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Adenine-Based Acyclic Nucleotides as Novel $P2X_3$ Receptor Ligands

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A new series of acyclic nucleotides based on the adenine skeleton and bearing in 9-position a phosphorylated four carbon chain has been synthesized. Various substituents were introduced in 2-position of the adenine core. The new compounds were evaluated on rat $P2X_3$ receptors, using patch clamp recording from HEK transfected cells and the full $P2X_3$ agonist α,β-meATP as reference compound. The results suggest that certain acyclic nucleotides, in particular compounds **28** and **29**, are endowed with modest partial agonism on $P2X_3$ receptors. This is an interesting property that can depress the function of $P2X_3$ receptors, whose activation is believed to be involved in a number of chronic pain conditions including neuropathic pain and migraine. In fact, the new acyclic nucleotides are able to persistently block (by desensitization) $P2X_3$ receptor activity after a brief, modest activation, yet leaving the ability of sensory neurons to mediate responses to standard painful stimuli via a lower level of signaling.

Introduction

$P2X_3$ receptors, which belong to a large family of purinergic receptors, are integral membrane proteins expressed by sensory ganglion neurons to sense painful stimuli.[1−3] Extensive activation of such receptors is believed to be involved in a number of chronic pain conditions including neuropathic pain and migraine.[2] Because these states are notoriously resistant to treatment, new molecules targeted to $P2X_3$ receptors could be useful to achieve better pain control.

$P2X_3$ receptors are ligand-gated channels activated by extracellular ATP to induce a rapid increase in membrane permeability to mono- and divalent cations.[4−7] One important property of $P2X_3$ receptors is the rapid transition to a state of desensitization, which curtails their persistent signaling in physiological conditions.[8−10] $P2X_3$ receptors regain their ATP sensitivity relatively slowly via a multistep process regulated by inflammatory substances.[11−14] $P2X_3$ receptor desensitization is surprisingly agonist-specific,[5] opening up the possibility of blocking receptor activity with long-acting agonists, ideally of low efficacy, to avoid pain stimulation. Attaining this goal is made further feasible by the recent observation that low concentrations of agonist can inactivate a fraction of the receptors even in the absence of prior activation.[9,15] These data highlight the importance of identifying ATP analogues that can shape $P2X_3$ receptor function by either desensitization or pharmacological antagonism.

Adenosine-based agonists and xanthine-based antagonists bind adenosine purinergic A_1 receptors with similar electrostatic properties.[16,17] This observation led Fischer and coworkers to design a set of modified xanthines (theobromine and theophylline) in order to identify new purinergic ligands. In particular, they synthesized xanthine-triphosphate derivatives, bearing an alkyl spacer instead of the ribose moiety, and termed them "mini-nucleotides".[18] These compounds proved to be useful selective agents for activation of P2X receptors. On the other hand, 9-ethyladenine was used as the prototype agent for a series of nonxanthine adenosine receptor antagonists. Previous studies from our group showed that many derivatives of this scaffold are endowed with high binding affinity toward P1 receptors and behave as antagonists.[19−21] The first reported adenosine receptor antagonists are derivatives of natural xanthines, caffeine or theophylline, whose substitution at different positions led to highly potent and selective P1 receptor antagonists.[22]

Starting from these observations, our search for new $P2X_3$ receptor ligands led us to design and synthesize a series of "acyclic nucleotides" based on the adenine skeleton and bearing at the 9-position a functionalized four carbon chain, which could mimic the ribose moiety of natural ligands and it is suitable to be phosphorylated. Hence, various substituents with different electronic and steric properties were introduced in 2-position of adenine such as chlorine, iodine, amino, phenylethoxy group, and hexynyl chain (general formula, Figure 1).

In most cases the mono-, di-, and triphosphate derivatives were synthesized. The new compounds were evaluated for their biological activity on rat $P2X_3$ receptors by using patch clamp recording from transfected human embryonic kidney (HEK) cells.

Results and Discussion

Chemistry. Adenine based acyclic-nucleotides **14**−**29** were synthesized using commercially available adenine (**1**), 2,6-dichloropurine (**2**), and 2-amino-6-chloropurine (**3**) as starting materials (Scheme 1). The introduction of a protected four

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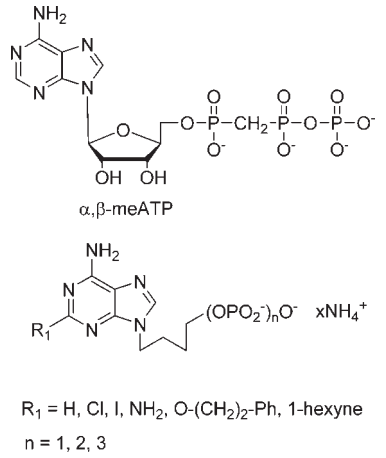


Figure 1. Structure of α,β-meATP and general structure of synthesized adenine "acyclic nucleotides".

carbon chain was performed by reacting **1**−**3** with 4-bromobutylacetate in the presence of potassium carbonate and dry DMF[a]. Both substituted N-9 (**4**−**6**) and N-7 (**4a**−**6a**) isomers were obtained in different ratios, depending on the used starting purine derivative, and ranging from about 4:1 ratio in the reaction with **1** to give **4** and **4a**, to 5:1 ratio in the reaction with compounds **2** and **3** to give **5**, **5a** and **6**, **6a**, respectively.

In all cases, the two isomers were isolated and characterized by their ^{1}H NMR spectra, which showed significant differences, and 1D NOE experiments. In the ^{1}H NMR spectra, the N-9 substituted compound **4** showed upfield signals for H-8 and CH_2-N compared to the corresponding N-7 isomer **4a**. The same differences were observed both comparing **5** with **5a**, and **6** with **6a** (Table 1). These data are in agreement with previous observations on N-9 and N-7 alkyl purine derivatives.[19,23] Furthermore, the alkylation site on the adenine derivatives **4** and **4a** was confirmed by 1D NOE experiments. In fact, after irradiation of the CH_2-N in compound **4**, NOEs were observed for the H-2 and H-8 and the vicinal CH_2 (Table 2). The same experiment performed on compound **4a** showed NOE only for the H-8 and the vicinal CH_2, while no effect was observed for H-2 signal, so unequivocally confirming **4** and **4a** as N-9 and N-7 isomers, respectively.

