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Nucleotides maintain the activity of Cav1.2 channels in guinea-pig ventricular myocytes

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ABSTRACT

The activity of Cav1.2 Ca^{2+} channels is maintained in the presence of calmodulin and ATP, even in cell-free patches, and thus a channel ATP-binding site has been suggested. In this study, we examined whether other nucleotides, such as GTP, UTP, CTP, ADP and AMP, could be substituted for ATP in guinea-pig ventricular myocytes. We found that all the nucleotides tested could re-prime the Ca^{2+} channels in the presence of 1 μM calmodulin in the inside-out mode. The order of efficacy was ATP > GTP > UTP > ADP > CTP ≈ AMP. Thus, the presumed nucleotide-binding site in the channel seemed to favor a purine rather than pyrimidine base and a triphosphate rather than a di- or mono-phosphate group. Furthermore, a high concentration (10 mM) of GTP, UTP, CTP, ADP and AMP had inhibitory effects on the channel activity. These results provide information on the putative nucleotide-binding site(s) in Cav1.2 Ca^{2+} channels.

1. Introduction

ATP, an important biomolecule produced mainly in mitochondria, plays a crucial role in the maintenance of physiological functions, including both electrical and contractile events in the heart [1,2]. Indeed, a reduction in ATP concentration is related to the pathogenesis and progression of heart disease such as arrhythmia, ischemic injury and heart failure [3]. Research aimed at determining the working mechanism(s) of ATP in the heart are necessary to understand the regulation of cardiac physiological function and pathophysiological changes.

Ca^{2+} influx through cardiac membrane voltage-gated Ca^{2+} channels (Cav1.2) plays essential roles in the generation of cardiac action potentials and excitation–contraction coupling [4,5]. The Ca^{2+} channel is regulated by a variety of intracellular factors, including Ca^{2+}, Mg^{2+}, protein kinases and phosphatases, redox agents, and calmodulin [5,6]. In addition, ATP has also been reported to be an essential factor for the regulation of voltage-dependent Ca^{2+} channels [7–10].

The effects of ATP on its target molecules are mediated through three major pathways: 1) providing chemical energy, 2) kinase-mediated phosphorylation, and 3) direct binding to proteins [11]. Hydrolysis of ATP is necessary for the first two, but is not necessarily required for the last. Because Cav1.2 Ca^{2+} channels are regulated by phosphorylation mediated by cAMP-dependent protein kinase (PKA) [5], the effects of ATP on Ca^{2+} channels have been attributed mainly to the promotion of protein phosphorylation. However, accumulating evidence has shown that a phosphorylation-independent effect of ATP on Ca^{2+} channels may exist [7–10]. We have previously shown that ATP, together with CaM, induces Ca^{2+} channel activity in the inside-out patches in a manner that is not affected by protein kinase inhibitors [8]. Moreover, we have recently reported the concentration-dependent binding of ATP to the Cav1.2 channel [9]. These findings strongly suggest that ATP modulates channel activity by directly interacting with the Ca^{2+} channel.

The aim of the present study is to elucidate the properties of the putative ATP-binding site(s) in Cav1.2 Ca^{2+} channels by examining the effects of various nucleotides on channel activity.

