In dispersed rat parotid gland acinar cells, the β-adrenergic agonist (-)-isoproterenol, but not its stereoisomer (+)-isoproterenol, induced a transient 1.6-fold (at maximum stimulation, $2 \times 10^{-8} \text{M}$) increase in cytosolic free calcium ([Ca$^{2+}$]$_i$) within 9 s, which returned to resting levels (-180 nM) by 60 s. This [Ca$^{2+}$], response was not altered by chelating extracellular Ca$^{2+}$ with ethyleneglycol bis(2-aminoethyl ether) $N,N'$-tetra-acetic acid (EGTA) and could be completely blocked by the β-adrenergic antagonists propranolol ($\beta_1 + \beta_2$) and ICI 118,551 ($\beta_1$) but not by atenolol ($\beta_1$). The muscarinic-cholinergic agonist carbachol (at maximum stimulation, $10^{-6} \text{M}$) induced a 3-4-fold elevation in [Ca$^{2+}$], within 6 s, which slowly returned to resting levels by 8-10 min. The peak carbachol [Ca$^{2+}$], response was not substantially altered by the addition of EGTA to the extracellular medium. However, if the cells were first stimulated with isoproterenol in the EGTA-containing medium, the peak carbachol response was decreased (-54%). When carbachol was added to cells in the presence of high extracellular calcium, at the isoproterenol-stimulated [Ca$^{2+}$], peak, the resulting [Ca$^{2+}$], level was equal to that achieved when carbachol was either added alone or added after propranolol and isoproterenol. 8-Bromo-cyclic AMP induced a [Ca$^{2+}$], response similar to that elicited by isoproterenol, which was not additive to that by carbachol.

Carbachol induced a ~3.5-fold increase in inositol trisphosphate (IP$_3$) production in parotid cells within 30 s. 8-Bromo-cAMP, N$^\gamma$,O$^\beta$-dioctanoyl-cAMP, and isoproterenol consistently induced a significant stimulation in IP$_3$ production. The half-maximal concentration of isoproterenol required for [Ca$^{2+}$], mobilization and IP$_3$ production was comparable (~$10^{-6}$ M). Isoproterenol-induced IP$_3$ formation was blocked by propranolol. The data show that in rat parotid acinar cells, β-adrenergic stimulation results in IP$_3$ formation and mobilization of a carbachol-sensitive intracellular Ca$^{2+}$ pool by a mechanism involving cAMP. This demonstrates an interaction between the cAMP and phosphoinositide second messenger systems in these cells.

A variety of autonomic stimuli regulate the processes of protein and electrolyte secretion in the parotid gland acinar cell (for reviews, see Refs. 1-5). Muscarinic-cholinergic and α$_1$-adrenergic receptor stimulation results in phosphatidylinositol 4,5-bisphosphate (PIP$_2$) turnover leading to the formation of inositol 1,4,5-trisphosphate (IP$_3$), which induces mobilization of Ca$^{2+}$ from non-mitochondrial intracellular Ca$^{2+}$ stores. It has been suggested that the resulting elevation in cytosolic Ca$^{2+}$ mediates electrolyte movements (K$, \text{Cl}$), resulting in fluid secretion (6-9). Relatively less protein secretion is achieved by these agonists as compared to that induced by β-adrenergic receptor stimulation. The regulatory cascade initiated by β-adrenergic agonists leads to the formation of cAMP, activation of cAMP-dependent protein kinases, and phosphorylation of specific target proteins. Various studies have suggested a requirement for intracellular Ca$^{2+}$ in β-adrenergic receptor-mediated protein secretion (9, 10). β-Adrenergic activation has also been reported to directly alter Ca$^{2+}$ flux mechanisms in parotid basolateral membranes and to mobilize Ca$^{2+}$ in parotid acinar cells (11-17). However, the intracellular events associated with this Ca$^{2+}$ mobilization process have not been characterized. In the study described here, we have assessed the alterations in cytosolic Ca$^{2+}$ induced by the β-adrenergic receptor agonist isoproterenol. The data presented suggest that in rat parotid acinar cells, there is an interaction between the cAMP and phosphoinositide intracellular signalling systems. Isoproterenol stimulates PIP$_2$ turnover and mobilizes Ca$^{2+}$ from an intracellular, carbachol-sensitive Ca$^{2+}$ pool via a mechanism involving β$_1$-adrenergic receptors and cAMP.

EXPERIMENTAL PROCEDURES

Materials—Male Wistar-derived rats were from Harlan Sprague-Dawley, Collagene (CLSPA, specific activity 395 units/mg) was from Cooper Biochemical. Isoproterenol, 8-Br-cAMP, propranolol, carbachol, bovine testicular hyaluronidase (type 14, 270 units/mg), and bovine serum albumin (BSA) were purchased from Sigma. Quin2-AM and dioctanoyl-cAMP were obtained from Calbiochem. Quin2-AM was stored in dithionite as a 50 mM stock solution. ICI 118,551 was a kind gift of the Imperial Chemical Industries. myo-[H]$^1$inositol (specific activity 12 Ci/mM) was from Du Pont-New England Nuclear. ACS scintillation fluid was from Amersham Corp. Hanks' balanced salt solution was obtained from Gibco. All other reagents were of the highest grade available.