Reaction of N-9 isomers **4**−**6** with methanolic ammonia, at 120 °C for 16 h, gave deprotection of the hydroxyl alkyl chain and, in compounds **5** and **6**, simultaneous substitution of the chlorine atom in 6-position with an amino group to obtain the desired derivatives **7**−**9**, respectively.

The 2-phenethoxy derivative **10** was synthesized by treating **8** with phenethyl alcohol, in the presence of sodium hydroxide (Scheme 1), while the synthesis of the 2-iodo and 2-hexynyl derivatives **12** and **13** was performed using **6** as starting material. Hence, **6** was reacted in the Sandmeyer conditions to obtain **11**, which was then treated with methanolic ammonia to give **12**. Subsequent reaction of **12**, using Sonogashira coupling conditions[21,24] with bis-triphenylphosphine palladium dichloride, copper iodide, triethylamine, and 1-hexyne, gave the desired 2-hexynyl derivative **13** (Scheme 2).

The adenine derivatives **7**−**10**, **12**, and **13** were phosphorylated by reacting them with phosphorus oxychloride in trimethyl phosphate at room temperature, according to Yoshikawa procedure.[25] After purification on a diethylaminoethyl resin (Sephadex DEAE A-25, HCO_3^- form) eluting with a gradient of water and ammonium bicarbonate, the corresponding monophosphates **14**−**19** were obtained in good yield ranging from 35 to 78%. The syntheses of the di- and triphosphate derivatives were carried out by a modification of the Hoard−Ott method.[26] Hence, the tri-*n*-butylammonium salts of the monophosphate derivatives **14**−**19** were converted to phosphorimidazolates with 1,1′-carbonyldiimidazole. The phosphorimidazolates obtained from compounds **14**−**17** were coupled with tri-*n*-butylammonium phosphate to give the corresponding diphosphate derivatives **20**−**23**.

The triphosphates **24**−**29** were prepared by reacting the phosphorimidazolates obtained from **14**−**19** with bis(tri-*n*-butylammonium)pyrophosphate (Scheme 3).

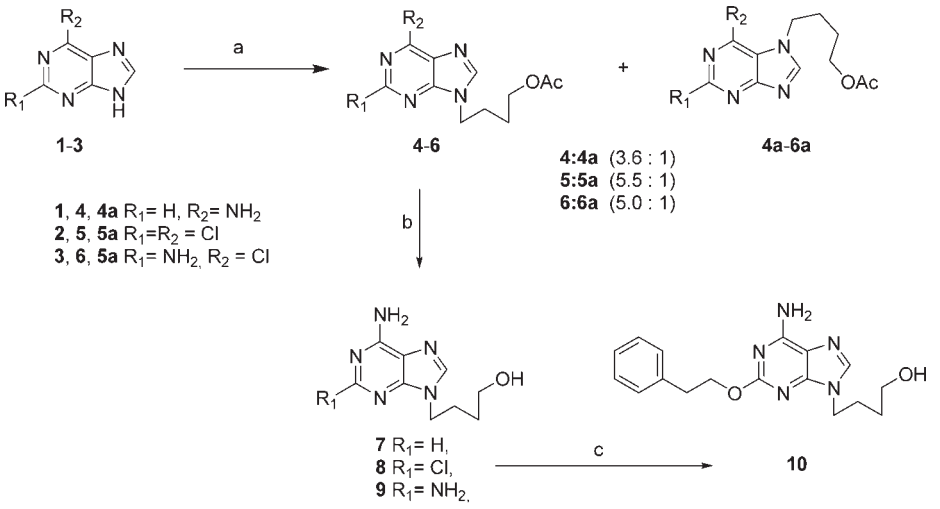
Biological Data. The newly synthesized adenine acyclic nucleotides were evaluated for their biological activity on rat $P2X_3$ receptors by using patch clamp recording from HEK-transfected cells. The full agonist α,β-meATP was selected as a reference compound because of its stability and high selectivity for $P2X_3$ receptors. Because we studied recombinant receptors on unexcitable epithelial cells, which do not constitutively synthesize and express them, we avoided interference by constitutive receptors and ion channels and enhanced the reliability of our results by measuring ionic currents exclusively caused by activation of $P2X_3$ receptors via a very rapid delivery system of the tested chemical agents.

Examples of inward currents induced by 2 s pulse application of α,β-meATP (100 μM) or certain acyclic nucleotides (100 μM) are shown in Figure 2. The inward current observed in the presence of α,β-meATP possessed all the characteristics typical of $P2X_3$ receptor mediated responses, namely rapid onset, large amplitude, and fast decay in the continuous presence of this agonist (desensitization). While the monophosphates **16**, **18**, and **19** lacked significant agonist activity, the di- and triphosphate derivatives **22** and **26**, bearing an amino group in 2-position of the adenine core, evoked responses smaller than those elicited by α,β-meATP and accompanied by minimal desensitization during the 2 s pulse application. The 2-iodo triphosphate derivative **28** did evoke a rapid current of lower amplitude than the one observed with α,β-meATP but still associated with rapid desensitization, while the triphosphate **29**, bearing a hexynyl chain in 2-position, induced a small response with characteristics intermediate between those of the triphosphates **26** and **28**. Hence, the presence of at least two phosphate groups seemed to be essential for receptor activation, while the nature of the substituent present in 2-position of the adenine moiety appeared to mostly influence the grade of receptor activation and desensitization.

The average $P2X_3$ agonist activity of the tested acyclic nucleotides in relation to α,β-meATP (taken as 100%; dotted line) is summarized in Figure 3 (A; open bars). Most of the tested monophosphate derivatives **14**−**19** were virtually devoid of any agonist action. Among compounds endowed with significant agonist activity, the triphosphates **24**−**29** were in general more active than the diphosphate **20**−**23** and all of them behaved as much weaker agonists with

[a] Abbreviations: CDI, 1,1′-carbonyldiimidazole; DEAE, diethylaminoethyl; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; α,β-meATP, α,β-methyleneATP; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic sodium salt; EGTA, glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MEM, minimum essential medium; NOE, nuclear Overhauser effect.

Scheme 1. Synthesis of 2-Substituted 9-Hydroxybutyladenines **7**−**10**[a]



[a] Reagents and conditions: (a) DMF, $Br(CH_2)_4OCOCH_3$, rt, 16 h; (b) NH_3/CH_3OH, 120 °C, 16 h; (c) $C_6H_5CH_2CH_2OH$, NaOH, 80 °C, 4 h.