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2. Materials and methods

2.1. Solutions and reagents

The tyrode solution contained (in mM) NaCl 135, KCl 5.4, NaH_2PO_4, 0.33, $MgCl_2$, 1.0, glucose 5.5, $CaCl_2$, 1.8 and Hepes-NaOH buffer 10, pH 7.4. The storage solution was composed of (in mM) KOH 70, glutamic acid 50, KCl 40, KH_2PO_4, 20, taurine 20, $MgCl_2$, 3.0, glucose 10, EGTA 0.5, and Hepes-KOH buffer 10, pH 7.4. The pipette solution contained (in mM) $BaCl_2$, 50, TEACl 70, EGTA 0.5, BayK 8644 3 μM, and 10 mM Hepes-CsOH buffer 10, pH 7.4. The basic internal solution consisted of (in mM) potassium aspartate 120, KCl 30, $MgCl_2$, 0.5, EGTA 1.0, $CaCl_2$, 0.5 (free $[Ca^{2+}] = 80$ nM) and Hepes-KOH buffer 10, pH 7.4. Bay K8644 and MgATP were purchased from Sigma (St. Louis, MO, USA), Na_2GTP and Na_2CTP from Carbosynth (Compton, UK), Na_2UTP from Jena Bioscience (Jena, Germany), and Na_2ADP and Na_2AMP from Oriental Yeast (Tokyo, Japan). The total $[Mg^{2+}]$ in the nucleotide-containing solutions was calculated to fix the free $[Mg^{2+}]$ at 0.5 mM using self-made software.

The human CaM was expressed in *Escherichia coli* BL 21, and purified. The purity of CaM was confirmed by SDS-PAGE and quantified by the Bradford method with bovine serum albumin as the standard and a correction factor of 1.69.

2.2. Preparation of single myocytes

Single ventricular myocytes from guinea pig hearts were dispersed by collagenase and protease dissociation as described previously [12]. The myocytes were stored in storage solution at 4 °C.

The experiments were carried out after approval of the Committee of Animal Experimentation of Kagoshima University, Kagoshima, Japan and that of China Medical University, Shenyang, China.

2.3. Patch–clamp method and data analysis

Cav1.2 channel activity was monitored with the patch–clamp technique. The myocytes were perfused with the basic internal solution at 31–35 °C using a patch pipette (2–4 MΩ) containing 50 mM Ba^{2+}. After Ca^{2+} channel activity was recorded, the membrane patch was excised to establish the inside-out patch configuration. The Ca^{2+} channel currents were elicited by depolarizing pulses from −70 to 0 mV for a 200 ms at 0.5 Hz, recorded with a patch-clamp amplifier (200B, Axon Instruments; Foster, CA, USA) and fed to a computer at a sampling rate of 3.3 kHz. The mean *NPo* values during the period 5–105 ms after the onset of the test pulses was measured, where *N* is the number of channels in the patch and *Po* is the time-averaged open-state probability of the channels. Data are presented as the mean + S.E.

The concentration-dependent relationship between Cav1.2 channel activity (Y) and nucleotide concentration (X) were fitted to the Hill equation:

$$Y = (Base + Max \cdot [X]^{n_a} / (Kd_a^{n_a} + [X]^{n_a})) \cdot Kd_i^{n_i} / (Kd_i^{n_i} + [X]^{n_i}),$$

where *Base* is the basal channel activity without nucleotide, *Max* is the maximal channel activity induced by the nucleotides; Kd_a and Kd_i are dissociation constants, and n_a and n_i are Hill's numbers for activation and inhibition, respectively. The parameters for the curve fitting of nucleotides are listed in Table 1.

Table 1
Parameters for the curve fitting of the effects of nucleotides on Ca^{2+} channel activity. Data shown in Fig. 4 were fitted to a Hill equation (Methods). *Base*: basal channel activity (fixed at 17.2); *Max*: the maximal channel activity; Kd_a and Kd_i: dissociation constants and n_a and n_i: Hill's numbers for activation and inhibition, respectively (n_i was fixed at 4). R_a^2: squared correlation coefficient for the activation phase of the nucleotide (<10 mM for all nucleotides except for AMP < 3 mM).