Cell Preparation—Animals were maintained on NIH Purina Lab Chow and water ad libitum until killed (~10:00 a.m.). Cells were obtained by enzymatic digestion of cleaned and finely minced parotid
glands as described previously (18). Briefly, the method involved incubation of 10% minced tissue in Hanks’ balanced salt solution buffered with Hepes to pH 7.5 (HBSS-H) containing 0.01% BSA, 100 units/ml collagenase, 0.2 mg/ml hyaluronidase (7, 13, 18). The incubation was performed in a metabolic shaker at 37 °C (110 rpm) for 80 min. Every 20 min the tissue was dispersed by pipetting and then gassed with 95% O2, 5% CO2 for 10 s. After the incubation period, the tissue was washed three times with HBSS-H by centrifugation at ~400 x g for 10 s and maintained in the shaker at 37 °C with gassing every 20 min until experimental use.

Measurement of Cytosolic Ca2+—Dispersed cell preparations were washed once with HBSS-H containing 0.01% BSA, maintained for a few minutes at 25 °C, resuspended in the same medium with 20 μM Quin2-AM and then incubated at 25 °C for 45 min with gassing (95% O2, 5% CO2) every 20 min. After incubation the cells were washed 3 times with HBSS-H, resuspended in the same medium, and maintained at 25 °C with gassing every 20 min until used for fluorescence studies. Fluorescence measurements were performed in an SLM 8000 spectrofluorimeter. Excitation and emission wavelengths were 339 and 495 nm respectively, and the slit width was adjusted to 4 nm. The cells were stirred gently in the cuvette during the assay, and the cuvette temperature was maintained at 37 °C. [Ca2+]i, was calculated according to Tsien et al. (19), 10 mM EGTA was added to correct for extracellular Quin2, and only corrected values have been given in the present study. The addition of 10% of the fluorescence intensity (F) was changed by the addition of 10 mM EGTA. Fmax was obtained by lysing the cells with 0.07% Triton X-100, and Fmax was obtained by the addition of 10 mM EGTA and Tris to increase the pH above 8.3. Details of individual experiments are presented in the tables and figure legends. Data are either the mean ± S.E. of results from experiments performed (number of times indicated) or are representative examples of experiments performed 3–5 times.

Measurement of Amylase Release—Amylase release from parotid acinar cells was determined as described previously (13, 18). Isoproterenol was used at concentrations from 10−8 to 10−5 M, while 8-Br-cAMP and diacetyl-cAMP were used at 2 and 0.5 mM. Propranolol (2 x 10−5 M), when used, was added at the same time as the agonist. Incubations were carried out at 37 °C for 30 min. Results are expressed as the mean ± S.E. (number of experiments indicated). The values given represent the percent of amylase released, i.e. amount of amylase in medium relative to total amylase (cells plus medium).

Permeabilization of Rat Parotid Acinar Cells—Acinar cells, prepared as described above, were washed once in intracellular medium (ICM) containing 120 mM KCl, 20 mM NaCl, 10 mM Hepes, pH 7.2, 1 mM MgCl2, and 2% BSA and resuspended in the same medium containing 50 μg of saponin/ml. The suspension was shaken in a bath at 37 °C for 15 min. Aliquots were withdrawn after various times and checked for permeability using trypan blue uptake. The dye was observed in almost all cells after 15 min of saponin treatment, at which time they were washed 3 times in saponin-free ICM (without BSA) and then maintained on ice for further use.

Measurement of Phosphatidylinositol 4,5-Biphosphate Turnover—To measure a PIP2 moiety and the method modified from Berridge et al. (20). Acinar cells were washed 3 times in HBSS-H, resuspended in HBSS-H containing 40 μCi/ml myo-[3H]inositol. Cells were then incubated in this medium at 37 °C with gassing every 20 min. Thereafter, the cells were washed 3 times in HBSS-H without BSA and allowed to recover for 10 min. In experiments with permeabilized cells, cells were permeabilized at this point according to the method described above. LiCl (10 mM) was added 5 min prior to the addition of agonists. Agonists (isoproterenol, carbachol, diacetyl-cAMP, and 8-Br-cAMP) were prepared as 100 × stock solutions and added to cell suspensions to yield the concentration indicated in the text. After 30 s, incubations were stopped by the addition of sufficient cold 100%CCI3H2O to yield a 10% (v/v) solution which was kept on ice for 30 min. The resulting precipitate was separated by centrifugation and CCl3COOH was removed from the supernatant by ether extraction. This procedure was repeated 4 times. The sample volume was made up to 2 ml with H2O and the pH was adjusted to 7.0 by the addition of 1 N KOH. Each wash was chromatographed on a 1-cm Bio-Rad AG-8 (formate) column. The column was eluted stepwise with 1) H2O, 2) 5 mM disodium tetraborate, 60 mM sodium formate, 3) 0.1 M formic acid, 0.2 M ammonium formate, 4) 0.1 M formic acid, 0.4 M ammonium formate, and 5) 0.1 M formic acid, 1.0 M ammonium formate. Fractions were collected, and an aliquot of each fraction was added to 10 ml of acidified ACS scintillant and the radioactivity determined by liquid scintillation spectrometry. The water soluble hydrolisis products of PIP2 (PIP1, IP3, and IP4) were quantitated relative to inositol.