Table 1. ^1H Chemical Shifts (ppm) of H-8 and CH_2−N Signals of Compounds **4**−**6** and **4a**−**6a** in DMSO-d_6

compd	H-8	CH_2-N
4	8.13	4.14
4a	8.34	4.32
5	8.78	4.26
5a	8.86	4.46
6	8.13	4.05
6a	8.37	4.29

Table 2. One-Dimensional NOE Data after Irradiation of CH_2−N Signal of Compounds **4** and **4a**

compd	H-8	H-2	CH_2CH_2-N
4	yes	yes	yes
4a	yes	no	yes

respect to α,β-meATP on $P2X_3$ receptors. The nature of the 2-substitution modulates the receptor activation; hence, the 2-unsubstituted acyclic nucleotides di- and triphosphate **20** and **24** showed responses that were 32 and 36% of the maximal effect elicited by α,β-meATP.

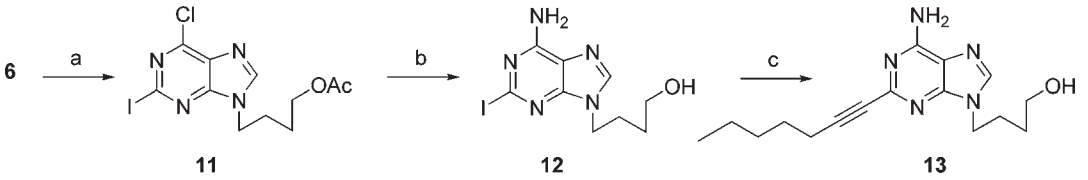
It is worthwhile to note that the diphosphate **20**, lacking substitution at the 2-position, is the only diphosphate derivative endowed with notable activity, comparable to that of the triphosphates. Considering the triphosphate derivatives **24**−**29**, it is evident that the substitution in the 2-position of lipophilic substituents like a chlorine and an iodine atom or a hexynyl group (compounds **25**, **28**, and **29**) was well tolerated. In particular, the 2-iodo triphosphate **28** and the 2-hexynyl triphosphate **29** showed responses of 60 and 38% of the maximal effect elicited by α,β-meATP, resulting the most active compounds of the series. However, the presence of a lipophilic sterically hindered phenethoxy group was not tolerated, leading to compound **27**, endowed with very low activity. Also the presence of a hydrophilic amino group produced a decrease of receptor activation ability (compare **26** with **24**). From these data, it is possible to hypothesize that the new acyclic nucleotides interacted with a receptor binding region so that the C-2 portion of the purine scaffold was found in proximity of a small $P2X_3$ receptor hydrophobic pocket (capable of binding halogens or the partially flexible hexynyl chain), where the small hydrophilic amino group is tolerated but the bulky phenethoxy substituent could not be allocated.

We next evaluated whether continuously applied acyclic nucleotides could modify subsequent responses to α,β-meATP. To this end, we preapplied these compounds for 20 s prior to the pulse of α,β-meATP, as shown in the examples of Figure 4 with **18** and **26**. The monophosphate **18** did not produce an inward current (see Figure 4, top), although it did depress the subsequent response to α,β-meATP (on average, the response became 80% of control; see B histograms in Figure 3), showing that **18** was a weak antagonist. Similar results were obtained with the monophosphates **16**, **17**, and **19** (Figure 3).

Compound **26** produced an initial small current (25% of the α,β-meATP response, indicating partial agonism; Figure 3A) that fully desensitized after 2 s pulse application and strongly decreased the subsequent response to α,β-meATP: on average, the residual $P2X_3$ receptor currents after applying compound **26** were 17% of control (Figure 3B). This pattern of effects was clearly observed also with triphosphate derivatives like **24** and **25** that generated a 36 and 30% response of the α,β-meATP maximum effect, yet they could inhibit potently receptor function (α,β-meATP responses had become 14 and 10%, respectively, of control). On the other hand, compounds **28** and **29**, which elicited the higher currents, completely inhibited the subsequent response to α,β-meATP.

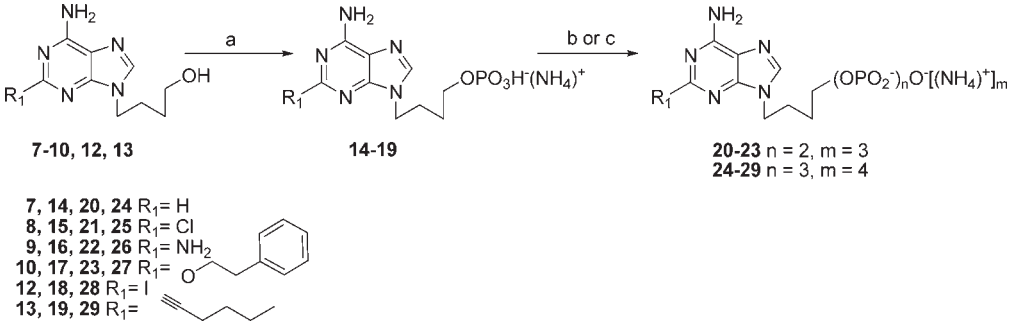
All these effects were reversible on washout. Hence, most of the tested acyclic nucleotides could persistently block (by desensitization) receptor activity after a brief, modest activation. In practice, these compounds behaved like weak partial agonists.[27] Full pharmacological demonstration of partial agonism would require the construction of complete dose response curves for each novel compound, and for validation of the same maximum amplitude attained with 100 μM α,β-meATP, a very large amount of the same compounds would have been required, taking into account that they are less potent than α,β-meATP and should be added by continuous application. Notwithstanding this limitation, the early, low amplitude receptor activation followed by desensitization (see Figure 2) suggests that compounds endowed with activity like the one produced by **28** and **29** acted as partial agonists on $P2X_3$ receptors. Thus, they were clearly not

Scheme 2. Synthesis of 2-Iodo and 2-Hexynyl-9-hydroxybutyladenines **12** and **13**[a]



[a] Reagents and conditions: (a) $C_5H_{11}ONO$, CH_2I_2, 85 °C, 1 h; (b) NH_3/CH_3OH, 120 °C, 16 h; (c) 1-hexyne, $(Ph_3P)_2PdCl_2$, Et_3N, CuI, DMF, 90 °C, 3 h.