	Max	Kd_a	n_a	Kd_i	n_i	R_a^2
ATP	173.9	0.83	1.31			0.981
GTP	117.6	0.82	2.23	5.86	4	0.997
UTP	85.2	1.91	1.51	6.77	4	0.999
CTP	34.9	0.81	2.60	7.40	4	0.992
ADP	72.7	1.33	1.18	6.80	4	0.998
AMP	36.4	1.42	1.49	2.59	4	0.642

3. Results

3.1. Effect of ATP on Cav1.2 channel activity

To confirm the existence of the activating effect of ATP observed in our previous studies [7–9], we examined the effect of ATP on Cav1.2 channel activity in cardiac myocytes over a wide range of concentrations (0.1–10 mM). In the inside-out mode, CaM (1 μM) was supplemented in the perfusion solution to prevent the run-down of channel activity and free Mg^{2+} and free Ca^{2+} were fixed at 0.5 mM and 80 nM, respectively. Application of ATP (0.1 and 0.3 mM) after patch excision produced concentration-dependent channel activity with *NPo* (relative to that in the control cell-attached mode) of 19.0 ± 5.2% (n = 6) and 64.1 ± 7.6% (n = 6), respectively (Fig. 4). At higher concentrations of ATP (1, 3, and 10 mM), the channel activity was further enhanced with an averaged *NPo* of 105.2 ± 9.4% (n = 6), 173.2 ± 8.8% (n = 6), and 180.2 ± 17.6% (n = 7), respectively (Fig. 1A and B, Fig. 4).

3.2. Effects of GTP, UTP and CTP on Cav 1.2 channel activity

We then examined the effect of GTP on Cav1.2 channel activity in the presence of CaM. GTP (0.1 and 0.3 mM) induced minimal channel activity, with relative activities of 19.0 ± 4.0% (n = 8), 28.3 ± 4.7% (n = 7), respectively (Fig. 4A). After addition of higher concentrations of GTP (1 and 3 mM), the channel activity was increased to relative activities of 89.0 ± 16.9% (n = 6) and 128.7 ± 24.2% (n = 5), respectively (Figs. 1C and 4A). However, in the presence of 10 mM GTP, the channel activity was sharply decreased to 9.1 ± 1.1% (n = 10) (Fig. 1D).

We next examined the effects of UTP and CTP on Cav1.2 channel activity. When the patch was perfused with UTP (0.1, 0.3, 1, 3 and 10 mM) and CaM-containing solution, the averaged relative activities were 16.5 ± 3.6% (n = 6), 22.8 ± 2.7% (n = 6), 40.4 ± 5.4% (n = 6), 73.8 ± 7.7% (n = 7), 16.1 ± 2.3% (n = 7) of that in the cell-attached patches, respectively (Fig. 2A and B, Fig. 4A). After perfusion of the patch with CTP (0.1, 0.3, 1, 3 and 10 mM), the averaged relative activities were 14.0 ± 1.6% (n = 8), 20.1 ± 2.7% (n = 8), 39.3 ± 5.6% (n = 6), 51.0 ± 3.9% (n = 6), 11.8 ± 4.0% (n = 6) of that in the cell-attached patches, respectively (Fig. 2C and D, Fig. 4A).

The effects of various nucleotides were plotted against their concentrations. As shown in Fig. 4A, perfusion with ATP effect yielded a typical S-shaped concentration-dependent curve. However, the effects other nucleotides (GTP, UTP and CTP) were weaker than that of ATP and the curves were bell-shaped (Table 1). When these data were fitted to the Hill equation, they appeared as continuous lines. These results indicated that the nucleotide triphosphates (GTP, UTP and CTP) had both repriming and inhibitory effects on Cav1.2 channels.