Statistical Analysis—Data obtained were analyzed for statistical significance using the Student’s t test.

RESULTS

Isoproterenol-induced [Ca2+]i Elevation—The addition of isoproterenol to dispersed parotid acini, loaded with Quin2, resulted in a prompt rise in fluorescence, indicating an increase in [Ca2+]i. In the presence of 1.37 mM total extracellular Ca2+, our dispersed cell preparations typically had resting [Ca2+]i levels of 211 ± 24 nM (Table I). Typical baseline fluctuations during a single experiment were minimal and can be seen in Fig. 1A. Isoproterenol, at concentrations between 5 x 10−6 and 2 x 10−4 M, elicited a dose-dependent rise in

<table>
<thead>
<tr>
<th>Table I</th>
<th>Effect of extracellular calcium on the isoproterenol-induced [Ca2+]i response</th>
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<tr>
<td>Control</td>
<td>1.37 mM Ca2+ (n = 7)</td>
</tr>
<tr>
<td>Control + EGTA (10 mM) (n = 5)</td>
<td>194 ± 19</td>
</tr>
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</table>

FIG. 1. The effect of isoproterenol on intracellular calcium in rat parotid acinar cells. Dispersed acinar cells were prepared from rat parotid glands and loaded with Quin2 as described under “Experimental Procedures.” Cells were maintained at 37 °C in HBSS-H, with gentle stirring, during the assay. Fluorescence emission of Quin2 was measured at 495 nm. Addition of isoproterenol, 2 x 10−4 M (I), propranolol, 1 x 10−5 M (P), and carbachol, 1 x 10−4 M (C), are indicated (†) in the traces. The traces are representative of similar results obtained with at least 6 different cell preparations. A shows a typical baseline Quin2 fluorescence recorded for 300 s. In B, note that the [Ca2+]i responses were recorded on a different time scale.
[Ca\(^{2+}\)]. A half-maximal [Ca\(^{2+}\)], change was achieved by \(\sim 10^{-6}\) M isoproterenol, while concentrations lower than \(5 \times 10^{-7}\) M elicited no observable increase in [Ca\(^{2+}\)]. Maximal isoproterenol stimulation (2 \(\times\) \(10^{-4}\) M) increased [Ca\(^{2+}\)], to 337 \(\pm\) 36 nM (i.e. a 1.6-fold increase). This change in [Ca\(^{2+}\)], is statistically significant (\(p < 0.025\)). This [Ca\(^{2+}\)], elevation, as shown in Fig. 1B, was transient in nature, peaking at \(\sim 9\) s and returning to resting levels after \(\sim 60\) s. We examined the ability of the \(\beta\)-adrenoceptor antagonist propranolol to attenuate the isoproterenol-stimulated [Ca\(^{2+}\)], elevation. As shown in Fig. 1C, the addition of \(10^{-6}\) M propranolol to the cells before isoproterenol completely blocked the subsequent isoproterenol-stimulated [Ca\(^{2+}\)], response but did not alter the ability of the muscarinic agonist carbachol to increase [Ca\(^{2+}\)]. (see Table II).

To localize (as intracellular or extracellular) the Ca\(^{2+}\) pool, which is involved in the \(\beta\)-adrenergic receptor-stimulated [Ca\(^{2+}\)], response, we examined the effect of isoproterenol on [Ca\(^{2+}\)], in the presence (1.37 mM) or virtual absence (\(+\) 10 mM EGTA) of extracellular Ca\(^{2+}\). These results are shown in Table I. Without [Ca\(^{2+}\)], both the resting [Ca\(^{2+}\)], 194 \(\pm\) 19 nM, and the stimulated [Ca\(^{2+}\)], reached after addition of 2 \(\times\) \(10^{-7}\) M isoproterenol, 303 \(\pm\) 42 nM, are not significantly different from that seen with control [Ca\(^{2+}\)]. The isoproterenol response induced with low [Ca\(^{2+}\)], was also completely blocked by \(10^{-4}\) M propranolol (data not shown). Thus the status of [Ca\(^{2+}\)], does not alter substantially the isoproterenol-induced [Ca\(^{2+}\)], response, indicating that an intracellular Ca\(^{2+}\) pool is being mobilized by \(\beta\)-adrenergic receptor stimulation.

**Receptor Specificity of Isoproterenol Stimulation**—We have assessed the stereoselectivity of the isoproterenol response by using (+)- and (−)-isomers of isoproterenol (see Fig. 2). The (+)-isomer, at 2 \(\times\) \(10^{-4}\) and 5 \(\times\) \(10^{-4}\) M, did not induce any [Ca\(^{2+}\)], change above base line. The complete blockage of the (−)-isoproterenol-induced [Ca\(^{2+}\)], elevation by propranolol shows that this mobilization of intracellular Ca\(^{2+}\) is mediated by \(\beta\)-adrenergic receptors. However, parotid acinar cells possess two types of \(\beta\)-adrenergic receptors, \(\beta_1\) and \(\beta_2\). Since propranolol is a nonspecific \(\beta\)-adrenergic antagonist, interacting with both receptor types, it is not possible, based on the propranolol inhibitory effect, to identify the specific \(\beta\)-adrenergic receptor subtype involved. We therefore tested the effects of specific antagonists directed against \(\beta_1\) and \(\beta_2\) adrenergic receptors (atenolol and ICI 118,551, respectively).