Scheme 3. Synthesis of 2-Substituted 9-Hydroxybutyladenine Mono- (**14**−**19**), Di- (**20**−**23**), and Triphosphates (**24**−**29**)[a]



[a] Reagents and conditions: (a) $POCl_3$, $(CH_3O)_3PO$, rt, 3 h; (b) CDI, tri-*n*-butylammonium phosphate, DMF, rt, 16 h; (c) CDI, bis(tri-*n*-butylammonium) pyrophosphate, DMF, rt, 16 h.

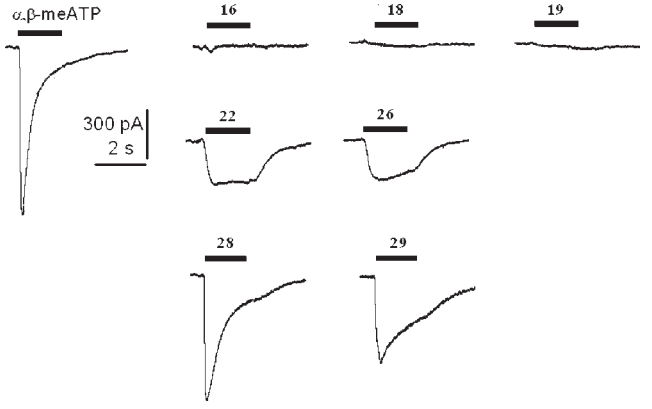


Figure 2. Effects of acyclic nucleotides on $P2X_3$ receptors expressed by HEK cells. Traces are sample records of inward currents (obtained with patch clamp recording in the whole cell configuration) elicited by 2 s pulse application of the indicated compounds. The response evoked by α,β-meATP is taken as a standard reference. Note that only **28** induces an effect partly similar in amplitude and kinetics to the response to α,β-meATP. While **18** and **19** are virtually inactive, **22** and **26** produce current responses without decay or with slow decay, respectively, suggestive of minimal desensitization. All compounds are applied by fast superfusion at 100 μM concentration.

typical antagonists in view of their observed ability to induce receptor activation directly measured in terms of inward current (Figure 3).

Partial agonism at ionotropic receptors has been recently identified as due to a rapid conformation change ("flipping") of the receptor that occurs once the agonist is bound while the channel still shut.[28] This property applied to $P2X_3$ receptors would be interesting to modulate their function without generating full suppression of receptor signaling; indeed, one might argue that a very strong antagonism of $P2X_3$ receptors can produce undesirable in vivo effects such as lack of response to standard painful stimuli. Conversely, administration of a partial agonist might depress receptor function yet leaving the ability of sensory neurons to mediate responses via a lower level of signaling. This concept has been applied, for instance, to the use of β-receptor partial agonists to inhibit overactivity of β-receptors in hypertension and cardiac arrhythmias without excessive depression of blood pressure and heart beating.[29] Indeed, the very recent study of the molecular mechanisms of partial agonism predicts that this phenomenon has implications for the design of partial agonists for therapeutic use.[28]

Our experimental data from this relatively ample series of acyclic nucleotides did not disclose any potent antagonist because among the compounds devoid of significant agonist activity at 100 μM concentration (**15**−**19**, Figure 3A). the maximum degree of inhibition of α,β-meATP induced responses was about 20% (Figure 3B). Nevertheless, the general low ability to induce currents showed by the new acyclic nucleotides may help to propose some hypothesis on the molecular groups necessary to bind the receptor and to activate it partially or fully. In fact, the lack of an intact ribose moiety in these molecules could be responsible of their inability to fully activate the $P2X_3$ receptor, being the sugar moiety interactions necessary for full agonist activity.[30]

Of equal interest is the question of the mechanisms of $P2X_3$ receptor inactivation, which our present study can help to address. A recent scheme of $P2X_3$ receptor function based on experimental investigations and kinetic modeling[15] has proposed that for equivalent degrees of receptor activation and subsequent desensitization, recovery depends on the chemical nature of the agonist that dissociates from the bound receptor at different rates. The present study suggests that the extent of receptor desensitization was mostly influenced by the structure of the substituent present in 2-position of the new acyclic nucleotides; however, our investigation shows that the level of phosphorylation of the compound also seemed to play a role. In fact, the 2-amino diphosphate **22** displayed weak agonist activity with minimal desensitization, yielding square-shape inward currents in contrast with the sharp transients induced by the full agonists, while the

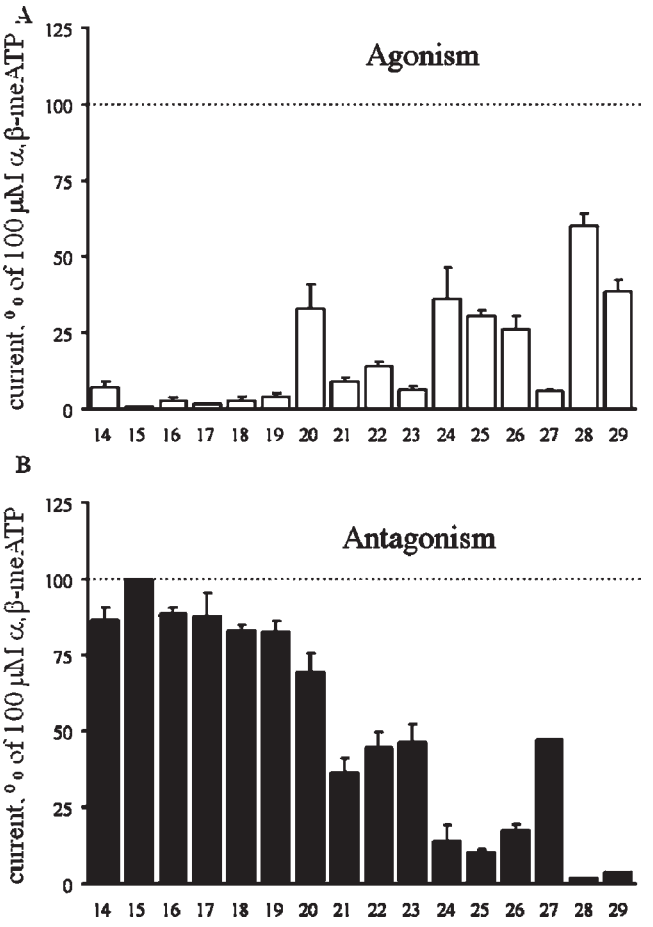


Figure 3. Summary of agonist or antagonism activity of acyclic nucleotides on $P2X_3$ receptors. (A) Open bars show average inward currents elicited by acyclic nucleotides applied for 2 s (100 μM) and expressed as % of the response induced by α,β-meATP (100 μM), $n = 7$, $P < 0.05$. (B) Filled bars show average inward currents induced by α,β-meATP (100 μM; 2 s) after preapplication of acyclic nucleotides (100 μM) for 20 s; data are % values of control α,β-meATP effects, $n = 7$, $P < 0.05$ for all acyclic nucleotides, except **15**. For structures see Scheme 3.

chemically related triphosphate **26** elicited responses of similar amplitude and characterized by more desensitization (see Figure 2). Assuming that analogous current response peaks indicated equivalent degree of receptor activation by different compounds, it is likely that $P2X_3$ receptors activated by **22** desensitized only very slowly because the dissociation of such an agonist and, consequently, receptor return to the resting state were intrinsically retarded due to the chemical properties of this compound. Further studies with **22** might, therefore, help to measure the time course of receptor inactivation without the complication of intervening desensitization, and to improve existing receptor models.