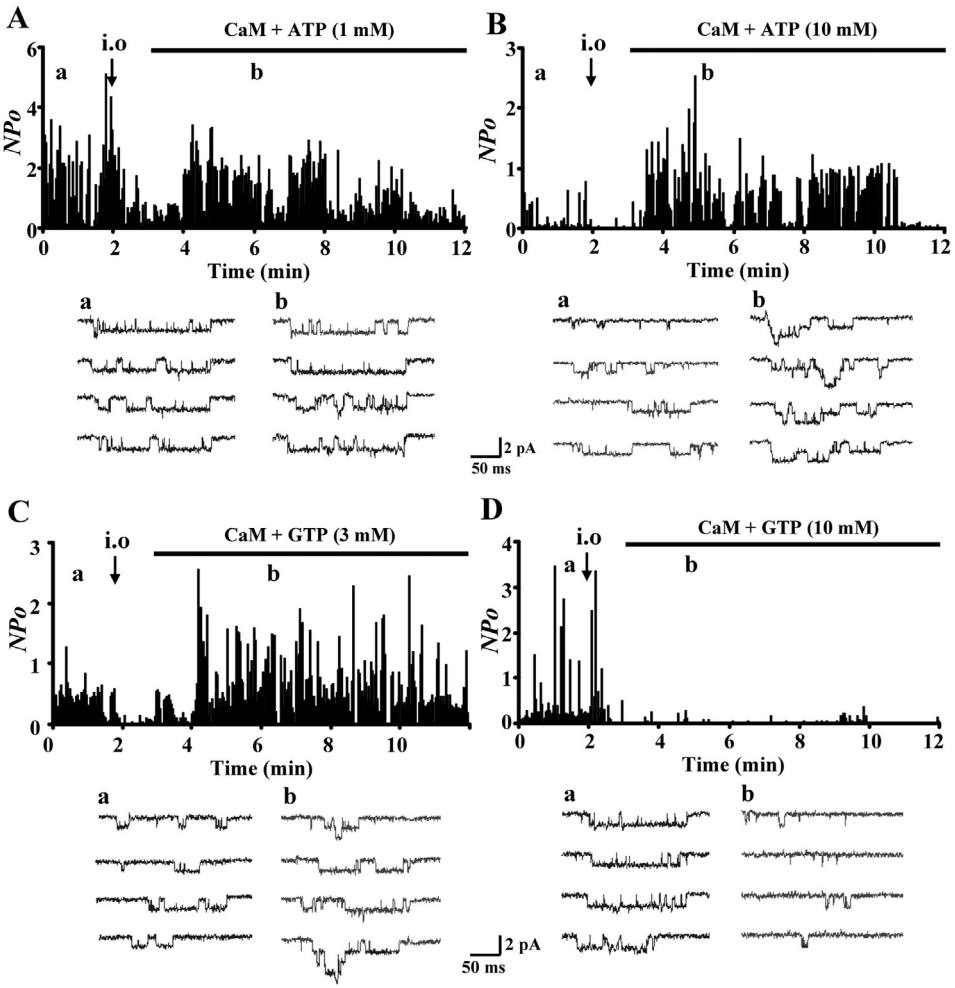

Fig. 1. Effect of ATP and GTP on Cav1.2 channel activity. Time course of channel activity (*NPo*) with different concentrations of ATP (A: 1 mM; B: 10 mM) and GTP (C: 3 mM; D: 10 mM) with CaM (1 μM). After recording in the cell-attached mode, the patches are excised to form the inside-out mode (as indicated by i.o). In the lower panels in A and B, examples of current traces for the control in the cell-attached mode (a) and with ATP in the i.o mode (b) are shown. In the lower panels in C and D, examples of current traces for the control in the cell-attached mode (a) and with GTP in the i.o mode (b) are shown.

3.3. *Effects of ADP and AMP on Cav1.2 channel activity*

We also examined whether other adenosine nucleotides (ADP, AMP) would affect Cav1.2 channel activity. ADP at 0.1, 0.3, and 1 mM induced channel activity in the inside-out patch, with an averaged channel activity of 19.0 ± 3.0% (n = 6), 28.8 ± 4.2% (n = 9), and 47.0 ± 6.0% (n = 7) of the control, respectively (Fig. 4B). When the concentration of ADP increased to 3 mM, the channel activity further increased to 69.8 ± 9.6% (n = 6) of the control (Figs. 3A and 4B). However, when the concentration of ADP increased to 10 mM, the channel activity was sharply decreased with a relative activity of 14.2 ± 1.2% (n = 9) (Figs. 3B and 4B). Similarly, AMP (0.1, 0.3, 1, 3, and 10 mM) induced the channel activity in the inside-out mode, with an averaged relative channel activity of 15.5 ± 5.3% (n = 6), 16.9 ± 2.0% (n = 11), 32.1 ± 4.6% (n = 8), 15.9 ± 1.9% (n = 5), and 12.3 ± 3.2% (n = 6) of the control, respectively. (Fig. 3C and D, Fig. 4B).