The results, summarized in Fig. 2, show that ICI 118,551 (\(10^{-6}\) M) inhibited the isoproterenol-stimulated [Ca\(^{2+}\)], increase by 86\%, an inhibition comparable to that obtained with propranolol. Atenolol, at the same concentration, was much less effective (~20\% inhibition). These observations suggest that \(\beta_2\)-adrenergic receptors are involved in the isoproterenol-induced [Ca\(^{2+}\)], response observed in rat parotid acinar cells.

**Characterization of the Intracellular Ca\(^{2+}\) Pool Mobilized by Isoproterenol**—In order to characterize the intracellular Ca\(^{2+}\) pool which responds to \(\beta_2\)-adrenergic receptor activation, we compared the isoproterenol-stimulated [Ca\(^{2+}\)], change to that induced by the muscarinic-cholinergic agonist carbachol. It has been shown in several cell types, including parotid acinar cells, that carbachol stimulation results in [Ca\(^{2+}\)], elevation due to the mobilization of a non-mitochondrial, intracellular Ca\(^{2+}\) pool. We have observed, with the Quin2 technique, that in both rat parotid and submandibular gland acinar cells (21, 22), epinephrine (an \(\alpha_2\)-adrenergic agonist) and carbachol rapidly mobilize the same intracellular Ca\(^{2+}\) pool and induce 3-4-fold elevations in [Ca\(^{2+}\)]. After stimulation by one of these agonists, [Ca\(^{2+}\)], cannot be further increased by the addition of the second agonist, thus indicating the involvement of the same Ca\(^{2+}\) pool in both agonist-induced responses. A similar approach was used here to characterize the isoproterenol response. Isoproterenol and carbachol, at 2 \(\times\) \(10^{-4}\) and \(10^{-5}\) M, respectively, independently induced [Ca\(^{2+}\)], elevations. The results are shown in Fig. 3. The isoproterenol response was quantitatively and temporally distinct from the carbachol-induced [Ca\(^{2+}\)], change (Fig. 3, A and B). [Ca\(^{2+}\)], was increased 3-4-fold when stimulated with carbachol (peak response = 745 \(\pm\) 73 nM), while it increased \(\sim 1.6\)-fold (peak response = 337 \(\pm\) 36 nM) when stimulated by isoproterenol. Peak [Ca\(^{2+}\)], after carbachol stimulation, was reached in 6.11 \(\pm\) 0.84 s, while peak [Ca\(^{2+}\)], after isoproterenol stimulation,

![Fig. 2. Receptor specificity of the isoproterenol-induced elevations of [Ca\(^{2+}\)]. The experimental conditions were similar to those described for Fig. 1. Stereoisomers (+) and (−)-isoproterenol were both added at 2 \(\times\) \(10^{-4}\) M. The data show the change in [Ca\(^{2+}\)], above base line. Antagonists were added to the cell suspension 30 s before the addition of 2 \(\times\) \(10^{-7}\) M (−)-isoproterenol. ICI, A, and P show, respectively, the effects of 1 \(\times\) \(10^{-5}\) M ICI 118,551, a \(\beta_2\)-specific antagonist, 1 \(\times\) \(10^{-5}\) M atenolol, a \(\beta_1\)-specific antagonist, and 1 \(\times\) \(10^{-5}\) M propranolol, a mixed \(\beta_1\) + \(\beta_2\) antagonist, on the change in [Ca\(^{2+}\)], induced by (−)-isoproterenol. The data represent the mean \(\pm\) S.E. of at least 5 separate experiments. The [Ca\(^{2+}\)], changes by (−)-isoproterenol (Iso) in the presence of ICI 118,551 effect differs significantly from that by (−)-isoproterenol alone (\(p < 0.005\)) and not from that with propranolol. The [Ca\(^{2+}\)], change with atenolol does not differ significantly from the control (−) Iso.

![Fig. 3. Carbachol- and isoproterenol-induced changes in [Ca\(^{2+}\)], in rat parotid acinar cells. Quin2 fluorescence in acinar cells was measured as described for Fig. 1. Arrows (†) indicate the time of the addition of agonists, C, 1 \(\times\) \(10^{-4}\) M carbachol; and I, 2 \(\times\) \(10^{-5}\) M isoproterenol. These traces are representative of similar data obtained in 12 individual experiments.]
was attained in 8.77 ± 0.77 s. This temporal difference is statistically significant (p < 0.05, n = 43). The estimated rate of the [Ca^{2+}]i change after stimulation with carbachol was 82 nM/s, and after stimulation with isoproterenol it was 13 nM/s, a 6-fold difference. In the case of carbachol stimulation, [Ca^{2+}]i peak was maintained for ~20 s and then slowly decreased to resting levels after ~10 min. This pattern is very different from the isoproterenol-induced transient [Ca^{2+}]i response (Figs. 1B and 2B).