Conclusion

The present data suggest that certain acyclic nucleotides, in particular compounds **28** and **29**, obtained with de novo synthesis and endowed with modest partial agonism on $P2X_3$ receptors are potentially useful drugs to regulate the sensitivity of such receptors to painful stimuli. This goal may be achieved by partially desensitizing the receptors so that they would signal less effectively but would still retain a certain degree of responsiveness to large concentrations of agonists. Because the transient concentration of extracellular ATP

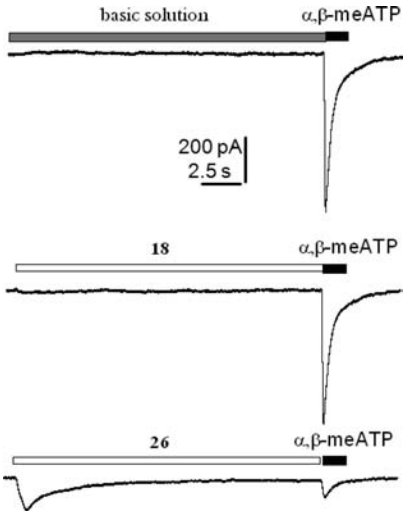


Figure 4. Example of protocol to test the blocking action of acyclic nucleotides on $P2X_3$ receptor responses induced by α,β-meATP. Application of **18** (100 μM; 20 s) does not evoke inward current and partly inhibits the amplitude (84%) of subsequent response to α,β-meATP (100 μM). Application of **26** (100 μM; 20 s) elicits a transient inward current and largely depresses subsequent response to α,β-meATP (12% residual response). All records are from the same cell.

activating $P2X_3$ receptors is envisioned to be large,[1,5] this system would preserve sufficient sensitivity to respond to natural stimuli. Future work is required to validate these suggestions with an in vivo model of pain.

Experimental Section

Chemistry. Melting points were determined with a Buchi apparatus and are uncorrected. UV spectra were collected on a Varian Cary-1E UV−visible spectrophotometer. ^{1}H NMR spectra were obtained with Varian Mercury 400 MHz spectrometer; δ in ppm, J in Hz. All exchangeable protons were confirmed by addition of D_2O. ^{31}P NMR spectra were recorded at room temperature by using a Varian Mercury 400 MHz spectrometer. Mass spectra were recorded on an HP 1100-MSD series instrument. All measurements were performed using electrospray ionization (ESI-MS) on a single quadrupole analyzer. TLC were carried out on precoated TLC plates with silica gel 60 F-254 (Merck). For column chromatography, silica gel 60 (Merck) was used. For ion exchange chromatography, Sephadex DEAE A-25, HCO_3^- form was used. Elemental analyses were determined on a Fisons model EA 1108 CHNS-O model analyzer and are within ±0.4% of theoretical values.

General Procedure for the Preparation of Compounds 4−6 and 4a−6a. To a solution of the suitable purine **1−3** (10 mmol) in dry DMF (13.0 mL), K_2CO_3 (1.66 g, 12.0 mmol) was added. After stirring for 5 min, 4-bromo-butylacetate (1.57 mL, 11.0 mmol) was added and the reaction was left at rt for 16 h. Reactions completion were checked by TLC eluted with $CHCl_3$−CH_3OH (9:1): the N-9 isomers showed R_f values ranging from 0.2 to 0.6, while N-7 isomers showed 0.1−0.5 R_f values, respectively. The mixture was concentrated under vacuum and chromatographed over flash silica gel column using the appropriate eluent to obtain compounds **4−6** and **4a−6a** as white solids.

9-Acetoxybutyladenine (4) and 7-Acetoxybutyladenine (4a). Compounds **4** and **4a** were obtained from **1** after chromatography eluting with $CHCl_3$−MeOH (98:2 to 95:5, v/v) as white solids, respectively. **4**: yield 54%, mp 176−178 °C. UV (EtOH) λ_{max}: 205 nm (ε 23335 dm^3 mol^{-1} cm^{-1}), 259 nm (ε 9404 dm^3 mol^{-1} cm^{-1}). ^{1}H NMR (DMSO-d_6) δ 8.13 (s, 1H, H-8), 8.11 (s, 1H, H-2), 7.19 (brs, 2H, NH_2), 4.14 (t, 2H, J = 7.0 Hz, NCH_2), 3.98 (t, 2H, J = 6.6 Hz, OCH_2), 1.95 (s, 3H, CH_3), 1.84 (m, 2H, NCH_2CH_2), 1.50 (m, 2H,

OCH_2CH_2). **4a**: yield 15%, mp 135−137 °C. UV (EtOH) λ_{max}: 211 nm (ε 13884 $dm^3\ mol^{-1}\ cm^{-1}$), 273 nm (ε 9175 $dm^3\ mol^{-1}\ cm^{-1}$). ^{1}H NMR (DMSO-d_6) δ 8.34 (s, 1H, H-8), 7.87 (brs, 2H, NH_2), 7.74 (s, 1H, H-2), 4.32 (t, 2H, J = 7.0 Hz, NCH_2), 3.99 (t, 2H, J = 6.4 Hz, OCH_2), 1.95 (s, 3H, CH_3), 1.91 (m, 2H, NCH_2CH_2), 1.54 (m, 2H, OCH_2CH_2). Anal. ($C_{11}H_{15}N_5O_2$) C, H, N.

