ATP or \(\beta,\gamma\)-methylene-ATP is consistent with the notion that the subtype of P2 receptor mediating the positive inotropic effect is a P2Y receptor or a 2-MeS-ATP-sensitive P2X receptor such as P2X\(_2\), P2X\(_4\), P2X\(_6\), or P2X\(_\alpha\) receptor (7).

Because UTP has a significant positive inotropic effect, it is possible that a UTP-sensitive P2 receptor, either one of the known P2Y receptors or a novel receptor, also mediates some of the ATP-stimulated positive inotropic effect (7, 17). Although the EC\(_{50}\) values for ATP- and UTP-stimulated PI hydrolysis were higher than those for the increase in contractile amplitude, 1 \(\mu\)M ATP or UTP was able to cause a significant increase in the inositol phosphate level (increase was 85 ± 10 and 90 ± 15% for ATP and UTP, respectively; \(n = 3\) experiments). Because UTP causes a pronounced increase in the level of inositol phosphates and Ins(1,4,5)P\(_3\) is known to stimulate the release of Ca\(^{2+}\) from the sarcoplasmic reticulum (34, 35), it is possible that the receptor-mediated increase in Ins(1,4,5)P\(_3\) causes the positive inotropic effect. On the other hand, because 2-MeS-ATP causes only a modest stimulation of inositol phosphate production, the 2-MeS-ATP-sensitive P2 receptor may be coupled directly to the stimulation of myocyte contractility independent of the increase in inositol phosphate production. According to this hypothesis, ATP causes its positive inotropic effect by activating both a PLC-coupled P2Y receptor and a 2-MeS-ATP-sensitive P2 receptor. Alternatively, the positive inotropic effect of UTP is due to its agonist activity, although modest, at the 2-MeS-ATP-sensitive P2 purinoceptor, whereas the stimulatory effect of 2-MeS-ATP on the inositol phosphate level is from its cross-activity at the UTP-sensitive P2Y receptor. Two lines of evidence support the latter notion. First, prior treatment of the myocyte with UTP desensitized both the UTP- and 2-MeS-ATP-induced stimulation of inositol phosphate production. On the other hand, prior treatment of the myocyte with 2-MeS-ATP had no effect on the increase in inositol phosphates caused by the subsequent stimulation with UTP or 2-MeS-ATP, suggesting that only the UTP-sensitive P2Y receptor is coupled to inositol phosphate production. Second, prior treatment of the myocyte with 2-MeS-ATP caused a significant desensitization of the positive inotropic response to either UTP or 2-MeS-ATP. On the other hand, prior exposure to UTP had no effect on the UTP- or 2-MeS-ATP-stimulated myocyte contractility, suggesting that only the 2-MeS-ATP-sensitive P2 purinoceptor is coupled to the stimulation of myocyte contractility.

That ATP can stimulate PI hydrolysis in cultured chick ventricular myocytes is similar to the findings obtained in mouse (36) and rat (18) ventricular myocytes. The present study showed that the stimulatory effect of ATP is mediated via a UTP-sensitive P2Y receptor. In contrast to previous suggestions (18), the present data suggest that the positive inotropic response to ATP, such as that mediated via the 2-MeS-ATP-sensitive P2 receptor, can occur independent of PLC activation. The formation of Ins(1,4,5)P\(_3\) is not necessary for the 2-MeS-ATP-stimulated positive inotropic response. This notion was further supported by the finding that although 1 \(\mu\)M 2-MeS-ATP caused a maximal positive inotropic effect, it had no effect on the inositol phosphate level. U-73122, a known PLC inhibitor, had no effect on the 2-MeS-ATP-stimulated increase in myocyte contractility even at 10 \(\mu\)M, providing further evidence against a role of PLC in mediating the inotropic response to 2-MeS-ATP.

Further investigation of the underlying cellular mechanism showed that the ATP agonists were able to cause a significant stimulation in transsarcolemmal Ca\(^{2+}\) entry. However, none of the ATP agonists caused an increase in the cell cAMP content, similar to the finding obtained in rat ventricular myocytes (26). The order of efficacy of ATP > 2-MeS-ATP > UTP > \(\alpha,\beta\)-methylene-ATP in stimulating Ca\(^{2+}\) entry is similar to that of the efficacy of the same analogs in stimulating myocyte contractility. These data suggest that a cAMP-independent, Ca\(^{2+}\) entry-stimulating mechanism underlies the 2-MeS-ATP-sensitive P2 purinoceptor-mediated increase in myocyte contractile amplitude. The present
data are compatible with prior findings that the classic P2Y agonist 2-MeS-ATP can stimulate Ca²⁺ entry via both a nonselective cation channel and an L-type Ca²⁺ channel (25, 28) and an increase in Ca²⁺ transients (4) in rat ventricular myocytes. It is unlikely that an ATP-induced acidification with a consequent stimulation of cytosolic Ca²⁺ contributes to the positive inotropic effect of ATP because ATP-induced acidification required a Mg-ATP complex at a concentration 100-fold higher than that causing the increase in myocyte contractility. The exact identity of the P2 purinoceptor that mediates the increase in myocyte contractility remains less clear. That some of the recently cloned P2X receptors can be potently activated by 2-MeS-ATP and that mRNAs encoding these receptors are expressed in abundant amount in the heart (16, 30) raise the possibility that a P2X receptor mediates the positive inotropic response within a few seconds (11) and because these two P2X receptors can be activated by α,β-methylene-ATP. Identification of the exact P2 receptor subtype mediating the positive inotropic response awaits the development of agonists and antagonists capable of potently coupling to stimulation of myocyte contractility, exists in the intact cardiac cell. This high-affinity stimulatory P2 purinoceptor pathway represents a potentially novel target for the development of new positive inotropic therapeutics.

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