Addition of 10^{-5} M carbachol after the isoproterenol-mediated [Ca^{2+}]i transient consistently resulted in a maximal [Ca^{2+}]i response, i.e., 3-4-fold [Ca^{2+}]i elevation (shown in Fig. 2B). When a maximal stimulatory concentration of isoproterenol (2 × 10^{-4} M) was added after the peak induced by a maximal stimulatory concentration of carbachol (10^{-5} M), no further elevation in [Ca^{2+}]i was seen (Fig. 3C). When a submaximal concentration of carbachol (1 × 10^{-7} M) was added, a small elevation in [Ca^{2+}]i was seen which was smaller than that induced by the addition of isoproterenol. Subsequent addition of 2 × 10^{-4} M isoproterenol then induced a further increase in [Ca^{2+}]i. This [Ca^{2+}]i could be further increased to the maximum response levels by the addition of 10^{-5} M carbachol (data not shown). 10^{-7} M carbachol when added at the peak of the isoproterenol response did not increase [Ca^{2+}]i further, while when >5 × 10^{-5} M carbachol was added, a further increase in [Ca^{2+}]i was observed (data not shown). It thus appears that isoproterenol stimulation does not facilitate the response induced by low carbachol concentrations.

The data, summarized in Fig. 4, show that the [Ca^{2+}]i peak induced by carbachol, when added after the isoproterenol-induced [Ca^{2+}]i peak, was less than the sum of the individual carbachol- and isoproterenol-induced [Ca^{2+}]i responses. The total [Ca^{2+}]i response, however, was equal to that seen when carbachol was either added alone or after isoproterenol and propranolol. As mentioned above, both the isoproterenol-induced and the initial phase of the carbachol-induced, [Ca^{2+}]i elevations are substantially independent of [Ca^{2+}]i status and primarily represent mobilization of an intracellular Ca^{2+} pool.

When carbachol was added to the cells in the EGTA-containing medium, following the isoproterenol-induced [Ca^{2+}]i transient, the peak carbachol [Ca^{2+}]i response was 54% lower than that seen when carbachol was added alone. These data are shown in Table II. This observation is also quite different from the trend shown in Fig. 3B, where in the presence of 1.37 mM Ca^{2+}, a similar carbachol stimulation after isoproterenol elicited a maximal response. In the presence of EGTA, addition of isoproterenol after carbachol stimulation, either at the [Ca^{2+}]i peak or subsequently, induces no further [Ca^{2+}]i change (data not shown). These observations suggest that isoproterenol partially depletes an intracellular Ca^{2+} pool that is also mobilized by carbachol. In the presence of physiological [Ca^{2+}]i, this intracellular pool can apparently be almost completely refilled within ~60 s.

Effect of 8-Br-cAMP on [Ca^{2+}]i—It has been reported that in rat parotid acinar cells, β-adrenergic receptor-regulated events are mediated by the intracellular messenger, cAMP (e.g., Refs. 1, 3, 15, 23). In order to characterize the intracellular signalling mechanism involved in the Ca^{2+} mobilization induced by isoproterenol, we tested the ability of the permeant cAMP analogue, 8-Br-cAMP, to mimic the isoproterenol-stimulated [Ca^{2+}]i response. The data are shown in Fig. 5. Addition of 2 mM 8-Br-cAMP to the cells induced a transient [Ca^{2+}]i alteration, similar to that seen with isoproterenol (Fig. 5A). However, quantitatively, at peak response, the 8-Br-cAMP-induced [Ca^{2+}]i increase was ~50% lower than that seen with isoproterenol. Addition of isoproterenol after the peak of the 8-Br-cAMP [Ca^{2+}]i transient resulted in a further rise in [Ca^{2+}]i (Fig. 5A). Addition of carbachol further increased [Ca^{2+}]i to maximum response levels when added to cells after isoproterenol and 8-Br-cAMP (Fig. 5A). When 8-Br-cAMP was added subsequent to either isoproterenol or carbachol stimulation of the cells, no further rise in [Ca^{2+}]i was observed (Fig. 5B). Fig. 5C shows the effect of 8-Br-cAMP on [Ca^{2+}]i in the absence of extracellular Ca^{2+} (i.e., +10 mM EGTA). The pattern of the 8-Br-cAMP-induced increase in [Ca^{2+}]i was similar in cells exposed to either 1.37 mM Ca^{2+} (Fig. 5A) or to medium without Ca^{2+} (Fig. 5C).

However, in the latter condition, addition of isoproterenol after 8-Br-cAMP did not induce a further increase in [Ca^{2+}]i, while addition of carbachol did induce a rapid, further rise in [Ca^{2+}]i. The data suggest that the same intracellular Ca^{2+} pool responds to both 8-Br-cAMP and isoproterenol and again suggest that this pool can be rapidly refilled in the presence of physiological concentrations of extracellular Ca^{2+}.

To assess whether the lower efficiency of 8-Br-cAMP was unique to mobilizing [Ca^{2+}]i, or was representative of a weaker potency of the cAMP analogue when compared to isoproterenol.