9-Acetoxybutyl-2,6-dichloropurine (5) and 7-Acetoxybutyl-2,6-dichloropurine (5a). Compounds **5** and **5a** were obtained from **2** after chromatography eluting with $CHCl_3$−MeOH (99:1, v/v) as white solids. **5**: yield 44%, mp 107−108 °C. UV (EtOH) λ_{max}: 213 nm (ε 21259 $dm^3\ mol^{-1}\ cm^{-1}$), 273 nm (ε 9151 $dm^3\ mol^{-1}\ cm^{-1}$). ^{1}H NMR (DMSO-d_6) δ 8.78 (s, 1H, H-8), 4.26 (t, 2H, J = 7.0 Hz, NCH_2), 3.99 (t, 2H, J = 6.6 Hz, OCH_2), 1.97 (s, 3H, CH_3), 1.87 (m, 2H, NCH_2CH_2), 1.55 (m, 2H, OCH_2CH_2). **5a**: yield 8%, mp 101−102 °C. UV (EtOH) λ_{max}: 212 nm (ε 27450 $dm^3\ mol^{-1}\ cm^{-1}$), 277 nm (ε 7194 $dm^3\ mol^{-1}\ cm^{-1}$). ^{1}H NMR (DMSO-d_6): δ 8.86 (s, 1H, H-8), 4.46 (t, 2H, J = 7.0 Hz, NCH_2), 3.99 (t, 2H, J = 6.6 Hz, OCH_2), 1.97 (s, 3H, CH_3), 1.84 (m, 2H, NCH_2CH_2), 1.58 (m, 2H, OCH_2CH_2). Anal. ($C_{11}H_{12}Cl_2N_4O_2$) C, H, N.

9-Acetoxybutyl-2-chloroadenine (6) and 7-Acetoxybutyl-2-chloroadenine (6a). Compounds **6** and **6a** were obtained from **3** after chromatography eluting with $CHCl_3$−MeOH (99:1, v/v) as white solids. **6**: yield 76%, mp 113−114 °C. UV (EtOH) λ_{max}: 202 nm (ε 3186 $dm^3\ mol^{-1}\ cm^{-1}$), 222 nm (ε 15007 $dm^3\ mol^{-1}\ cm^{-1}$), 246 nm (ε 3350 $dm^3\ mol^{-1}\ cm^{-1}$), 309 nm (ε 3652 $dm^3\ mol^{-1}\ cm^{-1}$). ^{1}H NMR (DMSO-d_6) δ 8.13 (s, 1H, H-8), 6.91 (brs, 2H, NH_2), 4.05 (t, 2H, J = 7.0 Hz, NCH_2), 3.97 (t, 2H, J = 6.6 Hz, OCH_2), 1.96 (s, 3H, CH_3), 1.80 (m, 2H, NCH_2CH_2), 1.51 (m, 2H, OCH_2CH_2). **6a**: yield 15%, mp 163 °C dec. (EtOH) λ_{max}: 221 nm (ε 39800 $dm^3\ mol^{-1}\ cm^{-1}$), 321 nm (ε 9154 $dm^3\ mol^{-1}\ cm^{-1}$). ^{1}H NMR (DMSO-d_6): δ 8.37 (s, 1H, H-8), 6.62 (brs, 2H, NH_2), 4.29 (t, 2H, J = 7.0 Hz, NCH_2), 3.98 (t, 2H, J = 6.6 Hz, OCH_2), 1.96 (s, 3H, CH_3), 1.81 (m, 2H, NCH_2CH_2), 1.53 (m, 2H, OCH_2CH_2). Anal. ($C_{11}H_{14}ClN_5O_2$) C, H, N.

General Procedure for the Preparation of Compounds 7−9 and 12. Compounds **4**−**6** or **11** (1.0 g, 4.01 mmol) were in turn added in a steel vial containing saturated methanolic ammonia (15 mL). The reaction was kept at 120 °C for 16 h unless otherwise stated. After volatiles removing, compounds **7**−**9** or **12** were obtained by crystallization from methanol.

9-Hydroxybutyladenine (7). Reaction of **4** gave **7** white solid. Yield 96%; mp 200−202 °C. ^{1}H NMR (DMSO-d_6) δ 8.12 (s, 1H, H-8/H-2), 8.11 (s, 1H, H-8/H-2), 7.19 (brs, 2H, NH_2), 4.44 (t, 1H, J = 5.2 Hz, OH), 4.12 (t, 2H, J = 7.2 Hz, NCH_2), 3.38 (m, 2H, OCH_2), 1.80 (m, 2H, NCH_2CH_2), 1.34 (m, 2H, OCH_2CH_2). Anal. ($C_9H_{13}N_5O$) C, H, N.

2-Chloro-9-hydroxybutyladenine (8). Reaction of **5**, at 80 °C for 16 h, gave **8** as white solid. Yield 90%; mp 188−190 °C. ^{1}H NMR (DMSO-d_6) δ 8.15 (s, 1H, H-8), 7.73 (s, 2H, NH_2) 4.44 (t, 1H, J = 7.0 Hz, OH), 4.09 (t, 2H, J = 7.0 Hz, NCH_2), 3.38 (m, 2H, OCH_2), 1.80 (m, 2H, NCH_2CH_2), 1.36 (m, 2H, OCH_2CH_2). Anal. ($C_9H_{12}ClN_5O$) C, H, N.

2-Amino-9-hydroxybutyladenine (9). Reaction of **6** gave **9** as white crystals. Yield 89%; mp 191−193 °C. ^{1}H NMR (DMSO-d_6) δ 7.67 (s, 1H, H-8), 6.61 (brs, 2H, NH_2), 5.75 (brs, 2H, NH_2), 4.44 (brs, 1H, OH), 3.92 (t, 2H, J = 7.0 Hz, NCH_2), 3.37 (t, 2H, J = 6.0 Hz, OCH_2), 1.73 (m, 2H, NCH_2CH_2), 1.34 (m, 2H, OCH_2CH_2). Anal. ($C_9H_{14}N_6O$) C, H, N.

9-Hydroxybutyl-2-phenethoxyadenine (10). To compound **8** (0.50 g, 2.06 mmol), phenethyl alcohol (2.5 mL) and sodium hydroxide (0.50 g) were added and the mixture was heated at 80 °C for 6 h. The solvent was removed under vacuum and the residue chromatographed on a silica gel column eluting with $CHCl_3$−MeOH (95:5, v/v) to give **10** as white solid. Yield 79%; mp 113−115 °C. ^{1}H NMR (DMSO-d_6) δ 7.92 (s, 1H, H-8), 7.32−7.18 (m, 7H, Ph-H and NH_2), 4.39 (m, 3H, OH and NCH_2), 4.03 (t, 2H, J = 7.0 Hz, OCH_2), 3.36 (m, 2H, $PhCH_2CH_2$), 2.99 (t, 2H, J = 7.0 Hz, $PhCH_2$), 1.79 (m, 2H, NCH_2CH_2), 1.34 (m, 2H, OCH_2CH_2). Anal. ($C_{17}H_{21}N_5O_2$) C, H, N.