The concentration–effect curves constructed for ADP and AMP were bell-shaped (Fig. 4B). The maximal effect of ADP and AMP was observed at 3 and 1 mM, respectively. These results indicated that ADP and AMP had both activation and inhibition effects on Cav1.2 channel activity.

4. Discussion

Intracellular ATP, together with CaM, is required for maintaining Cav1.2 Ca^{2+} channel activity [8]. It has been reported that the EC_{50} of the ATP effect is around 0.3–3 mM in ventricular myocytes [9,13] and smooth muscle cells [14]. In the present study, we confirmed the concentration-dependent re-priming effect of ATP on Cav1.2 channel activity with an EC_{50} of 0.8 mM. Furthermore, we found that GTP, UTP, CTP, ADP and AMP also partially re-primed the Cav1.2 channels, all in a concentration-dependent manner, and that nucleotides other than ATP have an additional inhibitory effect on the channels at high concentrations.

The Cav1.2 channels are believed to bind with ATP, which is a prerequisite for re-priming the channel [9,10]. The present study revealed that GTP, UTP and CTP can also support the channel re-priming. The efficacy of GTP, UTP and CTP relative to that of ATP, as estimated from their Max values (Table 1), was 68, 49 and 20%, respectively. Thus, the order of efficacy of the nucleotides on Cav1.2 channel activity was ATP > GTP > UTP > CTP. This suggests that nucleotides having purine bases (ATP and GTP) are more effective than those having pyrimidine bases (UTP and CTP) for re-priming the channels.

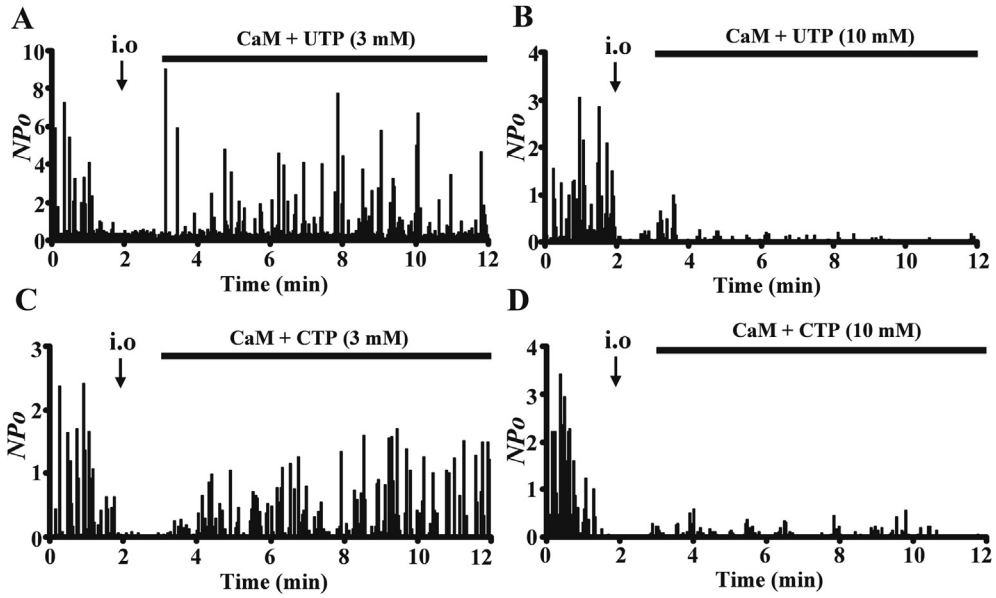

Fig. 2. Effects of UTP and CTP on Cav1.2 channel activity. Time course of channel activity (*NPo*) with different concentrations of UTP (A: 3 mM; B: 10 mM) and CTP (C: 3 mM; D: 10 mM) with CaM (1 μM). The patches are excised at the time indicated by i.o.