![Figure 4: Characterization of intracellular Ca^{2+} pool mobilized by isoproterenol and carbachol](image_url)

**Table II**

<table>
<thead>
<tr>
<th>Additions</th>
<th>High [Ca^{2+}]i (1.37 mM Ca^{2+})</th>
<th>Low [Ca^{2+}]i (+10 mM EGTA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 10^{-5} M Carbachol</td>
<td>745 ± 73 (n = 10)</td>
<td>770 ± 76 (n = 7)</td>
</tr>
<tr>
<td>2. 10^{-4} M Carbachol following 2 × 10^{-4} M isoproterenol</td>
<td>714 ± 80 (n = 6)</td>
<td>387 ± 83 (n = 5)</td>
</tr>
<tr>
<td>3. 10^{-4} M Carbachol following 10^{-4} M propranol + 2 × 10^{-4} M isoproterenol</td>
<td>757 ± 114 (n = 4)</td>
<td>ND</td>
</tr>
</tbody>
</table>
The effect of 8-Br-cAMP on [Ca\(^+\)]\(_i\). Experimental conditions were similar to that described in Fig. 1. 2 mM 8-Br-cAMP (8-B), 2 \times 10^{-4} \text{ M} isoproterenol (I), and 1 \times 10^{-4} \text{ M} carbachol (C) were added as indicated (†) in the traces. These represent similar results obtained in 4 separate experiments. Experiments were conducted either in HBSS-H (1.37 mM Ca\(^+\)) (A and B) or after 10 mM EGTA (C) was added to the extracellular medium 20 s prior to the addition of 8-Br-cAMP.

Stimulation of Phosphatidylinositol 4,5-Bisphosphate Turnover in Rat Parotid Acinar Cells—It has been established that IP\(_3\), generated by a phospholipase C-catalyzed breakdown of PIP\(_3\), is a second messenger mediating the intracellular Ca\(^+\)\(_i\) mobilization induced by a number of different hormones. As described above, 8-Br-cAMP can mimic the isoproterenol-mediated [Ca\(^+\)]\(_i\) response, suggesting that cAMP is likely involved in Ca\(^+\)\(_i\) mobilization in the rat parotid. Recently, reports in several mammalian systems have suggested that cAMP elevations can be correlated with IP\(_3\) production (24-26). Earlier reports by Aub and Putney (27) stated that \(\beta\)-adrenergic receptor activation does not lead to PIP\(_3\) breakdown in rat parotid acinar cells. However, in those studies cells were incubated with isoproterenol for 30 min before IP\(_3\) levels were determined. As described here, the isoproterenol-induced [Ca\(^+\)]\(_i\) response is rapid and transient (Fig. 1), the entire response occurring within 1 min. We therefore measured IP\(_3\) turnover (IP\(_1\), IP\(_2\), and IP\(_3\)) in rat parotid acinar cells incubated with the \(\beta\)-adrenergic agonist for 30 s. In parallel, we also determined the ability of carbachol to induce IP\(_3\) turnover in these cells. The data are shown in Fig. 6. After exposure of rat parotid acinar cells to carbachol (1 \times 10^{-5} \text{ M}) for 30 s, IP\(_1\), IP\(_2\), and IP\(_3\) were significantly elevated by 147, 238, and 365%, respectively, over control levels (n = 10, \(p < 0.005\), t test). A maximal isoproterenol concentration (2 \times 10^{-4} \text{ M}) significantly elevated IP\(_3\); IP\(_1\) (by 136 and 149%, respectively, over control levels (n = 8, \(p < 0.005\), t test), while IP\(_2\) was not altered to a statistically significant degree. Half-maximal stimulation by isoproterenol of IP\(_3\) production was seen at \(\sim 10^{-5} \text{ M}\). In these studies carbachol (10^{-5} \text{ M})-induced IP\(_3\) formation was \(\sim 5\)-fold greater than that induced by the maximal concentration of isoproterenol. When parotid cells were exposed to 8-Br-cAMP for 30 s, IP\(_1\) and IP\(_3\) formation were also significantly higher
β-Adrenergic Receptor-mediated IP3 Production and [Ca2+], Flux

12459

that the isoproterenol-mediated [Ca2+]i mobilization is specific, prompt, transient, stereoselective, 1.6-fold increase in cytosolic [Ca2+]i, the hydrophobic cAMP analogue dioctanoyl-cAMP (0.5 mM) was more effective than 8-Br-cAMP in increasing IP3 levels (134%).

To determine whether the isoproterenol-stimulated formation of IP3 was mediated through β-adrenergic receptors, we examined the effect of the mixed β-adrenergic antagonist propranolol on the isoproterenol-elicted PIP2 turnover under conditions in which the [Ca2+]i response was completely blocked. IP3 production in cells exposed to (i) propranolol alone, (ii) propranolol with isoproterenol, and (iii) propranolol with carbachol was determined and compared to that in control cells (without propranolol). The results are shown in Fig. 7. The isoproterenol-stimulated increase in IP3 production was almost entirely blocked by the presence of propranolol (80%, p < 0.025, t test). In parallel experiments, carbachol-stimulated IP3 formation remained unaltered by the inclusion of propranolol in the incubation medium. This demonstrates the receptor-specific effects of propranolol under our experimental conditions.