9-Acetoxybutyl-6-chloro-2-iodopurine (11). To a solution of **6** (0.20 g, 0.71 mmol) in dry DMF (3.0 mL), isoamylnitrite (0.30 mL) and diiodomethane (0.94 mL) were added and the mixture was heated at 85 °C for 1 h. After volatiles evaporation, the residue was chromatographed on a flash silica gel column eluting with $CHCl_3$−MeOH (99:1, v/v) to get **11** as white solid. Yield 37%; mp 127−129 °C. ^{1}H NMR (DMSO-d_6) δ 8.60 (s, 1H, H-8), 4.24 (t, 2H, J = 7.0 Hz, NCH_2), 4.00 (t, 2H, J = 6.6 Hz, OCH_2), 1.98 (s, 3H, CH_3), 1.85 (m, 2H, NCH_2CH_2), 1.54 (m, 2H, OCH_2CH_2). Anal. ($C_{11}H_{12}ClIN_4O_2$) C, H, N.

9-Hydroxybutyl-2-iodoadenine (12). Reaction of **11** gave **12** as yellowish crystals. Yield 85%; mp 184−186 °C. ^{1}H NMR (DMSO-d_6) δ 8.06 (s, 1H, H-8), 7.61 (brs, 2H, NH_2), 4.43 (t, 1H, J = 5.2 Hz, OH), 4.06 (t, 2H, J = 7.2 Hz, NCH_2), 3.37 (m, 2H, OCH_2), 1.77 (m, 2H, NCH_2CH_2), 1.34 (m, 2H, OCH_2CH_2). Anal. ($C_9H_{12}IN_5O$) C, H, N.

2-Hex-1-ynyl-9-hydroxybutyladenine (13). To a solution of **12** (0.70 g, 2.10 mmol) in dry DMF (8.0 mL), bistriphenylphosphine-palladium(II)chloride (31 mg, 0.043 mmol), copper iodide (30 mg, 0.16 mmol), triethylamine, and 1-hexyne (1.44 mL, 12.53 mmol (7.0 mL, 55.60 mmol) were added and the mixture was heated at 90 °C for 3 h. The reaction mixture was filtered, and after evaporation under vacuum of the filtrate, the residue was chromatographed on a flash silica gel eluting with $CHCl_3$−MeOH (98:2 to 91:9, v/v) to give **13** as yellowish powder. Yield 78%; mp 150−152 °C. ^{1}H NMR (DMSO-d_6) δ 8.16 (s, 1H, H-8), 7.30 (brs, 2H, NH_2), 4.44 (t, 1H, J = 4.2 Hz, OH), 4.09 (t, 2H, J = 6.8 Hz, NCH_2), 3.37 (m, 2H, OCH_2), 2.38 (t, 2H, J = 6.8 Hz, $CH_2C{\equiv}C$), 1.78 (m, 2H, NCH_2CH_2), 1.50 (m, 2H, OCH_2CH_2), 1.38 (m, 4H, $CH_2CH_2CH_3$), 0.90 (t, 3H, J = 7.4 Hz, CH_3). Anal. ($C_{15}H_{21}N_5O$) C, H, N.

General Procedure for the Synthesis of Monophosphates 14−19. Compounds **7**−**10**, **12**, or **13** (1.44 mmol) were in turn dissolved in trimethyl phosphate (3.0 mL), and then 4 equiv of $POCl_3$ (539 μL, 5.78 mmol) were added at 0 °C. The solution was stirred at rt for 3 h, and then H_2O (3.0 mL) was added to the reaction maintained at 0 °C and the solution neutralized by adding triethylamine. The reactions were monitored by TLC, using precoated TLC plates with silica gel 60 F-254 (Merck) and i-C_3H_7OH−H_2O−NH_4OH (5.5:1.0:3.5) as mobile phase; monophosphate derivatives showed 0.4−0.5 R_f values. The nucleotides were purified by means of ionic exchange chromatography on a Sephadex DEAE A-25 (Fluka) column (HCO_3^- form) equilibrated with H_2O and eluted with a linear gradient of H_2O/0.5 M NH_4HCO_3.

9-(4-Monophosphate-butyl)adenine (14). Reaction of **7** gave **14** as white solid. Yield 68%. ^{1}H NMR (D_2O) δ 8.15 (s, 1H, H-2), 8.10 (s, 1H, H-8), 4.16 (t, 2H, J = 7.0 Hz, NCH_2), 3.72 (m, 2H, OCH_2), 1.82 (m, 2H), 1.46 (m, 2H). ^{31}P NMR (D_2O) δ 1.07 (s). Anal. ($C_9H_{17}N_6O_4P$) C, H, N.

General Procedure of Phosphorylation. Preparation of Bis(tri-*n*-butylammonium) Pyrophosphate. Sodium pyrophosphate decahydrate 6.69 g (15 mmol) was dissolved in 150 mL of deionized water. Excess of Dowex ion-exchange resin 20−50 mesh, proton form, prewashed several times with water, was added to the solution of sodium pyrophosphate, and the mixture was gently stirred for 20 min. A mixture of 60 mL of EtOH and 7.14 mL of tri-*n*-butylamine in a flask was placed in an ice−water bath, and the pyrophosphate solution was filtered directly into the flask. The resin was repeatedly washed with water until the filtrate was no longer acidic. The solvent was then evaporated under reduced pressure at 40 °C to give a thick, nearly colorless syrup. This residue was treated twice with 100 mL of EtOH and then evaporated. The residue was taken up in 40 mL of anhydrous DMF and evaporated again. This residue was dissolved in 30 mL of anhydrous DMF, yielding 30 mL of a 0.5 M solution of bis(tri-*n*-butylammonium) pyrophosphate in DMF. The solution was stored, sealed, and cooled at 4 °C until use.[31]

Preparation of Tri-*n*-butylammonium Phosphate. The same procedure was used as in case of the preparation of

bis(tri-*n*-butylammonium) pyrophosphate using sodium monophosphate instead of sodium pyrophosphate.