In the present study, we also found that both ADP and AMP are partially effective in maintaining Ca^{2+} channel activity. The efficacy of ADP and AMP relative to that of ATP, as estimated from their Max values (Table 1), was 42 and 21%, respectively. This finding further supports that hydrolysis of ATP (which is associated with protein phosphorylation and energy transfer) plays no or, if any, a minor role in re-priming the channels in the time span examined in this study (<10 min). This idea is in accordance with previous reports that adenylyl [β,γ-methylene]diphosphate (AMP-PCP) and adenosine 5′-[β,γ-imido]triphosphate (AMP-PNP), both non-hydrolysable ATP analogs, are partially effective at maintaining channel activity [8,10]. However, the smaller effects of ADP and AMP compared with ATP suggest that the triphosphate group in ATP is important for re-priming the Cav1.2 channels [9].

In addition to the re-priming effects, the nucleotides (except ATP) showed an inhibitory effect on Cav1.2 channel activity at 10 mM resulting in bell-shaped concentration–response curves (Fig. 4). This result is surprising because ATP does not show any inhibitory effect at concentrations up to 10 mM. There are several possibilities for this observed inhibition. 1) The concentrations of Mg^{2+} or Ca^{2+} may not be tightly controlled, resulting in inhibition from higher than expected concentrations of Mg^{2+} or Ca^{2+}. 2) The presence of a high concentration of Na^{+} (20 mM) would inhibit the channels. Inhibition of a protein kinase or activation of a phosphatase might have occurred, resulting in inhibition of channel activity. 3) There might be an additional nucleotide-binding site in the channel which contributes to the observed inhibitory effect.

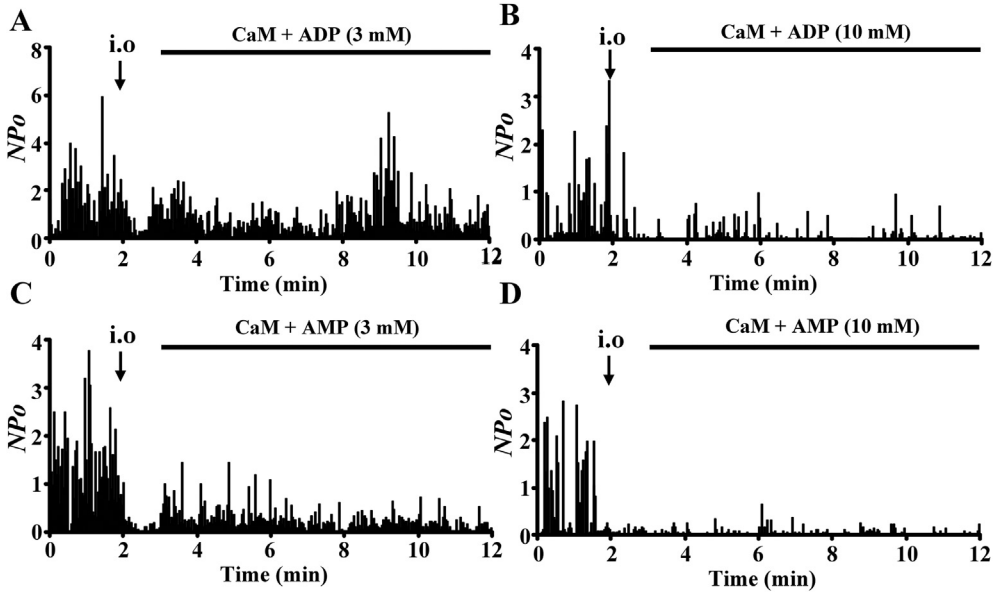

Fig. 3. Effects of ADP and AMP on Cav1.2 channel activity. Time course of channel activity (*NPo*) with different concentrations of ADP (A: 3 mM; B: 10 mM) and AMP (C: 3 mM; D: 10 mM) with CaM (1 μM). The patches are excised at the time indicated by i.o.