To assess whether the isoproterenol-induced increase in IP3 production was due to a direct stimulation by cAMP (i.e. generated at the membrane signalling complex) or a secondary stimulation due to the increase in [Ca2+]i, we measured IP3 formation in saponin-permeabilized cells (data not shown). The criteria used to establish permeabilization in these cells were (i) trypan blue uptake, (ii) ATP-dependent 46Ca2+ uptake into a non-mitochondrial pool, (iii) GTP-dependent Ca2+ release from a non-mitochondrial Ca2+ pool, and (iv) lack of measurable agonist stimulated IP3 response in cells which had been loaded with Quin2 prior to permeabilization.

In order to measure IP3 production, cells were labeled with myo-[3H]inositol for 1 h prior to permeabilization. Permeabilized cells were suspended in ICM medium (see “Experimental Procedures”) containing no added Ca2+. Carbachol (10−5 M) and isoproterenol (2 × 10−4 M) were added to the cells and the reaction was quenched after 30 s (n = 3). The control value for IP3 production in these experiments was 0.28% inositol. Carbachol increased IP3 accumulation by 2-fold over control levels, while isoproterenol increased IP3 by 1.36-fold. These results suggest that the isoproterenol-induced IP3 formation occurs directly in response to a signal (likely cAMP) generated at the β-adrenergic receptor signalling complex in the plasma membrane.

**DISCUSSION**

Our results show that in rat parotid acinar cells the β-adrenergic receptor agonist, (−)-isoproterenol, induces a prompt, transient, stereoselective, 1.6-fold increase in cytosolic Ca2+ at a maximum agonist concentration (2 × 10−4 M). Half-maximal [Ca2+]i response is induced by ∼10−8 M isoproterenol, which is a concentration approximately 2 orders of magnitude higher than that needed to induce in vitro amylase secretion (half-maximal response at ∼10−9 M) (28, 29). Earlier studies (30) have observed that in rat parotid acinar cells amylase secretion, stimulated by isoproterenol, involves the β2-adrenergic receptor subtype while CAMP generation is more pronounced following β2-adrenergic receptor stimulation. Our data show that the isoproterenol-mediated [Ca2+]i mobilization is specifically mediated by the β2-adrenergic receptor since it can be almost completely blocked by the antagonists propranolol (mixed β1 + β2) and ICI 118,551 (β2), while it is minimally affected by the β1 antagonist atenolol.

A number of reports have shown that β-adrenergic stimulation can induce Ca2+ mobilization in parotid cells and elevate the cytosolic Ca2+ level (12–17). It is clear from our data that the quantitative and qualitative pattern of the isoproterenol-stimulated Ca2+ flux is not affected by the status of extracellular Ca2+, suggesting the mobilization of an intracellular Ca2+ pool. In an earlier report, Takemura (14) had observed that the isoproterenol-mediated elevation of [Ca2+]i, elevation was blocked in the absence of external Ca2+. This result is different from our observation. However, the carbachol response obtained in that study was also ∼50% lower than that obtained by us (22) or reported by others using Fura 2 (31). Takemura (14) also employed a very high Quin2 concentration (50 μM), which could dampen the rise in [Ca2+]i, and this accounts for the variations in the agonist-induced [Ca2+]i responses.

Several earlier reports (11, 12) had also suggested that β-adrenergic receptor-mediated Ca2+ mobilization was distinct from both α-adrenergic and muscarinic stimulation. Our studies, however, suggest the involvement of a common intracellular Ca2+ pool during muscarinic and β-adrenergic stimulations. In the earlier studies calcium mobilization was assessed by measuring 45Ca2+ flux in cells, which is an indirect measure of cytosolic Ca2+ levels. Moreover, 45Ca2+ flux measurements represent the resultant of several different phenomena, the major one being the rate of Ca2+ transport across the plasma membrane. Therefore, 45Ca2+ flux measurements cannot be quantitatively compared to [Ca2+]i, changes directly measured by the Quin2 or Fura 2 (31) methods. It appears from our data that the intracellular Ca2+ pool mobilized by isoproterenol is part of the same pool that responds to carbachol. This conclusion is supported by several observations. The addition of isoproterenol or 8-Br-cAMP after the peak carbachol response induces no further [Ca2+]i increase. Also, at maximal stimulatory concentrations the isoproterenol and carbachol responses are not additive and the [Ca2+]i increase with carbachol alone is equal to that induced by the successive additions of both isoproterenol and carbachol. Moreover, the [Ca2+]i response induced by isoproterenol and the peak [Ca2+]i, induced by carbachol are not substantially altered by lowering the extracellular Ca2+. In the presence of 1.37 mM [Ca2+], carbachol can elicit normal [Ca2+], elevation when added after the isoproterenol induced [Ca2+]i transient. However, in the presence of low [Ca2+], (i.e. + EGTA), the carbachol-stimulated [Ca2+]i elevation seen after isoproterenol stimulation is decreased by about half. These observations, in aggregate, support the conclusion that the two agonists release Ca2+ from the same intracellular pool and that, in the presence of physiological concentrations of [Ca2+]i, this pool can be rapidly refilled. Judging from the quantitative aspects of the two responses it appears that, although under maximum stimulatory conditions, carbachol can completely deplete the isoproterenol sensitive pool, only part of the carbachol-sensitive pool is responsive to isoproterenol.