General Procedure for the Synthesis of Diphosphates 20−23 and Triphosphates 24−29. To 0.15 mmol of monophosphates **14−19**, in turn dissolved in dry DMF (1.0 mL), 36 μL of tri-*n*-butylamine (28 mg, 0.15 mmol) were added. The solution was stirred for 20 min at rt and then evaporated to dryness under anhydrous conditions. After resuspension in dry DMF (1.4 mL), *N*,*N*′-carbonyldiimidazole (122 mg, 0.75 mmol) was added and the mixture was stirred at rt for 24 h. The reaction was quenched by addition of dry CH_3OH (49 μL, 38.5 mg 1.2 mmol) and stirred for 30 min at rt. Then 6 mL (3 mmol) of a 0.5 M solution of tri-*n*-butylammonium phosphate or bis(tri-*n*-butylammonium) pyrophosphate in DMF were added and the mixture was stirred at rt for 16 h. The reactions were monitored by TLC, using precoated TLC plates with silica gel 60 F-254 (Merck) and *i*-$C_3H_7OH-H_2O-NH_4OH$ (5.5:2.0:2.5) for the diphosphate derivatives or *i*-$C_3H_7OH-H_2O-NH_4OH$ (4.5:2.0:3.5) as mobile phase for the triphosphate derivatives (0.4−0.5 R_f values), respectively. The solvent was removed under vacuum and the residue, dissolved in H_2O, was purified by means of ion exchange chromatography to give the diphosphates **20−23** or the triphosphates **24−29**, respectively.

9-(4-Diphosphate-butyl)adenine (20). Reaction of **14** gave **20** as white solid. Yield 15%, ^{1}H NMR (D_2O) δ 8.05 (s, 1H, H-2), 8.02 (s, 1H, H-8), 4.11 (t, 2H, J = 6.8 Hz, NCH_2), 3.78 (m, 2H, OCH_2), 1.80 (m, 2H, CH_2), 1.46 (m, 2H, CH_2). ^{31}P NMR (D_2O) δ −8.74 (d), −9.75 (m). ESI-MS: negative mode m/z 388.0 ([M − 2H + Na]$^-$), 366.0 ([M − H]$^-$), 182.6 ([(M − 2H)/2]$^-$). Anal. ($C_9H_{24}N_8O_7P_2$) C, H, N.

9-(4-Triphosphate-butyl)-adenine (24). Reaction of **14** gave **24** as white solid. Yield 22%, ^{1}H NMR (D_2O) δ 8.10 (s, 1H, H-2), 8.08 (s, 1H, H-8), 4.15 (t, 2H, J = 6.8 Hz, NCH_2), 3.83 (m, 2H, OCH_2), 1.83 (m, 2H, CH_2), 1.48 (m, 2H, CH_2). ^{31}P NMR (D_2O) δ −9.99 (m), −22.08 (m). ESI-MS: negative mode m/z 446.0 ([M − H]$^-$), 222.4 ([(M − 2H)/2]$^-$). Anal. ($C_9H_{28}N_9O_{10}P_3$) C, H, N.

Biology. Cell Culture and Transfection. HEK 293T cells, supplied by the in-house SISSA cell bank, were maintained in culture in MEM-Glutamax medium (Invitrogen) supplemented with 10% fetal calf serum (Sigma) and penicillin/streptomycin. For each transfection experiment, 5×10^{-5} cells were plated and transfected 24 h later with the calcium/phosphate method using 1 μg of high-quality purified plasmid DNA encoding for rat $P2X_3$ subunit (for details see reference; NCBI accession number: CAA62594).[32] Transfected cells were used 48 h later for further experiments.

Electrophysiological Recording. Full details of the wholecell patch clamp recording protocol can be found in previous reports.[9,31] In brief, HEK cells were continuously superfused at fast rate (3 mL. min^{-1}) with control solution containing (in mM): NaCl 152, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, HEPES 10; pH was adjusted to 7.4 with NaOH. Patch pipettes had 3−4 MΩ resistance when filled (in mM) with CsCl 130; HEPES 20; $MgCl_2$ 1, magnesium ATP 3; EGTA 5; pH was adjusted to 7.2 with CsOH. Cells were voltage-clamped at −60 mV. Currents were filtered at 1 kHz and acquired with pCLAMP 9.0 software (Molecular Devices).

Chemicals and Their Application. Novel compounds were applied via a rapid superfusion system (VC-6 with perfusion fast step SF-77B, Warner Instruments) placed near the recorded cell. Time for the solution exchange at cell membrane level was 20−30 ms. To screen the activity of the novel compounds, we used, as a reference response, the fast inward current evoked by the selective $P2X_3$ full agonist α,β-methyleneATP (α,β-meATP) usually applied for 2 s at 100 μM concentration that reliably produces a maximal response.[9,15,31] In each experiment, this concentration of α,β-meATP was applied at least 3 times (at 5 min interval to avoid cumulative desensitization) to obtain an average control response indicative of full activation of $P2X_3$ receptors. All compounds (used at 100 μM concentration) were examined for their intrinsic ability to generate an inward current (agonist property) whose peak amplitude was compared with the one elicited by the full agonist α,β-meATP. Furthermore, all agonist compounds were also evaluated for their ability to inhibit (after 20 s continuous application) the subsequent response to α,β-meATP via the process of desensitization, namely the depression of the subsequent α,β-meATP evoked current after any early agonist activity of the novel drug. Conversely, pure antagonism was defined as observed attenuation of α,β-meATP induced responses in the absence of any early inward current by the novel drug. We have previously reported that preapplication (20 s) of selective antagonists and agonists of $P2X_3$ receptors fully blocks subsequent α,β-meATP-induced currents.[5] All chemical reagents, including enzymes for cell culture, were from Sigma; culture mediums were obtained from Gibco BRL.

Data Analysis. All data are presented as mean ± SEM (n = number of cells) with statistical significance assessed with paired t test (for parametric data) or Mann−Whitney rank sum test (for nonparametric data). A value of $p < 0.05$ was accepted as indicative of statistically significant difference.

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Supporting Information Available: ^{1}H NMR spectral data for compounds **15−19**, **21−23**, and **25−29** and elemental analysis for compounds **4−13**, **4a−6a** (analytical appendix I), and **14−29** (analytical appendix II). This material is available free of charge via the Internet at http://pubs.acs.org.

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