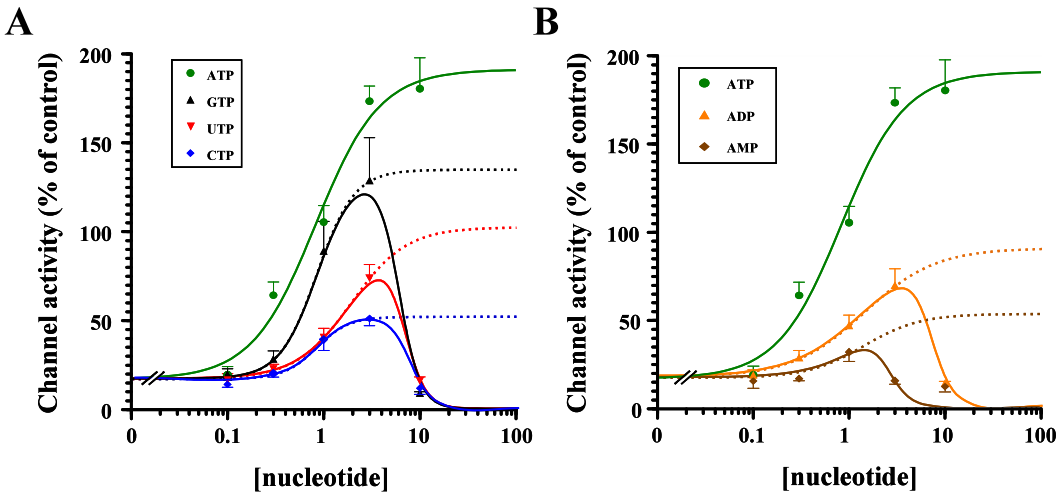

Fig. 4. Concentration-dependence of the effects of nucleotides. The relative channel activity (*NPo*) produced by the nucleotides with CaM (1 μM) in the inside-out mode was plotted against the concentration of nucleotides, in A, for ATP (green circles), GTP (black triangles), UTP (red nabra), and CTP (cyan diamond), and in B, for ATP (green circles), ADP (orange triangles), and AMP (brown diamond). Data are shown as the mean + S.E. (n = 5–11) and fitted with the Hill equation (see Methods). The dashed lines are fitted curves without the data at 10 mM to estimate *Max* values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The intracellular Mg^{2+} and Ca^{2+} concentrations have been reported to affect the availability and activity of Ca^{2+} channels and consequently heart and muscle contractility [3,15]. Intracellular Mg^{2+} inhibits L-type Ca^{2+} currents in cardiac myocytes [16], whereas intracellular Ca^{2+} regulates the Cav1.2 channel by Ca^{2+}-dependent facilitation (CDF) and inactivation (CDI) [5]. In this study, by using the inside-out configuration of the patch–clamp, the [Mg^{2+}] and [Ca^{2+}] were fixed at 0.5 mM and 80 nM, respectively. Therefore, it is unlikely that the inhibitory action of Mg^{2+} or Ca^{2+} happened during the application of the nucleotides.

The [Na^{+}] of the solution containing 10 mM nucleotides was 20 mM because we used the di-sodium salts of the nucleotides. However, to our knowledge, there are no reports that 20 mM Na^{+} inhibits L-type Ca^{2+} channels [17]. Thus, it is less likely that Na^{+} was involved in the inhibition observed at high concentration of the nucleotides.