The isoproterenol-elicted [Ca2+]i response is, however, distinct from the carbachol-elicted [Ca2+]i response in several respects. Elevation of [Ca2+]; by isoproterenol is (i) transient (lasting ∼60 s), while that by carbachol is maintained for longer periods (∼10 min); (ii) 3-fold slower in magnitude than that achieved by carbachol; (iii) 6-fold slower in rate than that of carbachol-induced [Ca2+]i increase; and (iv) essentially completely independent of [Ca2+]o, while the carbachol response has a component which is dependent on [Ca2+]o (the sustained phase, see e.g. Ref. 31). The isoproterenol-induced
[Ca²⁺], response is mimicked by the cAMP analog 8-Br-cAMP, indicating that the elevation of [Ca²⁺], resulting from isoproterenol stimulation is cAMP-dependent. In preliminary studies, we have observed similar effects by isoproterenol on [Ca²⁺], in Fura 2-loaded parotid acinar cells (data not given).

Furthermore, the data presented here show that isoproterenol stimulation also induces a dose-dependent, rapid turnover of PI(P)₂, measured as an increase in the formation of IP₃. In a prior report (27) which indicated that isoproterenol does not induce IP₃ formation, IP₃ levels were measured after long incubation periods (30 min). Our data show that 30 s after isoproterenol stimulation of rat parotid acinar cells there is a 1.5-fold increase in IP₃ levels. Moreover, our results show that IP₃ formation is also enhanced by 8-Br-cAMP and dioctanoyl-cAMP. Takuma and Ichida (32) have shown that addition of cAMP to permeabilized rat parotid acinar cells did not induce release of Ca²⁺ previously loaded into the endoplasmic reticulum, while IP₃ addition did induce Ca²⁺ release. Putney et al. (33) have reported that the phospholipase activity associated with PIP₂ breakdown in rat parotid and pancreatic acinar cells is not sensitive to Ca²⁺. We have also presented data to show that, in a permeabilized cell system, both carbachol and isoproterenol increase IP₃ formation in a medium free of added Ca²⁺. In view of these aggregate observations, it therefore seems likely that (i) CAMP-dependent IP₃ formation and CAMP-dependent elevation of [Ca²⁺]; in rat parotid acinar cells are not two independent events, and (ii) CAMP-dependent elevation of [Ca²⁺]; does not lead to IP₃ formation. Thus, it is possible that the CAMP-dependent increase in IP₃ production observed herein could account for the [Ca²⁺]; elevation. It is intriguing that qualitatively and quantitatively the [Ca²⁺]; response induced by isoproterenol is so different from that induced by carbachol. A direct enhancing effect of cAMP on the basolateral membrane ATP-dependent Ca²⁺ transporter in rat parotid acinar cells has been demonstrated (16). Such effects on plasma membrane Ca²⁺ transport were not observed after carbachol stimulation. This effect could contribute toward the regulation of [Ca²⁺];, following isoproterenol stimulation and in part explain the transient nature of the [Ca²⁺]; response. It has previously been reported that, for carbachol stimulation of rat parotid acinar cells, there is a quantitative correlation between the IP₃ formation and Ca²⁺ mobilization (34). The increase in [Ca²⁺]; induced by isoproterenol correlates rather well with the extent of the observed IP₃ increase. The dose dependence of isoproterenol for the two responses is also similar. However, considering that a maximal stimulatory concentration of isoproterenol was used, the IP₃ response appears small in comparison to that seen after maximal carbachol stimulation. The response is, however, highly reproducible, statistically significant, and β-adrenergic receptor-specific.

Our observation that CAMP leads to an increase in IP₃ formation in rat parotid acinar cells suggests an interaction between the adenylyl cyclase and phosphoinositide transmembrane signalling systems. A variety of agents influence cellular functions through these two signalling pathways, which appear to involve somewhat similar components (e.g. receptor, G protein, effector enzyme). Other signal pathway interactions have recently been observed. For example, in some cells adenylyl cyclase activity decreases as a result of phorbol ester treatment (suggesting protein kinase C involvement), while in other cells phorbol esters increase adenyl cyclase activity (35). On the other hand, in luteal and adrenal cells increased intracellular CAMP can be closely correlated with an increase in IP₃ levels (24, 25). Furthermore, it has been very recently reported (36) that β-adrenergic stimulation of guinea pig parotid cells induces the formation of 2,3-sn-diacylglycerol and the incorporation of fatty acids into phospholipids. Our data additionally show that in rat parotid acinar cells IP₃ levels are increased following β-adrenergic stimulation, by a mechanism involving cAMP, which results in [Ca²⁺]; mobilization. The exact molecular mechanism(s) involved in the interaction between cAMP and the inositol phosphate signalling systems, as well as the physiological relevance of such a concerted mechanism, still remains to be elucidated.

REFERENCES