Phosphorylation of the Cav1.2 channels by CaMKII or PKA is reported to be required for channel activity [18,19]. Thus the possibility that the other nucleotides except ATP might have inhibited a protein kinase critical for maintaining channel activity cannot be excluded. However, our previous work has shown that kinase inhibitors do not inhibit the effects of ATP on Ca^{2+} channel activity over a short time span (~10 min) [7,8]. Furthermore, the fact that similar concentration ranges for the inhibition of channel activity were observed for all the nucleotides tested (Table 1) argues against the involvement of protein kinases. The reason for this is that the *Ki* values of nucleotides for the inhibition of PKA, and perhaps other kinases, are largely variable among the nucleotides [20]. Activation of protein phosphatases (PP), such as PP type 1, type 2A and type 2B (calcineurin), by nucleotides, has not been reported to our knowledge. Thus, it is less likely that the nucleotides would activate a PP, which would suppress channel activity. Based on the above argument, we speculate that the nucleotides, except ATP, at high concentrations inhibit Cav1.2 channels by directly binding to the channels.

There are a number of channels that bind ATP and other nucleotides with variable affinities. The CFTR Cl^{-} channels, sulphonyl urea receptors (SUP) of ATP-sensitive K^{+} channels (K_{ATP}, Kir6) and the K_{ATP} channel itself have relatively high affinity ATP-binding sites (K_d < 100 μM) for activation or inhibition of the channels [21–23]. These sites have an ATP-binding cassette (ABC) consensus sequence consisting of a Walker A (GXXXXGKT/S; X, any residue) and B motif (R/KXXXXGXXXLhhhhD; h, any hydrophobic residue) or an 'FX4K' amino acid sequence (FXXXXK). Conversely, ROM K^{+} (Kir1.1), glial K^{+} (Kir4.1), Ks (KCNQ1) channels, TRP channels (TRPV and TRPM classes) and ryanodine receptor Ca^{2+} release channels (RyRs) have low affinity ATP-binding sites, some of which have a consensus sequence of GXG/AXXG [24–28].

Although ATP binding to the N- and C-terminus of Cav1.2 channels has been shown by a photo-affinity labeling study [9], no consensus sequence for an ATP-binding site has been detected. Considering the low-affinity nature of the ATP-binding site of Cav1.2 channels, it is possible that the site might have an amino acid sequence different from the known consensus sequences. The order of efficacy of the nucleotides for the effect on channel activity was ATP > GTP > UTP > ADP > CTP ≈ AMP, which is different from that for K_{ATP} channel inhibition [29], but similar to that for CFTR channel activation [30].

The main physiological function of ATP is to drive energy-requiring processes via its hydrolysis. The concentrations of ATP, ADP and AMP in intact myocytes are nearly 4.5, 0.5 and 0.02 mM, respectively [31]. Thus, under physiological conditions, ADP and AMP contribute only slightly or even negligibly to the re-priming of Cav1.2 channels. However, a decrease in ATP concentrations from around 5 mM by ischemia significantly reduces the Ca^{2+} channel activity and thereby attenuates the contraction, which results in energy savings for the myocytes. Thus, this mechanism provides a link between the function of the Cav1.2 channels and the metabolic state of the myocytes.

The physiological concentrations of the other nucleotides tested, GTP, UTP and CTP, are reported to be ~0.5, ~0.6, and ~0.3 mM, respectively [32]. Thus, GTP should slightly contribute (10–15 %) to Cav1.2 channel activity in intact myocytes, while UTP and CTP contribute only negligibly. It is therefore concluded that, in the intact myocytes, ATP plays a predominant role in re-priming the Cav1.2 channels.

It is difficult to find physiological relevance in the inhibitory actions of nucleotides other than ATP because concentrations higher than those found under physiological conditions are required for this inhibition. However, it might be possible that under ischemic conditions, increased concentrations of ADP and AMP act synergistically with reduced ATP to inhibit Cav1.2

channels. In summary, the present study characterized the concentration–response relationships between nucleotides and Cav1.2 Ca^{2+} channel activity.

Conflict of interest

The authors declare that there are no conflict of interest.

Acknowledgments

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